

Nutritional quality and physiological responses to transport and storage of live crustaceans traded in Portugal

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Resumo

Em Portugal, o consumo de produtos da pesca é o mais elevado de entre os países da UE. De entre estes, destacam-se os crustáceos, que são muito apreciados, especialmente se mantidos vivos até ao momento da sua confecção como garantia de frescura. Presentemente, a maioria dos crustáceos comercializados em vivo é importada, o que implica que a cadeia de comercialização seja longa e a sua logística complexa. Neste contexto, é expectável que desde o local de captura até ao consumidor final incluindo os restaurantes, estes animais sofram diversos factores de stress, que podem promover mortalidade e representar elevados custos económicos. Contudo, não existem até ao momento estudos publicados sobre a mortalidade de crustáceos neste sector e a sua extensão ao longo da cadeia de comercialização. Neste sentido, foi elaborado um inquérito e realizadas entrevistas presenciais aos principais comerciantes nacionais. De entre as espécies comercializadas, a sapateira *Cancer pagurus* e o lavagante Europeu *Homarus gammarus*, são as mais importadas, sendo principalmente provenientes do Reino Unido. Os preços variam consoante as espécies, sendo as lagostas normalmente mais caras e manuseadas com mais cuidado do que os caranguejos. Para além disso, as sapateiras capturadas no Canal da Mancha (EC) são por norma mais caras do que as sapateiras provenientes da costa Escocesa (SC). Em todos os estabelecimentos visitados, a maioria das espécies de caranguejo apresentava taxas de mortalidade (até 60 %) muito superiores às das lagostas (~10 %).

Os principais problemas que podem contribuir para o desencadear da mortalidade nos crustáceos foram registados nas entrevistas e, posteriormente observados durante um transporte de *C. pagurus* desde Inglaterra até Portugal. Assim, destacam-se: o manuseamento descuidado; períodos de exposição ao ar; qualidade da água de transporte inadequada (baixos níveis de oxigénio e elevados valores de amónia e nitritos); variações de temperatura; e elevada densidade (carga animal). Estes factores de stress foram correlacionados com alterações nos parâmetros da hemolinfa das sapateiras (e.g. glucose, lactato, pH e hemocianina), tendo-se concluído que o transporte em condições imersas promove o metabolismo anaeróbio.

Uma vez chegadas a Portugal, as sapateiras têm de ser redistribuídas pelos grossistas e retalhistas, pelo que sofrem transportes adicionais, em que a mortalidade pode atingir valores próximos de 40 a 60 %. Com objectivo de testar alternativas às actuais condições de transporte foram simuladas condições de transporte em ambiente semi-seco e submersas em água salgada com e sem anestésico. Concluiu-se que o transporte em semi-seco a baixas temperaturas (~ 8 °C) constitui uma alternativa eficaz, desde que sejam implementados alguns procedimentos que são discutidos.

O enorme esforço e a logística que o transporte e a manutenção de crustáceos vivos exigem, tem como principal propósito, fornecer animais frescos e de elevada qualidade aos consumidores mais criteriosos. No entanto, um animal vivo não é necessariamente sinónimo de um produto de elevada qualidade. Neste contexto, a qualidade nutricional dos tecidos edíveis (i.e. músculo, hepatopâncreas e gónadas) de *C. pagurus*, *H. gammarus* e *H. americanus* de ambos os sexos, foi

caracterizada. No caso de *C. pagurus*, as variações sazonais e as diferenças entre populações (sapateiras capturadas na costa Escocesa vs. sapateiras capturadas no Canal da Mancha) foram igualmente avaliadas.

Geralmente, o músculo das sapateiras e lavagantes apresentaram características típicas dos produtos da pesca, isto é, baixos teores de gordura e colesterol, fonte de proteínas de elevada qualidade e de ácidos gordos polinsaturados e de elementos essenciais dentro ou acima dos valores recomendados. Contudo, as generalizações relativas aos produtos da pesca não podem ser extrapoladas para todos os tecidos edíveis analisados. Na realidade, tanto lavagantes como sapateiras apresentaram uma grande diversidade na qualidade nutricional do músculo, hepatopâncreas e gónadas. O hepatopâncreas exibiu valores moderados a elevados de gordura, e comparativamente com o músculo apresentou valores mais elevados de ácidos gordos saturados, índice de aterogenicidade (IA) e de trombogenicidade (IT), bem como macro elementos e elementos traço, incluindo cádmio. No que respeita aos lavagantes, estes apresentaram um hepatopâncreas mais gordo, proporcionalmente com mais ácidos gordos saturados e valores de IA e IT mais elevados do que as sapateiras, mas menos cádmio. As gónadas apresentaram uma grande variação entre sexos, em que os ovários têm mais proteína, amino ácidos, gordura e colesterol do que os órgãos reprodutores masculinos. Geralmente, os três tecidos edíveis das três espécies de crustáceos analisados são excelentes fontes de elementos essenciais, sendo os únicos elementos limitantes o Mg, K e Mn.

As principais diferenças observadas entre as duas populações de sapateiras estudadas, ocorreram em relação à composição inorgânica, na medida em que as sapateiras da Escócia são mais ricas em elementos inorgânicos do que as do canal da Mancha. Além disso, o perfil em ácidos gordos do músculo das sapateiras da Escócia apresentou uma maior proporção de 14:0, 18:1n-9 e 18:2n-6, mas menor em 18:1n-7 e 16:4n-3 do que as sapateiras do Canal da Mancha.

Quanto às variações sazonais observadas nos tecidos edíveis de *C. pagurus*, estas foram mais pronunciadas no hepatopâncreas e gónadas do que no músculo. Do ponto de vista do consumidor, o Outono é a melhor época do ano para comer estes crustáceos, particularmente fêmeas, considerando que existe uma maior proporção de gónadas e hepatopâncreas, bem como teores mais elevados de taurina, Fe, Ca e Zn. Contudo, o Outono é também a estação do ano em que o hepatopâncreas é mais gordo e com menos ácidos gordos polinsaturados do tipo n-3, enquanto os valores de IA, IT e de colesterol são mais elevados no hepatopâncreas e gónadas. Por fim, as concentrações de cádmio determinada no hepatopâncreas, bem como de mercúrio tanto neste tecido como no músculo, foram superiores aos estabelecidos pelas agências internacionais.

Tendo em conta as três espécies estudadas, o único tecido que efectivamente pode representar riscos para a saúde humana é o hepatopâncreas devido ao elevado teor de cádmio. Deste modo, e como medida de precaução, este tecido deve de ser consumido moderadamente em todas as estações do ano, mas em particular sapateiras fêmeas no Outono.

Este estudo permitiu a obtenção de dados importantes, sobre a comercialização de crustáceos vivos em Portugal, bem como a qualidade nutricional de sapateiras e lavagantes. Os dados compilados e as recomendações sugeridas podem ser utilizadas por todos os intervenientes incluindo: a indústria, nutricionistas, agências e organizações que regulamentam os níveis de toxicidade nos alimentos e que podem fazer recomendações e implementar áreas de pesquisa. Estes dados também são importantes para o consumidor que estando melhor informado pode fazer escolhas e tomar decisões mais responsáveis.

Abstract

Portugal has one of the highest seafood consumptions in the EU. Among seafood crustaceans are most appreciated particularly if maintained alive until the culinary preparation as a guarantee of freshness. Nowadays, most live crustaceans are imported consequently the trade chain is long and, the logistics complex. In this context, it is predictable that from fishing grounds to Portuguese restaurants, crustaceans face several stressors that can lead to mortality and economical losses. However, there are no known reports of both mortality and its extension during the live trade of crustaceans. Therefore, a survey was elaborated and personal interviews were made to the main national traders. It was concluded that the edible crab, *Cancer pagurus* and the European clawed lobster, *Homarus gammarus* are the most imported live species, mainly from the UK. Prices are much variable between species, as lobsters are more expensive than crabs and usually more carefully handled. Also, edible crabs captured off the English Channel (EC) are more expensive than those captured off the Scottish coast (SC). In all national facilities, most crab species had higher rates of mortality (up to 60 %) than lobsters (~10 %).

Most problems pointed out in the interviews that might contribute to mortality were observed *in situ* during a consignment of *C. pagurus* from England to Portugal and were mainly: a) poor handling; b) periods of aerial exposure; c) poor water quality during transport (low oxygen, high ammonia and nitrites); d) variations in temperature and e) high animal densities. These stressors were correlated to changes in haemolymph parameters (D-glucose, L-lactate, pH and haemocyanine) and it was concluded that immersed transport elicited anaerobic metabolism.

Once in Portugal, edible crab must be redistributed to wholesalers and retailers, thus suffering an extra transport with mortality reaching 40 to 60 %. To test alternatives to the present transport conditions, experiments were carried out simulating national transport in seawater and in semi-dry environment with and without an anaesthetic. It was concluded that semi-dry transport at low temperatures (~ 8 °C) can be an efficient alternative as long as traders implement adequate procedures that are discussed.

The huge effort and logistics required to import and maintain live crustaceans has the purpose of supplying high quality fresh animals to demanding consumers. However, a live animal is not necessarily synonymous of a product with high quality. In this context, the nutritional quality of the edible tissues (*i.e.* muscle, hepatopancreas and gonads) of males and females *C. pagurus*, *H. gammarus* and *H. americanus* was characterized. In the case of *C. pagurus*, the seasonal variations and differences between populations (crabs captured off the Scottish coast vs. crabs captured in the English Channel) were also evaluated.

It was concluded that muscle of edible crab and homarids is a typical seafood product *i.e.* low in fat and cholesterol, a good source of high quality protein, polyunsaturated fatty acids and essential elements in the range of daily recommended intakes, or even above. However, generalizations about seafood nutritional quality cannot be assumed for all edible tissues. In fact, homarids and edible crab showed great diversity in the nutritional quality of muscle, hepatopancreas and gonads.

Hepatopancreas is a moderate to high fat tissue, rich in saturated fatty acids and macro and trace elements including cadmium, with higher indexes of atherogenicity (IA) and thrombogenicity (IT) comparatively to muscle. In general, the hepatopancreas of homarids is fattier, with proportionally more saturated fatty acids (SFA), IA and IT, but lower cadmium concentration than the edible crab. Gonads showed great variations between sexes as ovaries have more protein, amino acids as well as fat and cholesterol than testis. In general, all edible tissues of homarids and *C. pagurus* are excellent sources of most essential elements and the only limiting elements were Mg, K and Mn.

Differences between the two populations of *C. pagurus* were mainly observed in the elemental composition as crabs harvested in the SC were better sources of most elements than animals from the EC. Also, the fatty acids profile of crabs' muscle from the SC had higher proportion of 14:0, 18:1n-9 and 18:2n-6, and lower 18:1n-7 and 16:4n-3 than those of the EC.

Seasonal differences observed in the edible tissues of *C. pagurus* were more pronounced in hepatopancreas and gonads and less in muscle. From a consumer perspective, autumn is the best season to eat edible crab, particularly females, considering the higher brown meat yield (*i.e.* gonads and hepatopancreas) but also the high taurine concentration, Fe, Ca and Zn content. However, autumn is also the season when the hepatopancreas is fattier but with less n-3 fatty acids and when values of IA, IT and cholesterol are higher in both hepatopancreas and ovaries. Moreover, Cd in hepatopancreas, and Hg in both hepatopancreas and muscle were above the established levels set by international regulation agencies.

The only tissue in all three species that can pose risks to human health is hepatopancreas due to the high cadmium content. Therefore, consumption moderation of brown meat is advised in all seasons but, particularly in autumn and mainly of female edible crabs.

This research made available important information that fills a gap in the knowledge of live trade of crustaceans in Portugal and the nutritional composition of homarids and *C. pagurus*. The data compiled and the recommendations given can be used by all stakeholders including: a) the industry; b) nutritionists; c) regulation agencies and organizations that regulate maximum toxicity levels in food and that can advance further recommendations and research areas. These data are also important for the consumer who can be better informed and therefore be able to make choices and more reliable decisions.

Thesis Outline

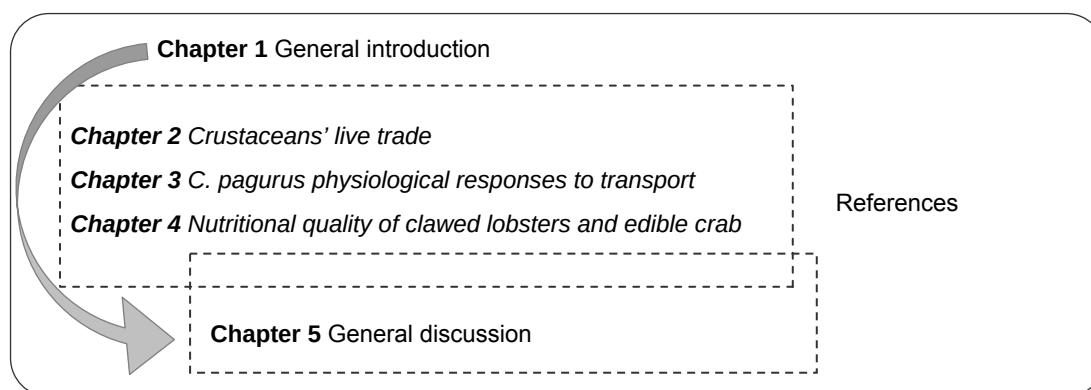
This thesis dissertation is the outcome of a three-year research period between 2007 and 2009 and is divided in six chapters. A general introduction about crustaceans is presented in chapter 1, including their biology, live trade in Portugal, and particularly the fisheries of *Cancer pagurus*, *Homarus gammarus* and *H. americanus*. Additionally, a brief bibliographic revision of the physiological challenges faced by crustaceans during live trade is provided, followed by the nutritional quality and safety of seafood in general, and crustaceans in particular. Finally, the main objectives are presented.

In chapter 2, the results of a national survey to Portuguese traders of live crustaceans are presented. This work was conducted with the specific aim of generating baseline information that enabled the identification of the major problems faced by this industry in Portugal.

Chapter 3 consists in the study of physiological stress responses of *C. pagurus* during one of the most important critical points of the trade chain of live crustaceans, *i.e.* transport. In this way, three experiments were made on the physiological responses of *C. pagurus* to stress during *in situ* live trade (sub-chapter 3.1) and under simulated conditions (sub-chapters 3.2 and 3.3).

Chapter 4 includes the nutritional quality of *H. gammarus*, *H. americanus* and *C. pagurus*. This chapter is divided in seven sub-chapters. In chapter 4.1 the biochemical composition of the edible tissues of both homarid species is characterized and compared, while in chapter 4.2 the inorganic elemental composition is described. In the following sub-chapters the biochemical composition (sub-chapter 4.3) and elemental composition (Sub-chapter 4.4 and 4.5) of female and male *C. pagurus* captured off the Scottish coast is compared to crabs of both sexes captured off the English Channel. The following last two chapters (Sub-chapters 4.6 and 4.7) cover the seasonal nutritional quality of female and male *C. pagurus* captured off the Scottish coast in relation to the biochemical and inorganic elemental composition, respectively.

Finally, the main results obtained in the previous chapters and conclusions drawn throughout the thesis are briefly discussed in the framework of the research objectives (Chapter 5) followed by the references list.



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List of units and abbreviations

°C	degree Celsius
%	percentage
/	per
atm	atmospheres (pressure unit)
eV	electronvolt
g	gram
<i>g</i>	relative centrifugal force or G force
Kcal	kilocalories
keV	kiloelectronvolt
kg	kilogram
kJ	kilojoule
kV	kilovolts
L	litre
M	molar
m	metre
mm	millimetre
mA	milliAmpere
mg	milligram
min	minute
mL	millilitre
mM	milimolar
nm	nanometre
pH	the negative logarithm (base 10) of the molar concentration of hydrogen ions
ppm	part per million
s	seconds
t	tonnes
UV	ultra violet light
α	alfa
μg	microgram
μL	microlitre
μm	micrometre
ω	omega
$\pm$	approximately

AA	arachidonic acid
AI	adequate intake
AL	action level
ALA	alfa-linolenic acid
Ala	alanine
AF	autumn female <i>C. pagurus</i>
AM	autumn male <i>C. pagurus</i>
ANOVA	analysis of variance
AOAC	Association of Analytical Communities
Arg	arginine
Asp	aspartic acid
BDL	below detection limit
CHD	coronary heart disease
CHH	crustacean hyperglycemic hormone
CL	carapace length
COX	cyclooxygenase
CVD	cardiovascular disease
CW	carapace width
Cys	cysteine
DGPA	Direcção Geral das Pescas e Aquicultura
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DRI	dietary reference intakes
EAA	essential amino acids
EC	European Commission
EC	edible contribution
EC	English Channel
EDXFR	energy dispersive X-ray fluorescence
EF	female <i>C. pagurus</i> from the English Channel
EFSA	European Food Safety Authority
EPA	eicosapentaenoic acid
EU	European Union
FAME	fatty acids methyl ester
FAAS	flame atomic-absorption spectrometry
FAO	Food and Agriculture Organization

FDA	Food and Drug Administration
G	gonads
Glu	glutamic acid
Gly	glycine
GSI	gonodossomatic index
H	hepatopaneas
HI	hepatossomatic index
HDL	high density lipoproteins
His	histidine
HPLA	high performance liquid chromatography
HSD	honestly significant differences
Hyp	hydroxyproline
IA	index of atherogenicity
Ile	isoleucine
IOM	Institute of Medicine
IT	index of thrombogenicity
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LA	linoleic acid
LDL	low density lipoproteins
Leu	leucine
LOX	lipxygenases
Lys	lysine
M	muscle
MAFF	Ministry of Agriculture, Fisheries and Food, UK
Met	methionine
ML	maximum level
MUFA	monounsaturated fatty acids
MY	claw muscle meat yield
ND	not determined
ND	no statistical difference
NEAA	non essential amino acids
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NS	value not set
n-3	omega-3 fatty acids

n-6	omega-6 fatty acids
ONU	Organization of the United Nations
p	p-value, probability of the test statistic
PCA	principal components analysis
Phe	phenylalanine
Pro	Proline
PTDI	provisional tolerable daily intake
PTWI	provisional tolerable weekly intake
PUFA	polyunsaturated fatty acids
RDA	recommended dietary allowance
SC	Scottish coast
sd	standard deviation
Ser	serine
SF	female <i>C. pagurus</i> from the Scottish coast
SFA	saturated fatty acids
SM	male <i>C. pagurus</i> from the Scottish coast
SUF	spring female <i>C. pagurus</i>
SUM	summer female <i>C. pagurus</i>
TAA	total amino acids
Tau	taurine
Thr	threonine
TI	thrombogenic index
TMY	total meat yield
Trp	tryptophan
Tyr	tyrosine
UKDH	United Kingdom Department of Health
UL	tolerable upper intake level
USA	United States of America
USDA	United States Department of Agriculture
USFDA	United States Food and Drug Administration
Val	valine
WF	winter female <i>C. pagurus</i>
WHO	World Health Organization
WM	winter male <i>C. pagurus</i>

Chapter 1

General introduction



Descarregadores
Rogério Chora (coleção privada)

1 Foreword

In the history of mankind, crabs, lobsters and shrimps have had a special but controversial role in gastronomy. Ancient civilizations such as the Egyptians banned crustaceans from their diet, while Romans had them on the menu of banquets. Religion has also influenced diet habits, and as far as crustaceans are concerned, both Islamism and Judaism prohibit its eating. After the Second World War crustaceans consumption increased, the demand expanded, prices rose and some species are nowadays associated to a high social status (Falciai and Minervini, 1995). Production also grew, contributing significantly to incomes of fishermen, processors and distributors; aquaculture of some shrimp species, such as *Penaeid* and *Palemonidae* intensified and for instance in Ecuador, the wealth generated by the production of *Penaeus vannamei* and *Penaeus stylirostris* exceeded that of petroleum in several years (Falciai and Minervini, 1995). Nowadays, the trade of crustaceans is so widespread and common that even in non coastal countries many people can easily distinguish a crab from a lobster.

1.1 Recognizing crabs and lobsters

Crustaceans are part of the most widespread animal group that includes about 97 % of all species colonizing oceans, riverbeds and the terrestrial environment - the invertebrates; animals without a backbone or spinal column. Crustaceans, such as crabs and lobsters along with insects and spiders all belong to the phylum Arthropoda (Bliss, 1990).

Crustaceans can be described as mandibulates with jointed appendages, two pairs of antennae and stalked compound eyes (Noga *et al.*, 2006). They have a hard external skeleton, often called exoskeleton or carapace, constituted by the nitrogen-rich polysaccharide chitin bound with proteins and inorganic salts, mainly calcium carbonate (Dando, 1996). The carapace in crabs and lobsters is shielded-like and often fused with some or all segments of the thorax. The carapace of these forms provides protection for the important anterior region of the body, with its numerous vital organs. In crabs and lobsters the carapace is composed by two regions, cephalothorax and abdomen. The appendages remain flexible because of pliable, non-calcified membranes of chitin at each joint (Bliss, 1990).

Crabs and lobsters belong to the order Decapoda (from the Greek, meaning “ten feet”) and have in general five pairs of legs. Most decapods are marine, but crayfish, some shrimps and crabs have invaded fresh water, while there are also some terrestrial crabs (Ingle, 1997). Members of the order Decapoda are divided in two groups of animals, those with long tails (lobsters, shrimps and prawns) and those with short tails located underneath the body (Bliss, 1990). Lobsters have often been referred to as macrurans, after the Greek words macros, meaning long, and oura, meaning tail. In general there are two kinds of lobsters: true lobsters of the infraorder Astacidea (e.g. European lobster, *Homarus gammarus*; American lobster, *Homarus americanus*) and spiny lobsters or rock lobsters, of the infraorder Palinura (e.g. common lobster, *Panulirus elephas*; South African rock lobster, *Jasus lalandii*). A true lobster has two large claws and a stiff tail fan, while a spiny lobster or rock lobster lacks large claws and has a flexible leathery tail fan (Figure 1.1; Bliss,

1990). Crabs belong to the infraorder Brachyura, after the Greek words brachys, meaning short and oura, meaning tail. The Brachyura, also commonly called true crabs, include well known species as the velvet crab (*Necora puber*), the green crab (*Carcinus maenas*) and the European edible crab (*Cancer pagurus*) (Bliss, 1990).

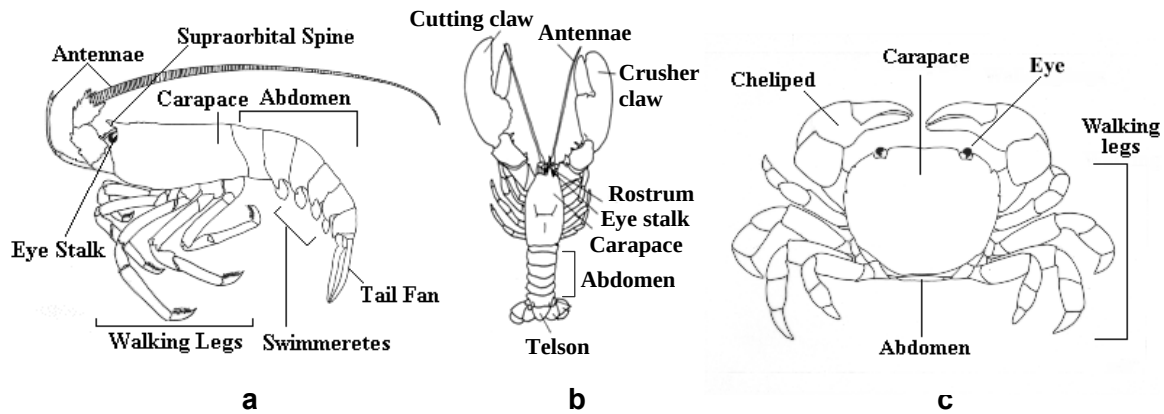


Figure 1.1 Schematic illustration of the principal external differences between (a) spiny lobster; (b) clawed lobsters and (c) crab (Illustration source: Taylor, 2009).

1.1.1 General physiological characteristics

Moulting and mating

Due to the hard exoskeleton, these animals can increase in size only periodically during moulting (period that follow the casting off of the old shell and precede the hardening of the new one). The shedding of the old shell is called ecdysis, after the Greek word ekdysis, meaning “a getting out”. Ecdysis is preceded and followed by an increase in the metabolic activity, where the old exoskeleton is selectively decalcified and the new one calcified. To these complex activities that constitute growth in crustaceans, the term moult is applied (Bliss, 1990). In most crustaceans, mating takes place when females are soft, which occurs after moulting, but fertilization is delayed until ovaries are ripen and female sexual cells are ready to descend the oviducts and be fertilized by the sperms. Meanwhile the sperm is stored in a “pouch” at the end of the oviducts called a spermatheca and can stay viable for several years depending on species. After moulting, the female new exoskeleton starts to harden, and muscles as well as the hepatopancreas build up. Ovaries start to develop and become ripped when the female sexual cells are fully developed and ready for spawning. In the spawning process the female sexual cells are fertilized with sperm stored in the spermatheca. Following spawning, the female usually carries fertilized eggs with her as they undergo development; this period of egg incubation is species dependent. Hatching occurs when eggs development is complete, and a tiny free-swimming larva emerges from each egg case. The development in these animals is not direct and therefore larvae face several metamorphoses, and moult several times before planktonic stages eventually settle and become juveniles (Edwards, 1979; Bliss, 1990). This is the general pattern of mating and spawning in decapod crustaceans, the details of these activities vary among the species.

Respiration and circulatory system

Respiration in decapod crustaceans generally takes place in gills that usually lie outside of the body within the branchial chamber, protected by the carapace. The oxygen-transport protein used by most crustaceans is a copper-containing pigment, called haemocyanine, which circulates in the extracellular fluid or haemolymph (Bliss, 1990, Chartois *et al.*, 1994).

The circulatory system is opened, meaning that although elastic arteries and thin-walled elastic capillaries occur in many species of crustaceans, there are no veins. Instead, the haemolymph returns to the heart by way of interconnecting spaces known as venous sinuses, which communicate with the pericardium (Bliss, 1990). As a consequence of its open circulatory system, these animals do not have two separate substances known as blood and lymph. Hence this substance is more accurately termed haemolymph, the first part of this word derived from a Greek word meaning “blood” (Bliss, 1990). The heart is located dorsally in the thorax, and is a compact single chambered sac with several openings known as ostia through which haemolymph enters coming from the pericardium (Figure 1.2).

Digestive and excretory systems

The digestive system includes the stomach, gastric mill (responsible for the mechanical digestion), midgut and hepatopancreas (or digestive gland). The midgut is long and extends to the rectum, where undigested wastes are eliminated through the anus (Arzel *et al.*, 1992).

Decapod crustaceans have a pair of excretory glands called the antennal glands that open in the base of the antennae. Nevertheless, gills are responsible for most of the excretion, eliminating ammonia (Arzel *et al.*, 1992).

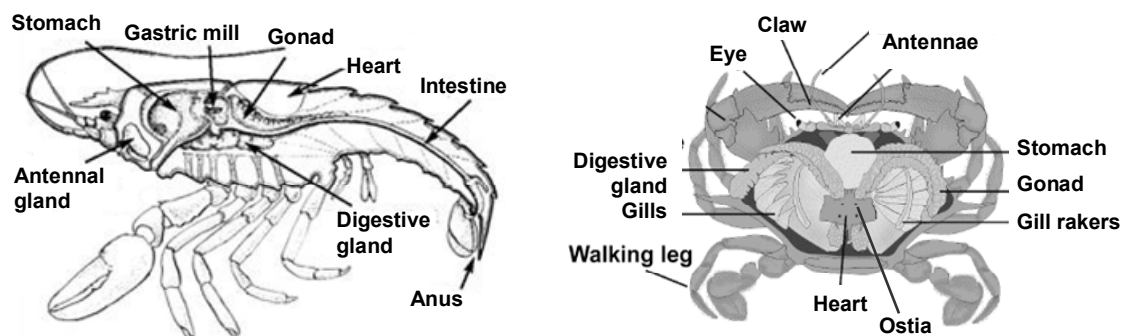


Figure 1.2 Basic internal anatomy of a clawed lobster and a swimming crab (from left to right), showing major organs of the digestive, circulatory and, excretory systems (clawed lobster diagram adapted from Maine Department of Marine Resources, 2009; crab diagram adapted from Smithsonian Marine Station at Fort Pierce).

1.2 Biology of the most important live crustaceans traded in Portugal

This thesis is focused on the major live imported species in Portugal, *C. pagurus* and *H. gammarus*. *H. americanus* was also included because of its similarity with European lobster and the increasing trend to import this species. *C. pagurus*, *H. gammarus* and *H. americanus* though different have several common biological and behavioural characteristics, for instance they are

nocturnal animals and act both as predators and scavengers. Like other cold-blooded animals, they can subsist without food for many days or weeks (Woll, 2006).

1.2.1 *Cancer pagurus*, (Linnaeus, 1758)

The scientific name of the European edible crab, *Cancer pagurus*, derives from the Latin word *cancer* meaning “a crab” and the Greek word *pagouros* also meaning “a crab” (Ingle, 1997). Some distinctive features include: carapace broadly oval; antero-lateral margins cut into broad lobes giving “pie crust” appearance; chelipeds robust and smooth, second to fifth pereopods stout, distal segments with tufts of short setae (Ingle, 1997; Figure 1.3). The carapace is reddish brown while small crabs are purple brown; pereopods are lighter, dactyl and propodal extremities of chelae are black. Carapace width (CW) rarely exceeds 16 cm and the commonly marked size is around 12-13 cm and 8-9 cm in length (CL), (Ingle, 1997).

Kingdom: Animalia
Phylum: Arthropoda
Class: Crustacea
Order Decapoda
Infraorder: Brachyura (true crabs)
Superfamily: Cancroidea
Family: Cancridae
Genus: *Cancer*
Species: *Cancer pagurus*

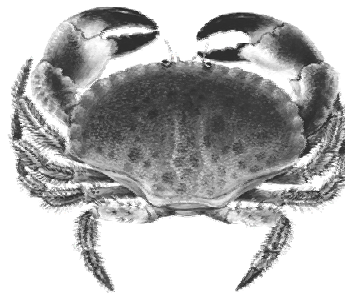


Figure 1.3 Taxonomic classification and illustration of *C. pagurus*, (Linnaeus, 1758), (Ingle, 1997; illustration source: FAO species, 2009).

The brown crab is a long-lived large decapod crustacean. Crabs live for at least 15 years and recruit to the fishery probably between the ages of 4-6 years, when crabs become sexual mature at a width size of 11-13 cm (Tully *et al.*, 2006). After maturation, gender characteristics become more pronounced during each moult. Female abdomen has four pairs of hairy appendages, the swimmerets, on which the eggs attach during spawning, and the external female genitals consist of a pair of large openings situated beneath the abdomen. The dorsal side of the carapace becomes more rounded during each moult giving more space for gonads. In contrast, males have a narrower abdomen and two abdominal appendages modified to form copulatory organs (Figure 1.4). Sexual mature males have larger claws and the carapace is flatter (Edwards, 1979; Woll, 2006).

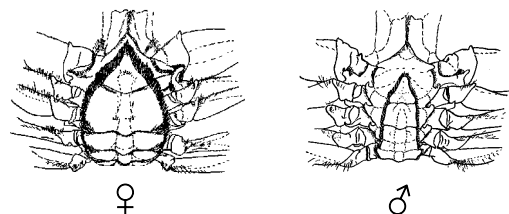


Figure 1.4 Ventral view of the abdomen of female (♀) and a male (♂) edible crab, (source: Edwards, 1979).

Mating takes place when the female crab is still soft immediately after moulting during summer months. Gonad development begins in early autumn, spawning occurs over winter and the incubation period takes about seven to eight months and during this period the female lies in the sand, partly buried and hardly eating (Edwards, 1979; Howard, 1982; Woll, 2003). The development of eggs and larvae is temperature-dependent, and the critical minimum temperature is 8-9 °C (Eaton *et al.*, 2003). The hatching starts at different times of the year, and closely follows the pattern of seabed warming. Fecundity is very high and each female crab may hatch between 1 and 4 million eggs. Post larvae are known to settle inshore (Tully *et al.*, 2006) and juvenile crabs are more common in shallow than in deep water. Adult crabs undertake extensive migrations, which may be associated with the reproductive cycle. Most of them stay in deeper water during the winter season and migrate to shallow water in the summer season (Woll, 2003). Differences in migration patterns are observed between female and male crabs. Large females tend to migrate long distances while males are more stationary (Woll, 2003).

1.2.2 *Homarus gammarus*, (Linnaeus, 1758)

The scientific name of the European clawed lobster, *Homarus gammarus*, derives from the old French word *homar* meaning “a lobster” and, the Greek word *kamamaros* meaning a “kind of lobster” (Ingle, 1997; Figure 1.5).

Kingdom: Animalia
 Phylum: Arthropoda
 Class: Crustacea
 Order Decapoda
 Suborder: Macrura Reptantia
 Infraorder: Astacidae
 Superfamily: Nephropoidea
 Family: Nephropidae
 Subfamily: Nephropinae
 Genus: *Homarus*
 Species: *Homarus gammarus*

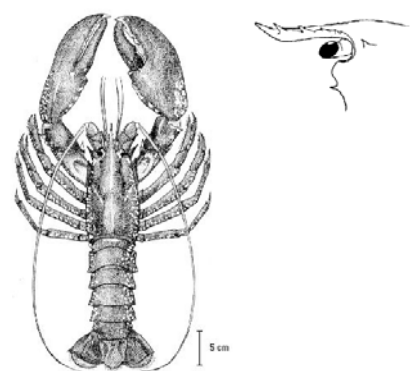


Figure 1.5 Taxonomic classification and illustration of *H. gammarus*, (Linnaeus, 1758) with a detail of the rostrum lateral view showing the absence of ventral teeth (source: Holthuis, 1991).

Some distinctive features include: a smooth carapace, rostral lateral margins with 4-5 teeth but lower margin without teeth, the medial groove is present throughout carapace length, abdominal segments and first pair of pereopods are smooth; chelae are dissimilar: one has irregular large teeth (crusher claw), while the other is narrower with smaller sharper teeth (pincher claw). The carapace is bluish to almost black, with lighter reticulations, but underside is white to yellowish (Ingle, 1997). The total length is commonly 35 to 40 cm, rarely exceeding 50 cm. The largest known had 62 cm and weighted 8.4 kg (Ingle, 1997). Females become mature at about 25 cm in length. Two main external differences distinguish both sexes in mature lobsters: a) the gonopores of males open at the basis of the coxae of the fifth pair of pereopods, while in females they are associated with the third pair of pereopods; b) in males, the first pair of pleopods (or swimmerets)

is modified for spermatophore transfer, being long, hard, grooved, and tapering, whereas in females these pleopods are small and soft (Figure 1.6). Pleopods of reproductive active females also bear long ovigerous setae for egg attachment. At sexual maturity, the carapace is shorter (relatively to body length) in females than males and females develop a wider abdomen to facilitate the carriage of eggs (Bliss, 1990).



Figure 1.6 From left to right, difference between a female and male clawed lobster. Male copulatory organs are hard while those of female are soft (adapted from Maine Department of Marine Resources, 2009)

Shortly after the female has moulted the male deposits the spermatophores into female's spermatheca. Spermatophores can remain viable for at least fifteen months. Spawning usually occurs during early autumn. The small fertilised eggs are incubated attached on the pleopods. From 10,000 to 100,000 eggs may be spawned depending upon size of the female. Incubation period usually lasts nine to ten months. Hatching of larvae occurs in late spring and early summer. These planktonic larvae moult several times increasing in size at each moult. Juveniles live in habitats similar to the adults *i.e.* holes and crevices (Ingle, 1997).

1.2.3 *Homarus americanus* H. Milne Edwards, 1837

The scientific name of the American clawed lobster, *Homarus americanus*, indicates the distribution of this lobster species, which is restricted to the North American continent (Ingle, 1997; Figure 1.7). Some distinctive features include: palm of first chelipeds without hair cover, the left and right first chelipeds are strongly different in shape and rostrum has one or more ventral teeth. The colour of the American lobster carapace can be surprisingly variable, from green or dark blue-green with small green-black spots and often red spines, to orange with green-black spots, yellow with purple-blue spots, and indigo blue.

Kingdom Animalia
Phylum Arthropoda
Class Crustacea
Order Decapoda
Suborder Macrura Reptantia
Infraorder: Astacidae
Superfamily Nephropoidea
Family Nephropidae
Subfamily Nephropinae
Genus *Homarus*
Species *Homarus americanus*

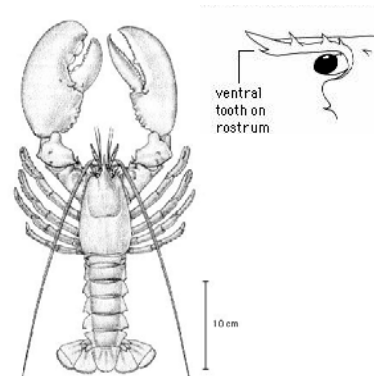


Figure 1.7 Taxonomic classification and illustration of *H. americanus* H. Milne Edwards, 1837, with a detail of the rostrum lateral view showing the ventral tooth (source: Holthuis, 1991).

Yet, they tend to be lightly pigmented or even cream-coloured underneath (Bliss, 1990). Maximum total body length reported was of 64 cm, but it is usually around 25 cm or less (Holthuis, 1991).

Differences between sexes of American lobsters are similar to those reported for European lobster (see above; Bliss, 1990; Talbot and Helluy, 1995). Female American lobsters mate 24 to 48 hours following the ecdysis, while still soft. Male inserts his first pair of pleopods, the copulatory appendages, into the spermatheca on the ventral side of the female thorax between the third and fifth pairs of thoracic legs. The female spawns after one month to two years after mating. The number of eggs spawned varies with the size of the female, a 18 cm (length) female lays approximately 3,000 eggs, while a 46 cm (length) female lays around 75,000 eggs. The fertilized eggs are carried by the female and the incubation period lasts about one year before eggs hatch (Bliss, 1990).

1.3 Live crustaceans in Portugal: fishing and marketing

Seafood has an important role in the socioeconomic and gastronomic history of Portugal. The average *per capita* supply of fish and fishery products from 2001 to 2003 was 57.1 kg/year, while in the rest of the world was only 16.4 kg/ year (Laurenti, 2004). The national seafood demand is so high that production is not sufficient, and therefore imports (€ 653,847,100) usually exceeds exports (€ 247,278,200) and consequently there is an inevitable trade deficit (data from January to July 2009; Datapescas, 2009).

In a country so devoted to seafood, crabs, lobsters and shrimps have a special place in the menu and are a national trade mark used as a lure by the tourism industry. Seafood festivals are becoming more popular across the country from the up North city of Bragança that in 2009 celebrated the second International Seafood Festival, to the Southern coast in Olhão, with already 24 years of festivals. In between, several coastal regions such as Ribamar, Peniche, Porto das Barcas, Murtosa and Sesimbra have their own seafood festivals. Special events are dedicated exclusively to one species, for example, in Aljezur barnacles are served with sweet potatoes and, in Santa Cruz edible crab is the main course. Besides for the sweet exquisite taste crustaceans are also famous for their high price. One of the most distinct characteristics that make the preparation of crustaceans in Portugal so well-known is that several species of crabs and lobsters are kept live until the culinary preparation as a guarantee of freshness and full taste. This practice was first attributed to the Romans, who transported live lobsters and other species in fishing vessels across Roman cities, in lead lined tanks with seawater (Soares, 2000; Aguilera, 2001). In Portugal, regulations regarding the harvest of spiny and clawed lobsters and stocking facilities to hold these live crustaceans were first documented in the nineteenth century but this practice might date far back (Portugal, 1897). The regulation included harvest restrictions and, the necessary conditions to obtain the legal permit for stocking facilities which was conceded on an annual basis (Portugal, 1897). During the first half of the twentieth century and until the seventies live stocking facilities of spiny and clawed lobsters proliferated, probably due to its high price and also because at the time these species were abundant in the Portuguese coast (Portugal, 1896; Figure 1.8).

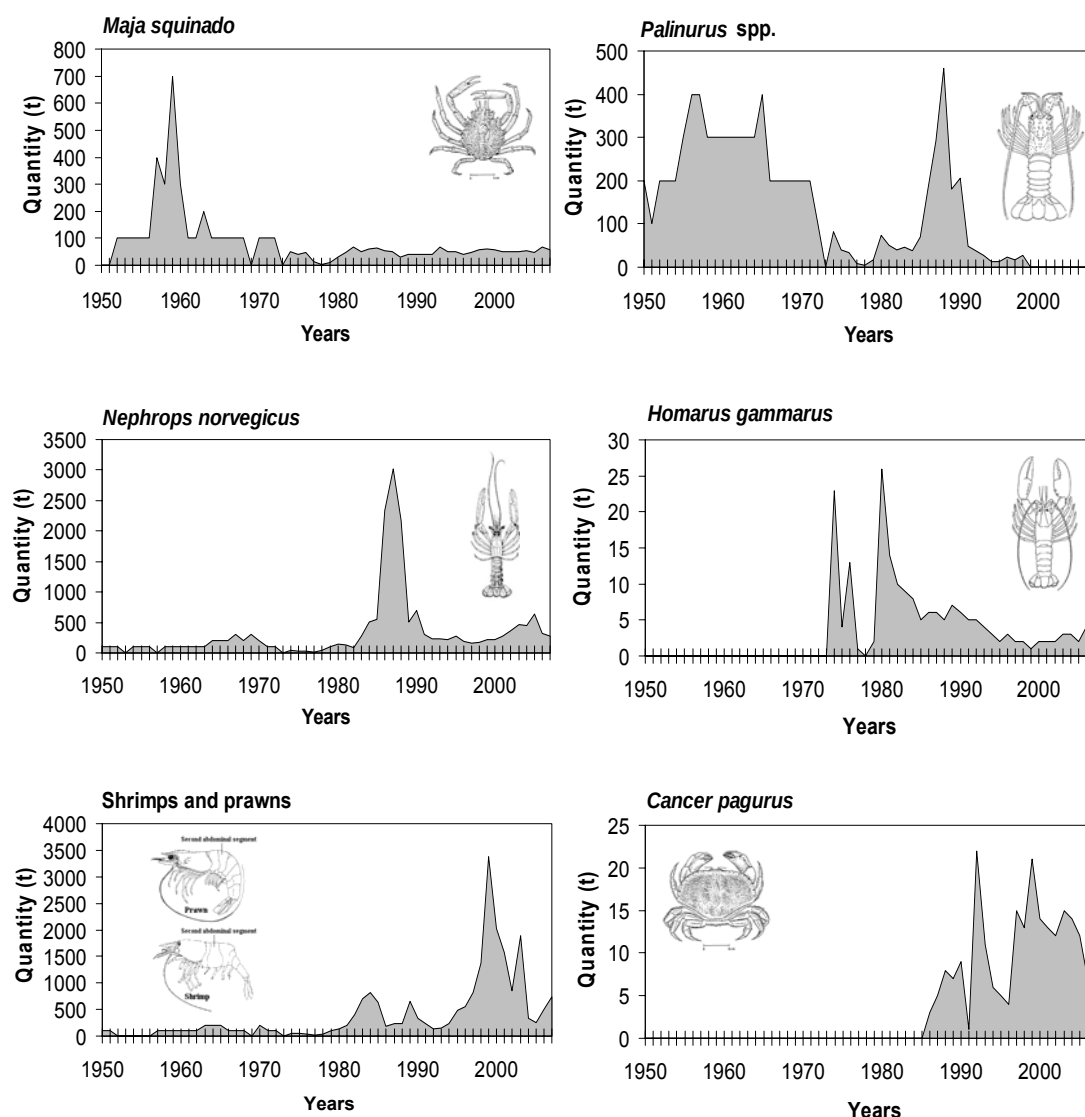


Figure 1.8 National production in tonnes (t) of the most commercially representative crustaceans of the Portuguese coast from 1950 to 2007 (data source: EUROSTAT, 2009; illustrations source: Chartois *et al.*, 1994; Holthuis, 1991).

Maja squinado (spider crab) and *Palinurus elephas* (spiny lobster), were the main targeted species during the three decades that followed the Second World War, and were mainly caught by traps (Leal, 1984). However, after 1974 stocks were poorly managed, fishing effort increased and trawling for highly priced species such as *Nephrops norvegicus* (Norway lobster), shrimps and prawns was implemented. Also, *H. gammarus* was heavily captured but mainly with traps. With the exception of shrimps and prawns, there was a steep decline of production in late eighties and beginning of the nineties (Leal, 1984). In order to cope with consumption, importation was a necessary alternative to the reduced amount of domestic landings. Consequently species that were common in our coast and dinner plates like the European lobster, *H. gammarus* and the spiny lobster, *P. elephas*, became less frequent. On the other hand, species harvested in other European regions such as *C. pagurus* and *H. gammarus* captured in the UK, and even in other continents like *H. americanus* and *Jasus* spp. became an alternative. In figure 1.9 the main supplier countries of several crustaceans to Portugal are presented in respect to import quantities.

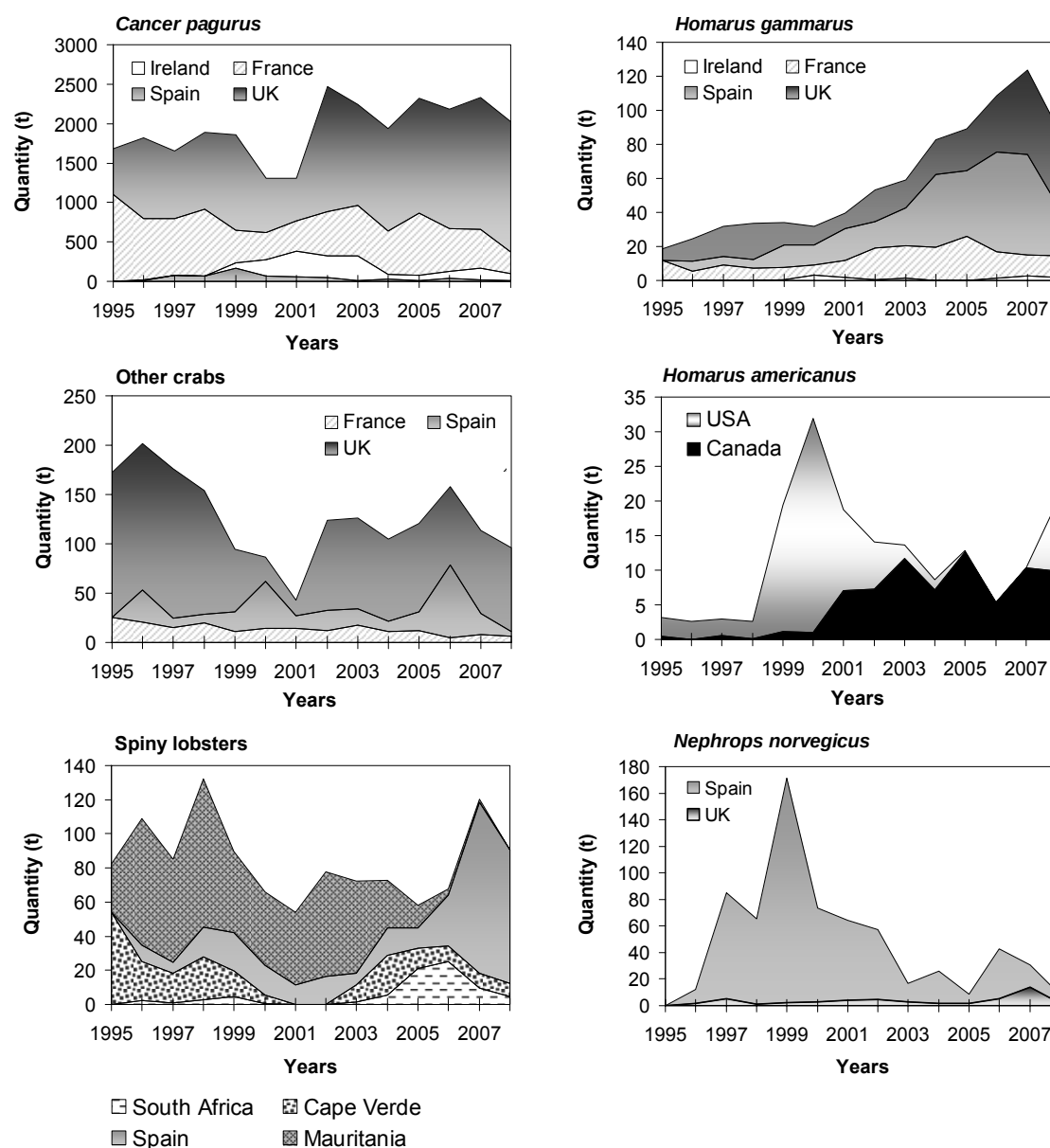


Figure 1.9 Main suppliers of crustaceans to Portugal, importation data in tonnes (t); (data source: EUROSTAT, 2009). *H. gammarus* and *H. americanus* refer exclusively to live imports. All remaining crustaceans are either in shell or not, live, dried, salted or in brine and includes crabs in shell cooked by steaming or by boiling in water.

The detailed classification codes used by Eurostat are presented in annex. Most non-frozen crustaceans that are traded in Portugal are supplied by many different countries according to the market demands.

By far the most important non frozen crustacean in terms of import volume is the European edible crab, *C. pagurus*, with 81 % of total imports in 2007, followed by *H. gammarus* (6 %) which are supplied mainly by the UK, France and Spain. Spiny lobsters, on the other hand, are mainly supplied by Southern countries such as South Africa (*Jasus* spp.), Cape Verde (*Panulirus regius*) and Mauritania (*Palinurus mauritanicus*).

Fluctuation in import quantities follows the market tendency and it can be seen that in 2008 most crustacean imports declined as a consequence of the global economical crises. With exception of *N. norvegicus* there is a marked imbalance between export and import. In 2007 the highest imbalance was obtained for *C. pagurus* (imports: € 7,333,879; exports: € 33,716) and homarids (imports: € 2,771,096; exports: € 34,494).

Comparing both homarid species traded in Portugal, the European lobster is more expensive, has a higher quality image, and is currently serving basically the high-end restaurants, while American lobster is mainly available at medium restaurants and in retailers for direct consumptions. However, considering the price differences and the market trend it can be expected that the American lobster will be more common in the national market in the near future.

1.3.1 *C. pagurus*

C. pagurus provides an important source of income for local fishermen and has been exploited for many centuries (Edwards, 1979). The edible crab is particularly abundant in coastal waters of northwest Europe, particularly off the coasts of Norway, Scotland, England and Brittany, where it lives in rocky, sandy or muddy bottoms at 0 to 300 m depths and temperatures vary between 4 and 16 °C (Chartois *et al.*, 1994; Metzger *et al.*, 2007). World captures have increased from 10,000 t after Second World War to nearly 60,000 t in 2007 (Figure 1.10).

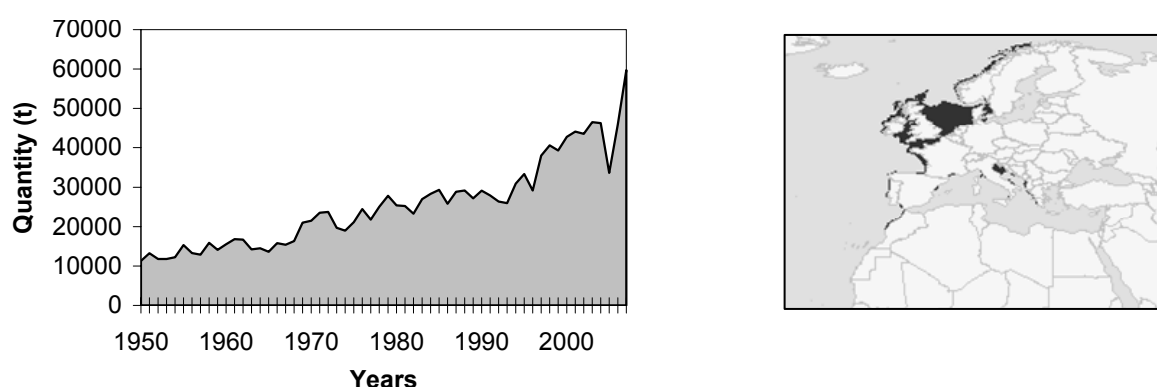


Figure 1.10 Edible crab world captures in tonnes (t) since 1950 to 2007 and its geographical distribution (data and map source: FAO species, 2009).

Crabs are fished with creel and pots by coastal vessels that work on a daily bases and by offshore boats that fish 6 to 10 days (Chartois *et al.*, 1994; Ingle, 1997). After crabs are removed from creels, some are “nicked”, a process consisting in the immobilization of the claws by cutting the tendons of the upper pincer in the dactyls of the claw, in order to avoid injuries to handlers and between crabs. Nicking is undertaken by incision of either the inner or outer tendon of the upper mobile pincer - colloquially referred to as the ‘French’ and ‘English’ nick, respectively. This practice was abandoned in clawed lobsters and presently, claws are immobilized with rubber bandages in order to prevent cannibalism and injuries to handlers (Chartois *et al.*, 1994; Figure 1.11).

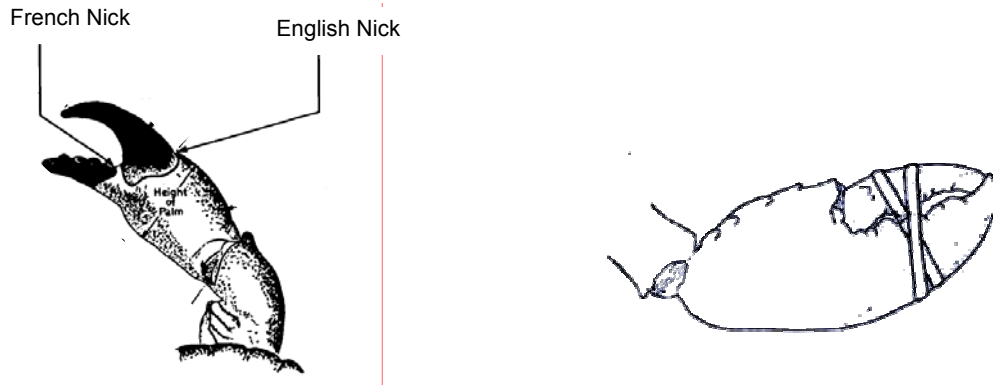


Figure 1.11 From left to right, diagram of crab claw describing location of nicking (adapted from Jackline and Lart, 1995) and representation of the immobilized (banded) claw of a homarid (adapted from Ingle, 1997).

Live *C. pagurus* are caught in pots and hauled aboard the vessel where they are stored in either a “nicked” or “un-nicked” condition. On larger vessels, nicked animals are stored in onboard vivier tanks whereas on smaller vessels animals are often stored dry in containers on deck (Jackline and Lart, 1995).

On landing, un-nicked crabs destined for processing are transported to the factory. Nicked animals destined for live export are placed into keep pots for storage or into vivier transport facilities for shipment to Europe (Jackline and Lart, 1995). Originally, all crabs were sold freshly cooked in the shell but the market for boiled crabs was limited by the lack of refrigeration facilities and the rather poor keeping quality of crab meat (Edwards, 1979). Nowadays, there is an important trade of live crabs, but processed crabs are also commercialized as whole cooked crabs either fresh or frozen. In southern Europe, crabs are especially appreciated because of brown meat, which consists of hepatopancreas and reproductive organs, and is used to dress the carapace in elaborate dishes. The preparation of a whole crab can be time consuming which is not compatible with the fast living of modern days. Therefore, convenience food is a concept also applied to the edible crab; presently white and brown meat are sold in separate as a fresh pasteurized product and minced brown meat is also sold frozen (Holmyard and Franz, 2006).

1.3.2 *H. gammarus*

The European lobster, *H. gammarus*, has a broad geographical distribution, occupying the Eastern Atlantic Ocean from north-western Norway (Lofoten Islands) to the Azores and, the Atlantic coast of Morocco, but is absent in the Baltic Sea probably due to lowered salinity and temperature extremes. It can also be found along the northwest coast of the Black Sea and in the Mediterranean Sea (but lacking in the extreme eastern part, east of Crete). This lobster can be found between 0 and 150 m depth but usually not deeper than 50 m on hard substrates, such as rock or hard mud where they are usually found in holes or crevices with temperatures fluctuating according to season and geographical location from 7 to 19 °C (Chartois *et al.*, 1994). The European lobster is a highly esteemed food source and is fished throughout its range, fetching very high prices (Holthuis, 1991). Within the past 70 years, total annual European landings have varied

between 2,000 and 4,800 tonnes (Figure 1.12). This crustacean is mostly fished with lobster pots, although it occasionally turns up in trammel nets and dredges.

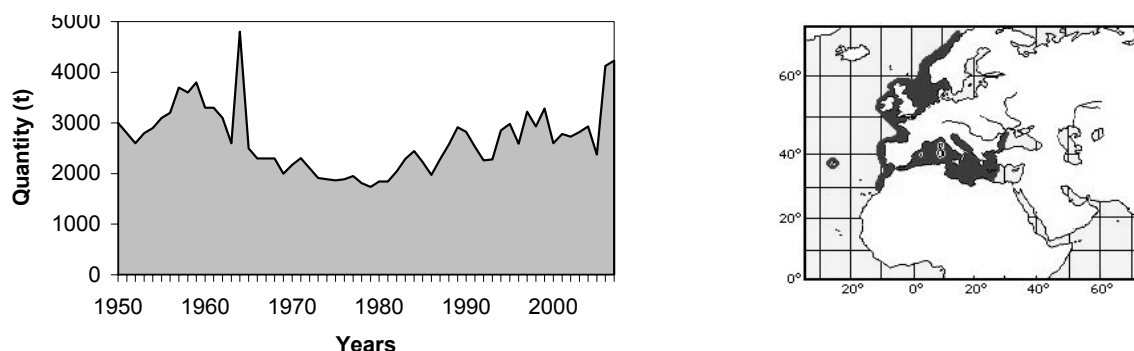


Figure 1.12 European clawed lobster world captures in tonnes (t) since 1950 to 2007 and its geographical distribution (data and map source: FAO species, 2009).

Catches are marketed whole and in some areas captured specimens are kept alive in enclosures. The wholesale price is affected by lobster live condition and therefore particular care is given to proper storage, packaging and transport (Ingle, 1997).

1.3.3 *H. americanus*

H. americanus have a restricted geographical distribution, from south-eastern Labrador to southern New England (but particularly in waters of Maine, southern Nova Scotia, and the southern Gulf of Saint Lawrence (Figure 1.13; Bliss, 1990). Lobsters can be found from sub-littoral to 480 m depth but most commonly between 4 and 50 m in hard bottom substrates (Holthuis, 1991). Temperatures can be as low as -1 °C in February and March till 24 °C in August (Chartois *et al.*, 1994).

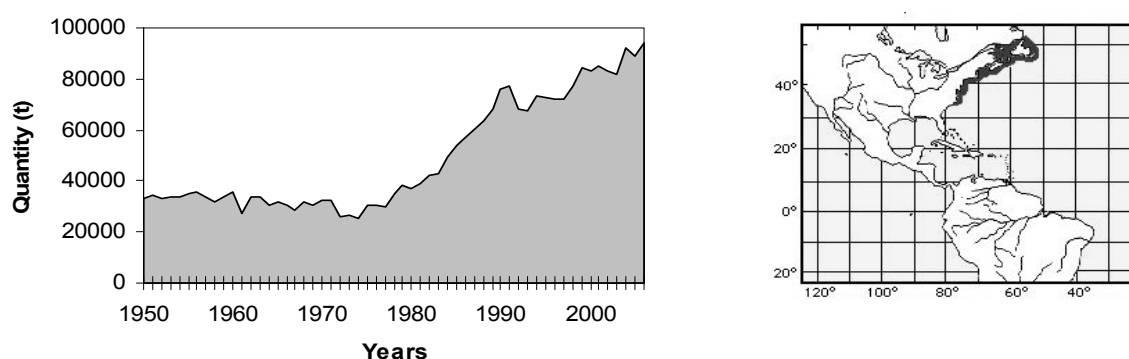


Figure 1.13 American lobster world captures in tonnes (t) since 1950 to 2007 and its geographical distribution (data and map source: FAO, species 2009).

This species is the subject of one of the most important Crustacean fisheries in the northwest Atlantic. According to FAO statistics the catches in 1980 amounted to 36,850 t increasing to a record value of 94,042 t in 2007. Lobster meat is generally hand picked and sold in tamper-proof containers of vacuum packs, and may contain a combination of tail and claw ready for use. Tail is also sold on its own as a higher value product. The meat is can be canned and the hepatopancreas

is processed as a green coloured paste/spread known as tomalley; and lobster roe is also used to produce red caviar (Holmyard and Franz, 2006).

1.3.4 Trade chain of live crustaceans: now and then

The recent market trend based on importation implicates a complex logistics based on transport of long duration including air freight, and holding facilities adapted for stocking of greater quantities and, of species from different geographical locations (Chartois *et al.*, 1994). Nowadays the trade chain of live crustaceans has more steps from capture to holding facilities because most species are no longer from national production (Figure 1.14). When trade chain was based in national production the stocking facilities to hold live crustaceans were mainly located in the intertidal region making use of natural rock pools.

Nowadays, it is more common to pump seawater directly from the ocean or estuary into a tank (flow through open system) usually without refrigeration or filtration systems. These systems are easy to operate. However, there is little or no control over the water quality. Some companies have re-circulating systems (closed systems), where the water is re-used after each pass through the tanks, first being treated to remove waste products (ammonia, nitrite and carbon dioxide) and waste solids before being returned to the tanks (Crear and Allen, 2002).

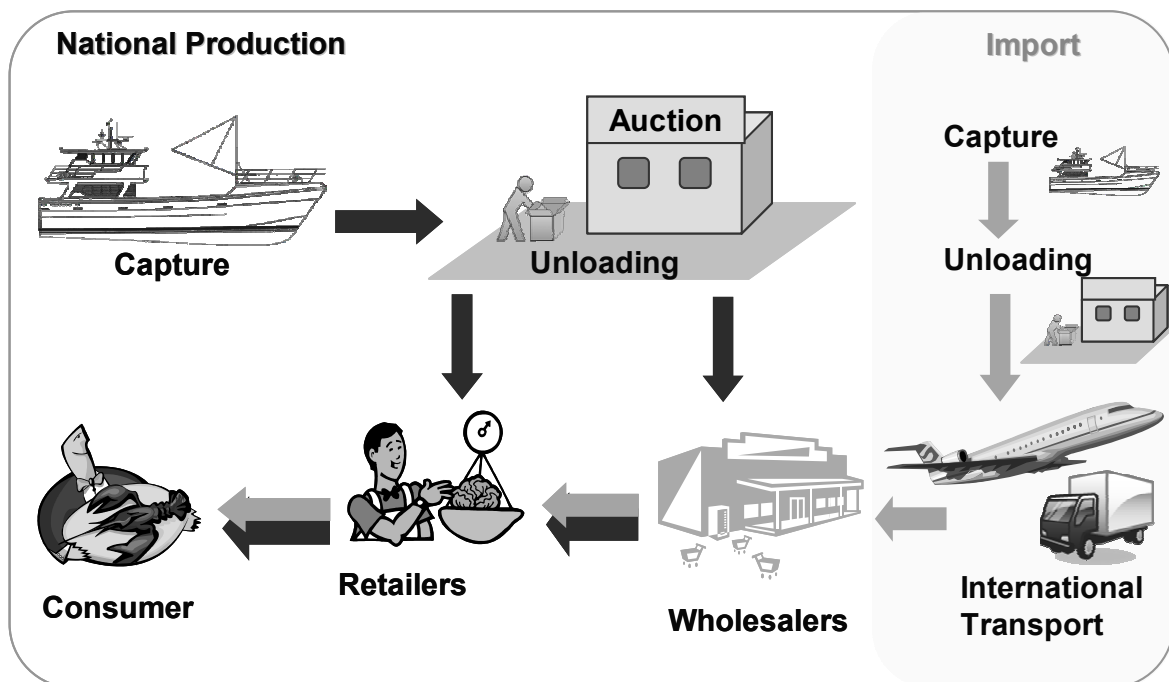


Figure 1.14 Live trade chain of crustaceans from capture to consumer. National production is represented by black arrows, while grey arrows represent external production and imports. Species imported from other continents are usually air freight (e.g. *H. americanus*), while species from European countries are usually transported in vivier trucks (e.g. *C. pagurus*).

Even though initial set up costs may be higher, re-circulating systems have practical application to a range of situations, including where seawater of optimal quality is not guaranteed (e.g. estuaries); where pumping costs from the sea are excessive (inshore holding facilities); where specific control over temperature or other environmental parameters is required or where crustaceans are being held outside their normal geographical range (Crear *et al.*, 2003).

As an alternative, semi-open systems are also used. In this case, water is pumped during high tide directly from the ocean or estuary into a deposit tank where it can be refrigerated, passes through filtration and is re-circulated during short periods, usually of one day.

1.4 Challenges during live trade

Worldwide, transport of live crustaceans is being increasingly used to maximize returns from most commercial fisheries. In Portugal, the tradition to hold some live crustacean species dates back the nineteenth century when national production sufficed demand (Portugal, 1897). Nowadays, we live in the era of globalized seafood, Portugal relies in imports, transport is transcontinental, shipments are no longer restricted to trucks and trains and, air freight is common to respond to market demands of top quality live crustaceans. However, a wild harvested animal, such as *C. pagurus* and homarids, faced years of evolution that adapted them to the specific environment where they live. Unless the holding and transport conditions provided are within the physiological tolerances of the animals, their deterioration and/or death are inevitable (Danford *et al.*, 2001a).

Most crustaceans are placed in an alien environment from the moment they are captured, which is reflected in the high mortality rates reported for a number of commercial important crustaceans (see below). Mortality vary depending on species, handling procedures, type of capture method, transport and stocking conditions and, duration of each of these steps (Otwell and Webb, 1977; Uglow *et al.*, 1986; Chartois *et al.*, 1994; Spanoghe and Bourne, 1997; Bezerra, 1998; Ridgway *et al.*, 2006; Albalat *et al.*, 2009).

Chartois *et al.* (1994), reported that the on board mortality for most live crustaceans commercialized in France was about 2 to 3 % (mainly *C. pagurus*, *M. squinado*, *H. gammarus*, *P. elephas*, *P. mauritanicus* and *P. regius*). But wide variations could occur for instance between fishing vessels which were related to handling procedures and conditions on board. These species are usually transported in refrigerated vivier trucks immersed in aerated seawater, where mortality was reported to be around 3 to 5 % but higher values could be expected in case of oxygen or cooling failure. However, most mortality occurred at stocking facilities as can be seen in figure 1.15 (data from Chartois *et al.*, 1994). According to these figures from catch to holding facilities in France, there is an expected mortality rate of 10 to 23 % for *C. pagurus* and of 8 to 20 % for *H. gammarus*. Much higher mortality values (50 %) have been reported at arrival of *C. pagurus* transported from UK to France, and it could reach 70 % after two days at the importer's premises (Uglow *et al.*, 1986).

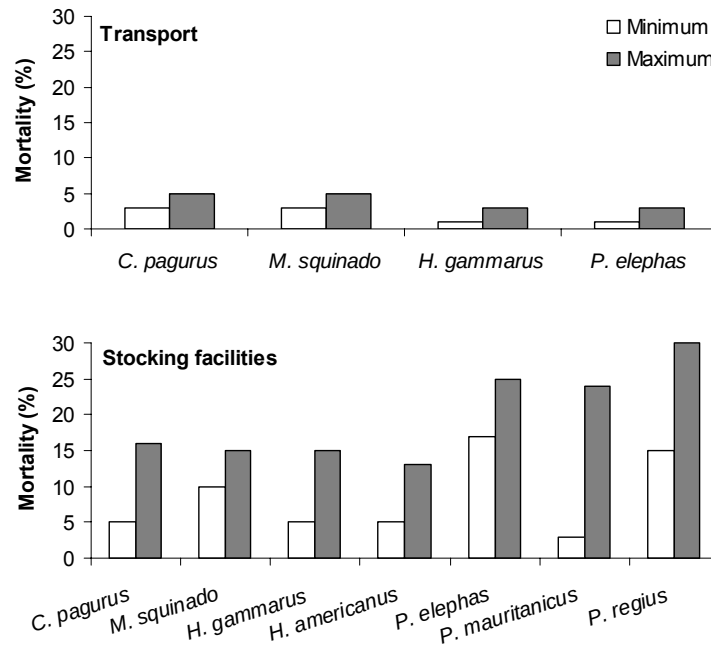


Figure 1.15 Minimum and maximum mortality rates registered during transport in vivier trucks and at stocking facilities for several live traded crustacean species in France (data adapted from Chartois *et al.*, 1994).

Stress associated with capture and handling has been blamed for these losses (Taylor *et al.*, 1997). The word “stress” has become a very ambiguous and oversimplified term that is often used to explain either events (stressor) or individual response to various life challenges. In the context of live transport and holding, potential stressors include capture, poor handling, physical damages (e.g. limb and haemolymph loss), emersion, hypoxia, desiccation, rapid temperature changes and, poor seawater quality in transport and in holding tanks. These challenges promote physiological responses, such as changes in the oxygen uptake, heart rate, pH and concentration of metabolites, hormones and ions (Taylor *et al.*, 1997). Such responses are species-specific and therefore the successful shipment can be better assured by knowledge of the facts pertaining to each species survival when facing stressors during live marketing and distribution. This can be achieved by identifying the type and magnitude of stress encountered for each individual species from capture to delivery.

Recent studies on the effects of commercial distribution procedures on crustaceans focus mainly on physiological changes elicited by emersion (DeFur, 1988; DeFur *et al.*, 1988; Taylor and Whiteley, 1989; Spicer *et al.*, 1990; Whiteley and Taylor, 1992; Morris and Oliver, 1999; Speed *et al.*, 2001; Lorenzon *et al.*, 2007; Bernasconi and Ugolow, 2008). The industry has shown a great interest in the possibility of transporting crustaceans out of water, not only because distribution can be extended to faraway places by flight transport but, it also can be an alternative to the traditional mainland immersed transport in vivier trucks. This interest is primarily motivated by economical reasons, because it costs just as much money to transport water as it does product. The pioneering studies on the air-freighting on lobsters were carried out in Canada by Mcleese (1958) on *H. americanus*. The major concern was the development of suitable packages for flight transport, as they had to be light weighted and leakage proof. On the completion of 30 h shipment (23 kg of

lobster and 5 kg of ice), 95 % of the specimens were in excellent condition (Mcleese, 1958). Several studies followed, mainly concerning lobsters such as *Panulirus argus* (Witham, 1970) and *H. gammarus* (Whiteley and Taylor, 1992), but also the blue crab *Callinectes sapidus* (Otwell and Webb, 1977). To support the industry, several codes of conduct were also especially developed in several countries (Australia: Crear and Allen, 2002; Crear *et al.*, 2003; New Caledonia: Prescott, 1980; UK: MAFF, 1966; Beard and McGregor, 2004; Jackline, 2007; USA: APEC, 1999; Estrella, 2002).

At the present time, challenges during live trade concern the physiological needs of each species of crustaceans that have to face several stressors, and consequently the industrial degree of understanding and awareness of these stressors. The industry cannot afford to have high mortalities in a product that must be alive, healthy and in a good condition in accordance with established requisites. Consequently, there is the need to evaluate the physiological responses to stressors which can be done subjectively (behaviour, vigour, simple postural tests), or expressed quantitatively by measured changes in physiological variables (Taylor *et al.*, 1997). This can give an insight into the types and magnitude of stress encountered from capture to delivery. If the developing fishery is to be successful, ways must be found to mitigate stress factors.

1.5 Evaluation of stress responses

The techniques used to evaluate stress responses in crustaceans are either subjective or objective. This issue is very important in several industries that need to grade animals for live export. The industry is interested to trial animals that are suitable for live transport from those that are already too weak to survive such journey. Some parameters are already used by the industry to grade mainly lobsters. In the rock lobster industry, for instance, the health assessment of animals is usually carried out on the basis of visual subjective estimates which evaluate the behavioural responses of animals to physical stimulation (Spanoghe, 1996). As a result, lobsters are graded as healthy or weak, in other words, retained or rejected for live export (Spanoghe, 1996). Such subjective tests have the advantage of being non-invasive, inexpensive and quick (Taylor *et al.*, 1997). In research, behaviour responses have also been used to evaluate pain and thermal tolerance (Gardner, 1997; Cuculesco *et al.*, 1998; Hopkin *et al.*, 2006).

Objective techniques such as measurements of oxygen uptake, heart rate, muscle glycogen, and haemolymph pH, haemocyanine, L-lactate, D-glucose, ammonia, urate, Ca^{2+} , Mn^{2+} , Cl^- , O_2 , CO_2 and of the crustacean hyperglycemic hormone (CHH), have the advantage of being quantitative variables (Taylor *et al.*, 1997). On the other hand, most of these measurements are invasive, time consuming, expensive and require a skilled operator. Therefore, objective techniques are mainly used in research to address several issues, such as environmental monitoring (Bamber and Depledge, 1997), thermal tolerance (Taylor and Whitley, 1979; Metzger *et al.*, 2007), emersion and hypoxia stress (Zou *et al.*, 1996; Morris and Oliver, 1999), and handling stress (Mercier *et al.*, 2006), just to name a few. Subjective and objective techniques have also been used simultaneously (Haupt *et al.*, 2006). Most recently, an objective but non invasive vitality sensor was

proposed by Bolton *et al.* (2007). The developed hand-held instrument consists of an optical sensor easy to operate that measures total haemolymph protein. The total protein in the haemolymph is believed to be related to health and long-term viability of lobsters (Bolton *et al.*, 2007).

Though there is a wide range of physiological parameters recognized as good indicators of physiological status, most concern energy and protein, including energy substrate utilization, metabolic and enzymatic activity, respiratory physiology, acid-base disturbance, osmotic and ionic regulation, and endocrine regulation (Spanoghe, 1996). However, with such a vast array of indicators it is rarely possible to study them all, and some are inter-related. In this study, glucose, lactate and haemocyanine concentration, as well as pH values were selected as objective techniques. Besides being practical and simple indicators they are the most often used in literature and can be easily related to anaerobic metabolism, which is one of the major physiological responses to oxygen deprivation that affects live crustaceans along the trade chain.

1.5.1 Aerobic versus anaerobic metabolism

Living organisms require energy to maintain metabolic processes which is obtained from the organic molecules that have chemical energy locked in their structure. This energy can be transferred to adenosine triphosphate (ATP) by the oxidation of foodstuff molecules to CO_2 and H_2O via the citric acid cycle (in the presence of O_2 , aerobic metabolism) or the Embden-Meyerhof pathway (in the absence of O_2 , anaerobic metabolism). Proteins, fats and carbohydrates can all provide energy for cells through ATP synthesis. From ATP, the energy can be transferred to operate energy-requiring cell functions e.g. muscle contractions, the active transport of molecules across membranes, synthesis of chemical compounds, etc.

Both anaerobic and aerobic pathways start with the same first step at the cytoplasm of cells: the process of glycolysis which is the breakdown of glucose (6 carbons) into two molecules of pyruvate acid. This molecule can either be oxidized to acetyl CoA to be catalysed in the citric acid cycle or be reduced to lactate (Mackenna and Callander, 1997). In crustacean tissues like in vertebrates, when oxygen is in short supply, the re-oxidation of NADH formed during glycolysis is impaired and it is under these circumstances that the reduction of pyruvate to lactate is coupled with the reoxidation of NADH. This allows further glycolysis to proceed. However, anaerobic glycolysis only produces 2 ATP molecules against 38 molecules yielded by the oxidative phosphorylation, thus the most efficient pathway to produce energy in the form of ATP is in the presence of oxygen. Consequently, to maintain the energy efficient aerobic metabolism, oxygen must be transported from the aquatic environment to tissue cells and, simultaneously, the produced CO_2 in the cells must be eliminated to the environment. Yet, dissolved oxygen is about 30 times higher in air than in an equivalent volume of water at the same oxygen pressure and, temperature (Randall *et al.*, 2002). Therefore, aquatic organisms have developed highly efficient organs to extract oxygen from water. In decapods, gas exchange is almost exclusively achieved through gills, which provide a considerable surface area (60 to 80 times that of the rest of the body) and whose epithelium shows great permeability to dissolved gases. Both oxygen and carbon dioxide diffuse across the gills to and

from the haemolymph. Oxygen is transported in the haemolymph to tissues either in solution or combined with the respiratory pigment haemocyanin (Hcy), which is able to reversibly bind oxygen (Ruppert and Barnes, 1994).

1.5.2 Glucose

The first step to obtain energy in the form of ATP starts with the glucose molecule. The glucose present in crustacean haemolymph comes from two main sources: from the direct absorption of dietary glucose through hepatopancreatic and intestinal epithelial cells, or from hepatopancreas, where it is stored as glycogen or synthesized by the gluconeogenic pathway (Randall *et al.*, 2002). Glucose levels in the haemolymph of crustaceans are controlled, particularly by CHH, a neuropeptide produced by the sinus gland of eyestalks (Verri *et al.*, 2001). If glucose levels drop, neurons of the sinus gland release CHH and this induces the hydrolysis of glycogen. On the contrary, if glucose concentration in haemolymph increases, CHH release is inhibited, reversing the process of glucose production in hepatopancreas and muscle (Verri *et al.*, 2001). Glucose levels in haemolymph are rigorously controlled and are much lower than in mammals' blood, with values of 0.9702 mM in *Procambarus clarkii*, 0.03 - 0.19 mM in *Orconectes limosus*; 0.1 - 0.3 mM in *C. maenas*, 0.8 mM in *C. pagurus*, 0.05 - 0.49 mM in *C. borealis*; 0.77 - 1.39 mM in *Penaeus monodon*, and 1.1 - 1.4 mM in *H. americanus* (Verri *et al.*, 2001).

Glucose concentration may depend on a number of factors, such as nutritional state, moult stage (Chang, 1995), time of day (Kallen *et al.*, 1990), handling, capture method and emersion (Uglow *et al.*, 1986; Bergmann *et al.*, 2001). Moulting in crustaceans involves a series of stages with different feeding requirements and, therefore, energy from available food. During inter-moult, crustaceans feed actively; while prior to moulting, feeding declines until it stops completely during moulting. Finally, feeding begins again in post-moult (Sánchez-Paz *et al.*, 2006). This feeding pattern is consistent with reports stating that haemolymph glucose increase markedly during pre-moult compared to post-moult (Chang, 1995). Also, in some decapod crustaceans, glucose reveals day/night rhythmicity, characterized by a low basal level during the day, and a peak in glucose content appearing at night (Kallen *et al.*, 1990). In addition, hyperglycaemia has been reported in decapod crustaceans as a result of endocrine-mediated mobilization of glucose from stored reserves as a response to handling, capture method, hypoxia and anoxia (Taylor and Spicer, 1987; Spicer *et al.*, 1990; Danford *et al.*, 2001; Lund *et al.*, 2009). *Palaemon elegans* and *P. serratus* under conditions of moderate (30 Torr) and severe (10 Torr) hypoxia exhibited an increase by a factor of two in the haemolymph glucose concentration during the first six hours. On return to normoxia, the concentration of glucose returned to resting levels within the first six hours. (Taylor and Spicer, 1997). Also, *N. norvegicus* experienced a marked hyperglycaemia when emersed on ice, glucose reaching a maximum level of 1.38 ± 0.21 mM after 24 h, whereas at 10 °C, emersion elicited a glucose maximum of 2.04 ± 0.15 mM after only 12 h (Spicer *et al.*, 1990). Glucose concentration of trawled-caught *Munida rugosa* (0.097 mM) and *Liocarcinus depurator* (0.140 mM) was higher comparatively to creel-caught, 0.059 and 0.079 mM, respectively (Bergmann *et al.*, 2001). Given these considerations, glucose concentration in the haemolymph is a good tool to

determine stress in crustaceans; yet, this parameter should be analyzed with caution as several factors can influence it.

1.5.3 Lactate

Numerous studies demonstrated that lactate is the main end product of anaerobic metabolism in crustaceans (Zebe, 1982). This metabolite is usually found in muscle tissues after short bursts of intense muscular activity (Henry *et al.*, 1994), such as tail flips used by decapods to escape (Haupt *et al.*, 2006), or in animals held in hypoxic environmental conditions or exposed to air (Spicer *et al.*, 1990; Speed *et al.*, 2001). Therefore, factors that promote sudden activity in crustaceans such as handling, high animal density and interaction between individuals, increased temperature and other stressors might trigger the use of anaerobic metabolism to support the extra activity levels and metabolic rates above basal levels. As a result, there is an accumulation of lactate in the tissues (e.g. hepatopancreas, heart and muscle; Childress and Seibel, 1998), which can then be released to the haemolymph (Chen and Chen, 1998; Schmitt and Uglow, 1997; Frederich and Pörtner, 2000). The shift to anaerobic metabolism and the degree of lactate accumulation is species specific.

Watt *et al.* (1999) measured *in situ* the basal lactate levels in three species of crabs with different rates of activity and found that *N. puber*, an active, fast moving crab had the lowest lactate concentration (1.1 mM), followed by *C. pagurus* (1.6 mM) and by *M. squinado* (2.5 mM) that is the less active crab. The authors concluded that less active crabs rely to a greater extent on anaerobic respiration comparatively to more active species. These authors also observed that *N. puber* had a higher oxygen carrying capacity than *M. squinado*, which might indicate that active species are more capable of transporting enough oxygen to meet their aerobic metabolic requirements, thus having lower basal level of anaerobic metabolism and, accumulating less lactic acid. Accordingly, when exposed to specific stressors species will respond in different ways and the same stressor might be more detrimental to one species than to another. For instance, emersion, temperature, and salinity changes occur more or less daily for intertidal animals, but are rarely experienced by sub-tidal animals. When exposed to air, crustaceans face the problem of impaired gill function. As a response to low oxygen (stressors), many decapod crustacean species switch to anaerobic metabolism (DeFur, 1988). However, anaerobic metabolism is an inefficient pathway to produce energy in the form of ATP. In this sense, species that are able to delay anaerobic metabolism are more tolerant to low oxygen environments. The shore crab, *C. maenas*, for instance, can survive 24 hours out of water without recourse to anaerobic metabolism whereas the sub-tidal *N. puber* crab rapidly switches to anaerobiosis upon emersion (Johnson and Uglow, 1985). Previous studies on the effect of aerial exposure in other decapods indicated considerable inter-specific variation in the rate of L-lactate accumulation in relation to habitat (Table 1.1).

When lactate is accumulated and transferred to the haemolymph, two major physiological effects are usually recorded in crustaceans: a) an increase in H^+ concentration, with the subsequent

acidosis; b) and an allosteric effect on the haemocyanin molecule, leading to an increase in the affinity of the pigment for oxygen (Gäde, *et al.*, 1986).

Table 1.1 Accumulation of L-lactate in haemolymph of several decapod crustaceans during emersion at different temperatures (T).

Species	Δ L-lactate	Emersion duration	T (°C)	Habitat	Reference
<i>H. gammarus</i>	4.7	48 h	6	subtidal	Lorenzon <i>et al.</i> (2007)
<i>H. gammarus</i>	14.1	48 h	15	subtidal	
<i>C. pagurus</i>	10.5	36 h	10-13	subtidal	Lorenzon <i>et al.</i> (2008)
<i>C. pagurus</i>	12.3	3 h	12	subtidal	Webster (1996)
<i>N. norvegicus</i>	6.0	3 h	10	subtidal	Spicer <i>et al.</i> (1990)
<i>C. maenas</i>	0.7	3 h	10	intertidal	Taylor and Butler (1978)
<i>C. sapidus</i>	1.5	4 h	20	intertidal	DeFur <i>et al.</i> (1988)

Δ L-lactate: concentration of L-lactate accumulated in the haemolymph over a period ranging from 3 h to 48 h. Values are in mM.

The lactate itself is not usually excreted (Gäde, *et al.*, 1986). Instead, when sufficient oxygen is available, this waste is converted back into other metabolic intermediates and either oxidized further to carbon dioxide or used to re-synthesize storage sugars such as glycogen (Gäde, *et al.*, 1986). Therefore, the rate of disappearance of lactate from the haemolymph and tissues in crustaceans is likely to depend on to which extent the ATP reserves have been depleted, the importance of glycogen store and the rate of oxygen supply (Spanoghe, 1996).

1.5.4 pH

Efficient metabolic processes require the maintenance of haemolymph and tissues physiological ranges within certain limits and in this instance; pH is a major factor for homeostasis maintenance. Several authors have reported low pH in crustacean haemolymph as a result of respiratory and metabolic acidosis due to increase of lactate and bicarbonate-carbonic acid during anaerobic metabolism (Spanoghe, 1996). Crustaceans have compensation mechanisms to act in response to acidosis by mobilizing buffer bases. One mechanism is the mobilization of divalent carbonate ions by a reversal of the mechanism for calcification of the exoskeleton (Cameron and Wood, 1985). Therefore, acidosis compensation in crustaceans is supported by an increase in HCO_3^- and Ca^{2+} concentrations in the haemolymph. However, there is a wide variation in the capacity of different crustacean species for acid-base regulation during air-exposure (Harry and Waldron, 1997).

Intertidal crabs like *Cyclograpsus lavauxi* exhibit excellent compensation by elevation of haemolymph buffers that prevent even short term perturbation of haemolymph pH (Innes and Taylor, 1986). Slightly less effective regulation is shown by *C. maenas* in which an initial haemolymph acidosis is fully corrected only within a few hours (Truchot, 1975). A rather lower compensatory ability is shown by the subtidal *H. gammarus*, in which pH falls during emersion but

reaches a plateau that maintains for many hours (Taylor and Whiteley, 1989). Moreover, Lorenzon *et al.* (2007) reported an increased Ca^{2+} during emersion, which might indicate the mobilization of divalent carbon ions to compensate the low pH. On the other hand, the lobster *J. edwardsii* was able to deal with emersion rather less efficiently than *H. gammarus*, as under similar conditions the latter maintained a pH plateau for at least 14 h and increased bicarbonate by 7.2 mM, while the former could only maintain pH relatively constant during the first 4 h followed by a sudden drop in pH with no further buffer compensation (Harry and Waldron, 1997). Other sub-tidal decapods showed almost no ability to regulate haemolymph pH on emersion, such as the lobster *P. argus* (Vermeer, 1987). When no further metabolic compensation occurs, the pH will drop below the physiological range and, as a result the haemocyanin oxygen affinity, rates of enzymatic reactions, ionic and osmoregulatory controls and cell membrane structure will be affected (Whiteley and Taylor, 1990). Poor health, morbidity, and mortality have been reported to be correlated to low pH values in crustaceans (Whiteley and Taylor, 1990; Ridgway *et al.*, 2006). Therefore, pH haemolymph is a good tool to determine stress in decapod crustaceans.

1.5.5 Haemocyanine role in the exchange of gases

Haemocyanin is a copper containing respiratory pigment responsible for the increased solubility of oxygen by a factor of 2 to 4 and accounts for 80 to 95 % of the total protein concentration in the haemolymph (Watt *et al.*, 1999). The relative amount of dissolved and combined O_2 (HcyO_2) found in haemolymph can vary with several factors, one of them being the pigment affinity with oxygen and its concentration (Pelster and Decker, 2004). Changes in the affinity of haemocyanin with oxygen occur and are described as two major shifts in the oxygen dissociation curves: a) the Bohr effect, which describes a decrease in pigment oxygen affinity with decreasing pH, corresponding to the shift of an oxygen dissociation curve towards the delivery of more oxygen to the tissues where pH is lower; and b) the Root effect, which describes the induced decrease in the oxygen-carrying capacity at low pH even at high PO_2 ; thus part of the pigment remains deoxygenated even at high PO_2 . In this sense, the Root effect has often been discussed as an exaggerated Bohr effect (Pelster and Decker, 2004). As a result of the Root effect, a reduction of the total amount of oxygen delivered to the tissues can occur, because full saturation at the gills is impaired when the haemolymph is too acidic. In this respect, it has been described that in fish severely stressed by handling, mortality can occur as a result of uncompensated acidosis, even when normoxic conditions were maintained or during recovery after exposure (Forthhead, 1993).

Haemocyanin, besides being a blue respiratory pigment functionally equivalent to haemoglobin, can also function as a storage protein and has antimicrobial properties (Adachi *et al.*, 2003; Decker and Jaenicke, 2004). Its concentration has been reported to depend on the nutritional state of the animal, hypoxia exposure, season and temperature (Baden *et al.*, 1990; Brower, 1990; Baden *et al.*, 1994; Powell and Watts, 2006). Haemocyanin concentration usually falls as nutritional state declines but it increases in hypoxic sites (Baden *et al.*, 1990; Baden *et al.*, 1994). However, when both hypoxia and starvation were tested in *N. norvegicus*, lobsters were in such a poor nutritional state that physiological changes normally associated with starvation were superimposed on

responses to hypoxia alone (Baden *et al.*, 1990). This suggests that, by starving crustaceans, one might reduce indirectly their ability to cope with situations where haemocyanin is required to maintain optimal oxygen supply to the tissues. In particular, this issue may become more important, when extended periods of fasting are one of the treatments to prepare crustaceans for live dry shipments.

1.6 The use of anaesthetics in crustaceans to minimize stress

The use of anaesthetics is fundamentally restricted to aquaculture procedures. Fish aquaculture production is the top group whether by quantity (47.4 %) or by value (53.9 %), respectively, whereas crustaceans are fourth by quantity (6.2 %) but second by value (20.4 %). Therefore, much less is known about anesthetizing crustaceans in comparison to fish mainly because it is not done as often. Among crustaceans, even less is known about anesthetizing crabs, considering that aquaculture is largely dominated by the penaeid shrimps, representing 86 % of all farmed marine crustaceans (FAO, 2006).

In fish aquaculture, anaesthetics are used to minimize stress, injury and mortality caused while handling animals during culture and management procedures including population estimates, age and growth studies, spawning, vaccination, grading and transport (Meinertz *et al.*, 2006). In research, the use of anaesthetics facilitates work with marine animals and is required for invasive studies such as surgical preparations for physiological investigations, where the animal must be held immobile for extended periods of time. Overdose of anaesthetics is also used routinely as an effective and humane means of euthanizing fish (Ackerman *et al.*, 2005).

Most operations in crustacean culture can be conducted without anaesthesia, the major critical points occur during holding and transport due to cannibalistic nature of some species. Consequently, there has been some interest in investigating crustacean anaesthetics, particularly for transport not only of cultured species but also of wild crustaceans that are more valued when traded live.

Anaesthesia is generally defined as a state caused by an applied external agent resulting in a loss of sensation through depression of the nervous system (Ackerman *et al.*, 2005). An ideal anaesthetic should fulfil several criteria: a) rapidly induce anaesthesia with minimum stress; b) be easy to administer; c) be biodegradable; d) have properties that allow the body to eliminate the anaesthetic from tissues following exposure; e) be effective at low doses; f) the toxic dose should greatly exceed the effective dose so that there is a wide margin of safety (Coyle *et al.*, 2004); g) once the animal stops contacting with the anaesthetic the recovery should be rapid; h) not having toxic side effects for the fish and the handler; and i) have no persisting physiological, immunological or behavioural effects which could reduce the likelihood of survival of the animal or interfere with later measurements (Ackerman *et al.*, 2005).

1.6.1 Anaesthetics legal aspects

Many chemical anaesthetics have been used on fish over the years. Most have now been discarded or are not widely used. The USA Food and Drug Administration (FDA) regulates which chemicals can be used on food fish. When fish are exposed to an anaesthetic, residues or metabolites of the substance remain in the flesh for a period of time until they are excreted or metabolized (Ackerman *et al.*, 2005). Therefore, FDA may require a specific withdrawal time before the animal can be used as food or released into the environment where it might be captured for food. Anaesthetics are licensed for use in food animals only after completing a full drug development program designed to protect the cultured animals, human users, the food chain and the environment (Ackerman *et al.*, 2005). The only anaesthetic drug currently approved by the FDA for use on food fish is tricaine methanesulfonate (MS-222). However, many compounds have been evaluated experimentally and some are being used on non-food fish and in research (Ackerman *et al.*, 2005). In the European Union, there are no anaesthetics currently approved for use on fish destined for human consumption. In case the product is not destined for human consumption, an overdose of anaesthetic could be considered a suitable way to humanely kill moribund or diseased seabream and seabass. At the moment only MS-222 is licensed for this purpose in EU (EFSA, 2008).

1.6.2 Crustaceans response to anaesthesia

Crustaceans respond differently to anaesthesia than fish, possibly because their synaptic receptor sites are not affected by certain anaesthetics. For example, MS-222 is not effective on many crustaceans (Coyle *et al.*, 2004). Several agents have also been shown to be ineffective or detrimental to various crustacean species, including carbon dioxide, 2-phenoxy ethanol and magnesium chloride (Noga *et al.*, 2006). It seems that much higher concentrations are required to anaesthetize crustaceans than fish. Aqui-S[®] has been reported to be effective on freshwater prawns (*Macrobrachium rosenbergii*), but only at concentrations 5 to 10 times higher (100 to 200 mg L⁻¹) than those used on fish (20 mg L⁻¹) (Coyle *et al.*, 2005). Aqui-S[®] is a relatively new anaesthetic for fish developed by the Seafood Research Laboratory in New Zealand. This compound is approximately 50 % isoeugenol and 50 % polysorbate 80. Aqui-S[®] has also been proven to be effective for short duration transport (< 2 h), such as local smolts to sea, or longer transports (> 40 h), such as live lobster, eel or cod to distant international markets (AQUI-S[®] NZ, 2009). Currently, Aqui-S[®] is approved for use on food fish in New Zealand, Australia, Chile, Korea, Costa Rica and Honduras. In the USA it is undergoing the New Animal Drug Approval process for use with no withdrawal time. This anaesthetic is primarily used for the “rested harvest” of commercial fish species, where low stress induction improves colour, texture and appearance of the product (Coyle *et al.*, 2004).

Cooling is also an effective way to immobilize crustaceans, but one must be careful since excessive cooling can also kill the animals (Coyle *et al.*, 2004).

1.7 Seafood: benefits and risks to human consumption

The recent investments and technological developments towards a globalised trade chain of live crustaceans are governed by the need to supply prime quality crustaceans to exigent consumers. Nowadays, seafood cannot be dissociated from its nutritional quality and, in this regard, the general perception is that seafood is healthy.

The awareness of seafood importance started in the seventies when Danish investigators reported that Greenland Eskimos had a low mortality rate from coronary heart disease (CHD) despite a high intake of fat (about 40 % of their total caloric intake) from their diet (Middaugh, 1990). This so-called 'Eskimos paradox' led to a series of epidemiological studies in the late 1970s by Bang and Dyerberg, which suggested a close correlation between the observed low incidence of CHD amongst Eskimos population and their high consumption of fish and fish-eating mammals (including seals and whales), resulting in a diet rich in long chain n-3 PUFA. (Bang *et al.*, 1971; Dyerberg *et al.*, 1975; Dyerberg *et al.*, 1978).

Other papers followed and attention was drawn to the rapid fall in ischemic heart disease incidence and mortality in Norway that occurred in parallel with dietary changes (*i.e.*, more fish, less meat) during German invasion in 1940 (Bang and Dyerberg, 1985). Studies of Japanese populations with different intakes of fish have produced broadly similar data, showing an inverse relationship between n-3 PUFA from fish intake and CHD death rates, thus suggesting a potential role of marine n-3 PUFA in the prevention of CHD (Hirai *et al.*, 1989; Sugano and Hirahara, 2000). Since then a considerable amount of epidemiological studies have repeatedly linked fish consumption and n-3 PUFA with prevention of cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, depression and improvement of several symptoms of cancer, asthma, autoimmune disorders and Crohn's disease (Kris-Etherthon *et al.*, 2002; Lee and Lip, 2003; Sidhu, 2003; Tribble, 2007). Seafood nutritional quality is also related to its high-quality protein, essential elements, antioxidants and vitamins. The intake of these nutrients has decreased in industrialized societies, and therefore, seafood is recommended in a healthy diet, for instance the American Heart Association recommends eating fish (particularly fatty fish) at least twice a week (Kris-Etherthon *et al.*, 2002; He *et al.*, 2004).

However, several uncertainties still remain. For example, the optimal intake of n-3 fatty acids is not firmly established nor is their mechanism of action fully understood. Moreover, some studies have produced conflicting results, whereas certain concerns have aroused regarding the levels of chemical contaminants in fish (Domingo *et al.*, 2007). To further complicate the equation of benefits *versus* risks of seafood consumption it should also be noted that chemical composition of wild marine species varies according to season, geographical origin, size, sex and state of reproductive cycle (Oehlenschläger, 1997).

1.7.1 Lipids

Dietary lipids consists primarily of triacylglycerol (98 %), which is composed of one glycerol molecule esterified with three fatty acid molecules, followed by smaller amounts of phospholipids

and sterols. Fish, crustaceans and molluscs contain approximately 0.7 % of phospholipids in the muscle tissue (deMan, 1999).

Fatty acids are hydrocarbon chains that contain a methyl (CH_3 -) and a carboxyl ($-\text{COOH}$) end. The fatty acids vary in carbon chain length and degree of unsaturation (number of double bonds in the carbon chain; IOM, 2005). The fatty acids can be classified into the following categories: a) saturated; b) monounsaturated; c) and polyunsaturated fatty acids.

Saturated fatty acids (SFA) are so called because their carbon atoms are a 100 % saturated with hydrogen atoms (Tribble, 2007). The majority of dietary SFA come from animal products such as meat and dairy products, while the remaining comes from plant sources (USDA, 1996). Seafood is not the major contributor of SFA, and the crustaceans muscle has low amounts of SFA (about 20 % of total fatty acids). In crustaceans, palmitic acid 16:0 is the principal SFA, usually followed by stearic acid 18:0 and to a less extent myristic acid 14:0 (Floreto *et al.*, 2000; Sirot *et al.*, 2008; Latyshev *et al.*, 2009; Limbourn and Nichols, 2009).

Unsaturated fats may be monounsaturated or polyunsaturated. Monounsaturated fatty acids (MUFA) are characterized by having one pair of carbon atoms that are not saturated with hydrogen atoms (hence the term *mono*) and therefore have one double bond in the carbon chain. Olive oil and canola oil are high in MUFA (IMO, 2005). In crustacean's muscle MUFA represents about 30 % of total fatty acids, and oleic 18:1n-9 and palmitoleic 16:1n-7 acids are usually the most abundant MUFA (Floreto *et al.*, 2000; Sirot *et al.*, 2008; Latyshev *et al.*, 2009; Limbourn and Nichols, 2009).

Polyunsaturated fatty acids (PUFA) have at least two pairs of carbons that are not saturated with hydrogen and therefore have at least two double bonds in the carbon chain (Tribble, 2007). Unsaturated fatty acids are classified by the position of the first double bond from the methyl end (ω) of the fatty acid carbon chain (Figure 1.16). Omega 3 or (n-3) fatty acids have a double bond between the 3rd and 4th carbon from the methyl end, whereas omega-6 or (n-6) fatty acids have a double bond between the 6th and 7th carbon from the methyl end.

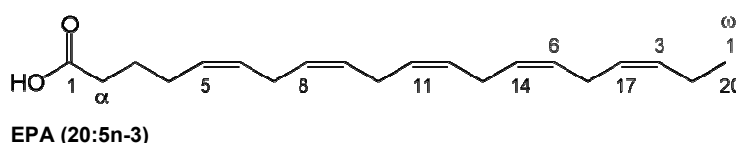


Figure 1.16 General representation of an unsaturated fatty acid in this case, EPA, showing the carbon chain, the methyl end (ω) and the carboxyl end (source: Lipidomics Gateway, 2009).

A PUFA with at least 20 carbon atoms is a long chain fatty acid (e.g. eicosapentaenoic acid - EPA, 20:5n-3; docosahexaenoic acid - DHA, 22:6n-3; arachidonic acid - AA, 20:4n-6). The long chain PUFA are found primarily in seafood (Tribble, 2007), and crustaceans muscle is no exception with PUFA accounting with 40 % of total fatty acids (Floreto *et al.*, 2000; Sirot *et al.*, 2008; Latyshev *et al.*, 2009; Limbourn and Nichols, 2009). The proportion of PUFA to SFA is usually above one and, vary with species and tissues (Table 1.2).

Table 1.2 Ratios of n-3 to n-6 and of PUFA to SFA in the muscle and hepatopancreas (hepat.) of several marine species.

Species	n - 3/n - 6		PUFA/SFA		Source
	Muscle	Hepat.	Muscle	Hepat.	
<i>Tetrasehnis suecica</i> (microalgae)	3.08*		0.70*	-	Albentosa <i>et al.</i> (1996)
<i>C. sapidus</i> (blue crab)	2.32	1.57	1.32	0.36	Çelik <i>et al.</i> (2004)
<i>H. americanus</i> (american lobster)	10.28	6.07	2.04	1.60	Floreto <i>et al.</i> (2000)
<i>Perna canaliculus</i> (New Zealand green-lipped mussels)	9.46	-	1.44	-	Taylor and Savage (2006)
<i>Sepia officinalis</i> (cuttlefish)	7.67 (S)	-	1.88 (S)	-	Özogul <i>et al.</i> (2008)
	7.82 (A)	-	1.75 (A)	-	
	9.11 (W)	-	1.74 (W)	-	
<i>Salmo trutta macrostigma</i> (salmon)	2.89 (♂)	2.59 (♂)	1.23 (♂)	1.50 (♂)	Akpınar <i>et al.</i> (2009)
	2.26 (♀)	1.97 (♀)	1.12 (♀)	1.16 (♀)	
<i>Scomber scombrus</i> (mackerel)	10.64	-	1.85	-	Özogul <i>et al.</i> (2007)

Abbreviations: (S) summer; (A) autumn; and (W) winter. Symbols: females (♀) and males (♂). The microalgae *T. suecica* were considered as a whole (*).

Synthesis of DHA occurs mainly in phytoplankton but not in plants. This means that DHA is absent from foods of plant origin, including vegetable fats and oils, grains, nuts and seeds. However, DHA is present in animal tissue lipids, with the richest dietary source being fatty fish such as mackerel and salmon (Innis, 2003). EPA can also be found in marine animals but it is not usually synthesized by them, being mainly obtained from the diet. The content of n-3 PUFA may considerably vary in relation to species, population, location, season and tissue (Schmidt *et al.*, 2000), as can be seen in table 1.2. Besides fatty fish, crustaceans are also good sources of EPA and DHA, having in general more n-3 than n-6 fatty acids.

1.7.2 Cholesterol

Cholesterol is a sterol that is present in all animal tissues but not in plants. Tissue cholesterol occurs primarily as free (unesterified) cholesterol, but is also bound covalently to fatty acids and to certain proteins. Animal fats contain cholesterol and, in some cases, minor amounts of other sterols such as lanosterol. Plant fats and oils contain several phytosterols (deMan, 1999). In seafood, cholesterol generally accounts 90 to 95 % of total sterols; yet, cholesterol content in some species like the shrimp *Squilla mantis* from the Mediterranean Sea, can have a much lower fraction of the total sterol content (55.7 %) (Serrazanetti *et al.*, 1990). Kritchevsky *et al.* (1967) reported that crabs contain 57 % cholesterol ($C_{27}H_{46}O$) and 37 % brassicasterol ($C_{28}H_{46}O$), which has a similar chemical structure

Shellfish are generally regarded as having a high cholesterol content. Mathew *et al.* (1999) analyzed seven species of Penaid prawns, three species of Portunid crabs and two species of

Scyllaridae crabs and concluded that cholesterol content in muscle was generally lower in crabs compared to prawns (Table 1.3). Oehlenschläger (2006) also reported that edible crab has lower cholesterol than Norway lobster and cold water shrimp in the muscle tissue. Regarding the cholesterol content of hepatopancreas and roe, Iwasaki and Harada (1984) analyzed several marine species of fish, molluscs and crustaceans and concluded that the cholesterol content of roe lies in the range 250-650 mg 100 g⁻¹ and is usually lower than that of liver in fish or hepatopancreas in crustaceans.

Table 1.3 Cholesterol content in the muscle of different marine species of crustaceans, molluscs and fish.

Species	Cholesterol (mg 100 g ⁻¹)	Source
<i>Penaeidae</i> (n=7 shrimp species)	118 - 163	Mathew <i>et al.</i> , 1999
<i>Portunidae</i> (n=3 crab species)	52.4 - 66.8	
<i>Scyllaridae</i> (n=2 crab species)	51.5 - 54.8	
<i>Cancer pagurus</i> (edible crab)	23 - 42	Oehlenschläger, 2006
<i>Nephrops norvegicus</i> (Norway lobster)	81 - 120	
<i>Mytilus edulis</i> (blue mussel)	18	
<i>Todarodes sagittatus</i> (flying squid)	75 - 227	
<i>Scomber scombrus</i> (mackerel)	14 - 55	

1.7.3 Fatty acids and cholesterol in health and in disease

Fatty acids and cholesterol carry out many functions that are necessary for normal physiological maintenance and good health. SFA are involved in energy production, energy storage, lipid transport, synthesis of phospholipids needed for membrane synthesis, and covalent modification of many regulatory proteins. MUFA are also involved in many of these processes and play a key role in maintaining optimal fluidity of the membrane lipid bilayer (Spector, 1999). PUFA are an integral part of the cell membrane, significantly impacting its fluidity and function, serving as the major constituents of phospholipids, triglycerides and cholesterol esters (Lee *et al.*, 2003). Moreover, AA and EPA are important for the synthesis of eicosanoids (Leonard *et al.*, 2004). Cholesterol plays an important role in steroid hormone and bile acid biosynthesis and serves as an integral component of cell membranes where it is involved in a wide variety of critical cell functions, including modulation of enzyme function, permeability, fusion and receptor function (Albert and Boesze-Battaglia, 2005; IOM, 2005).

Yet, there are also strong, consistent, and graded relationships between saturated fat intake, blood cholesterol levels, and the mass occurrence of coronary vascular disease. The relationships are accepted as causal (Graham, 2007). In recent years atherosclerosis has been more distinctly linked to elevations of low density lipoproteins (LDL) levels instead of total serum cholesterol (Parodi, 2009). Owing to its limited solubility in water, cholesterol is transported in blood in spherical particles, lipoproteins. The lipoprotein outer layer is formed of amphiphilic cholesterol and phospholipid molecules, studded with proteins, surrounding a hydrophobic core of triglycerides and cholesterol esters. The types of lipoproteins are named for their density and they are specifically targeted to cells by distinct apolipoproteins on their surface that bind to specific receptors. Low

density lipoprotein (LDL) contains the highest level of cholesterol. LDL receptors in peripheral tissues bind LDL, triggering its endocytosis, lysosomal targeting and hydrolysis. When cells have abundant cholesterol, LDL receptor synthesis is inhibited. When cholesterol levels become deregulated, more LDL exists in the blood than can be taken up by LDL receptors. Excess LDL is oxidized and taken up by macrophages, forming foam cells that can become trapped in the walls of blood vessels. The end result is an atherosclerotic plaque, the major cause of heart attacks and strokes. Although LDL levels correlate with heart attack risk, high density lipoprotein (HDL) has an inverse ratio of risk because these particles transport cholesterol to the liver for excretion. Modern cholesterol tests distinguish the LDL/HDL ratio as well as the overall level (Leah, 2009).

Human tissues have the ability to synthesize sufficient amounts of cholesterol and SFA for their metabolic and structural needs and consequently cholesterol and SFA do not need to be supplied by the diet (IOM, 2005). In this regard, the guidelines of the European Society of Cardiology recommend that in general, total plasma cholesterol should be below 5 mM (190 mg dL^{-1}), and LDL cholesterol should be below 3 mM (115 mg dL^{-1}).

On the other hand, animals are unable to form n-3 or n-6 fatty acids *de novo*; therefore these fatty acids must be obtained from their diet (Innis, 2008). Once obtained from the diet, a small proportion of α -linolenic acid (ALA; 18:3n-3) can be further metabolized to eicosapentaenoic acid (EPA, 20:5n-3), while linoleic acid (LA, 18:2n-6) is metabolized using the same enzymes of the 20 carbon chain n-6 fatty acid, arachidonic acid (AA, 20:4n-6). DHA (22:6n-3) is derived from EPA (Leonard *et al.*, 2004; Figure 1.17).

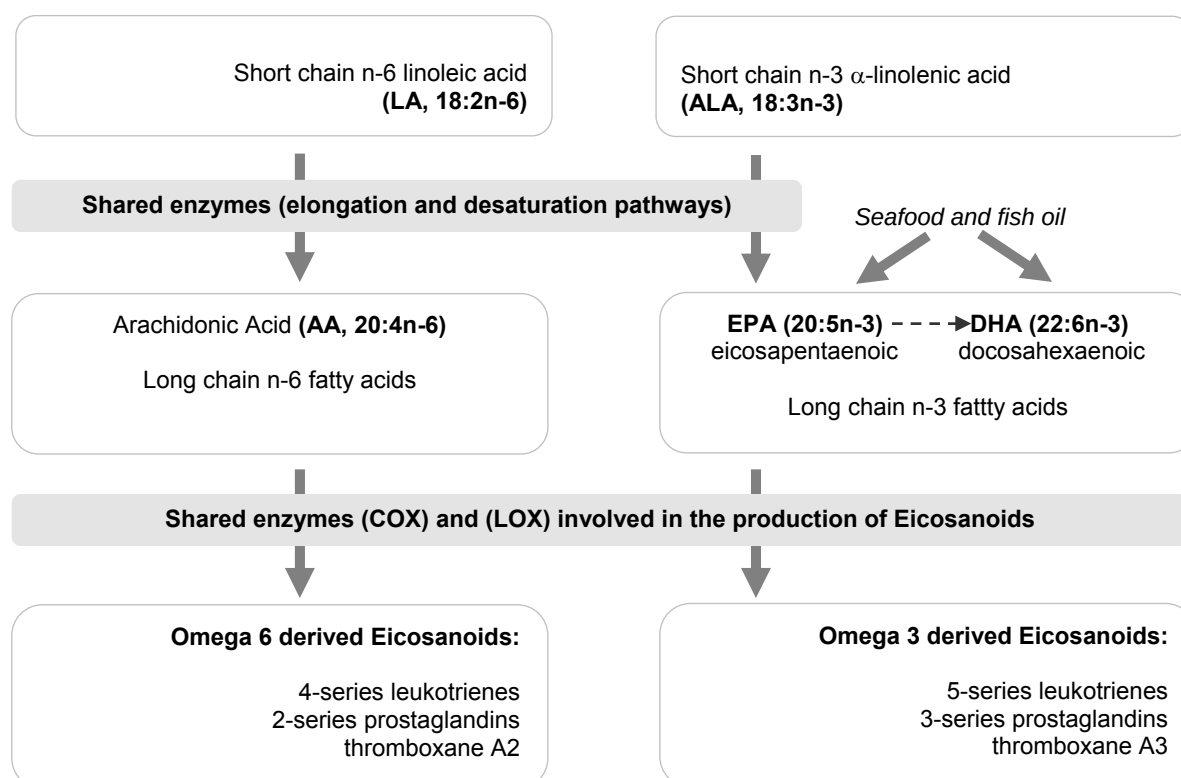


Figure 1.17 Desaturation and elongation pathways of the n-3 and n-6 PUFA; (adapted from: Lee and Lop, 2003; Tribble, 2007).

Studies generally agree that whole-body conversion of ALA to DHA is < 5 % in humans, and depends on the concentration of n-6 fatty acids and long chain PUFA in the diet (Tribble, 2007). Both AA and EPA are important for the synthesis of eicosanoids and compete for cyclooxygenases (COX) and lipoxygenases (LOX) involved in the production of prostaglandins and leukotrienes, respectively (Figure 1.17). AA is the primary substrate for the synthesis of the 2- and 4-series eicosanoids that promote pro-inflammatory and pro-thrombotic effects in response to physiological stressors.

In contrast, the corresponding 3- and 5-series produced from EPA exhibit much lower levels of biologic activity, are formed at a slower rate and, work to attenuate excessive series-2 prostaglandins (Jordan *et al.*, 2004). Thus, adequate production of the series-3 prostaglandins, which are derived from the n-3 fatty acid, EPA, may protect against heart attack and stroke as well as certain inflammatory diseases like arthritis, lupus, and asthma (Wang *et al.*, 2004). Excessive intake of LA relative to ALA increases production of AA compared with that of EPA and DHA, and large adipose tissue reservoirs of LA slow down conversion of ALA to EPA and DHA. Therefore, the ratio between LA and ALA, rather than the absolute amounts of ALA in the diet, may be critical for disease prevention, due to the 'competition' between the two essential PUFA for their entry into the elongation and desaturation pathways, leading to the synthesis of their respective eicosanoids (Lee and Lip, 2003; Le, *et al.*, 2009).

It has been estimated that the present Western (USA) diet is "deficient" in n-3 fatty acids with a ratio of n-3 to n-6 of 1:15: to 1:17 instead of 1:1 as is the case with wild animals and presumably human beings (Simopoulos, 2006). Compensation for this imbalance can be achieved in part by increasing consumption of EPA and DHA, but this does not completely satisfy the biologic requirements for n-3 fatty acids, indicating that ALA has important biologic effects that are independent of being a precursor for EPA and DHA.

1.7.4 Dietary factors and coronary heart disease

Nowadays, it is common knowledge that diet is a major environmental factor affecting health and disease and the expression "we are what we eat" is now part of our daily life. In this respect, several dietary factors are potentially linked with the incidence of coronary heart disease, either in its reduction or promotion (Ulbricht and Southgate, 1991). For instance, the ratio of PUFA to SFA has often been used as an indicator of diet atherogenicity, considering that SFA are associated to hypercholesterolaemia, while PUFA is associated to lowered cholesterol (Ulbricht and Southgate, 1991). Another factor usually used in literature is the ratio of n-3 to n-6. Given the fact that both fatty acids families compete for the same enzymes in the synthesis of eicosanoids having antagonistic effects in respect to pro-inflammatory and pro-thrombotic effects, the ratio n-3 to n-6 has been purposed as an indicator of a lower risk of thrombosis. Yet, according to Ulbricht and Southgate (1991), these indicators are minimalist approaches and other factors should be considered.

These authors take in consideration that different fatty acids can promote or prevent atherosclerosis and thrombosis based on their effects on the serum cholesterol and low density lipoprotein-cholesterol concentrations. Of the SFA, only 12:0, 14:0 or 16:0 have a cholesterol-raising effect and are thus atherogenic, while 14:0, 16:0 and 18:0 have been suggested to be thrombogenic. Both MUFA and n-6 PUFA are considered anti-atherogenic due to their cholesterol-lowering effects, but n-3 and n-6 act differently: n-6 also lowers HDL, which is believed to protect against coronary heart disease (CHD), while n-3 is believed to lower LDL but increases HDL. Moreover, many studies indicate that long chain unsaturated fatty acids slow down platelet aggregation and are thus antithrombotic (Ulbricht and Southgate, 1991). In an attempt to take into account these different effects of the various fatty acids, Ulbricht and Southgate (1991) proposed two indices: the index of atherogenicity (IA) and the index of thrombogenicity (IT).

$$IA = \frac{12:0 + 4(14:0) + 16:0}{(n-6 + n-3)PUFA + MUFA}$$

According to this equation, only saturated fatty acids with chain lengths of 12 to 16 are atherogenic and myristic acid is considered four times more atherogenic than the other two. All unsaturated fatty acids, regardless of their double bond number, position, or configuration, are considered equally effective in decreasing atherogenicity primarily for lack of reliable information to assign more suitable coefficients to the various structures. When applied to various fats and oils, this equation gives IA values of 14 for coconut oil, 0.3-2 for fish fats, 0.5-1 for meat fats, 0.2-0.3 for crustacean fats, 0.2 for dairy food, and 0.14 for olive oil (Ulbricht and Southgate, 1991; Rosa and Nunes, 2003a; Grigorakis, 2007; Turan *et al.*, 2007). In the following equation:

$$IT = \frac{14:0 + 16:0 + 18:0}{0.5 (MUFA) + 0.5 (n-6) + 3 (n-3) + (n-3/n-6)}$$

the SFA 14:0, 16:0 and 18:0 are equally thrombogenic, n-3 are six times more antithrombotic than MUFA and n-6, three times more antithrombotic than the ratio n-3 to n-6. When applied to various fats and oils, this equation gives IT values of 6 for coconut oil, 2 for dairy food, 1-2 for meat fats, 0.32 for olive oil, 0.2-0.6 for fish and 0.1-0.2 for crustacean fats (Ulbricht and Southgate, 1991; Rosa and Nunes, 2003a; Grigorakis, 2007; Turan *et al.*, 2007).

However, there are still several weaknesses to these indexes that are pointed out by the authors, namely the fact that PUFA have been weighted equally in their anti-atherogenic properties while MUFA and n-6 have been weighed equally in their anti-thombogenic properties.

Fehily *et al.* (1994) concluded that the proposed dietary scores of atherogenicity and thrombogenicity could be weak predictors of ischaemic heart disease risk, and both indices were unlikely to be better predictors than more simple approaches such as total SFA or PUFA:SFA ratios. To enhance their predictive ability, the authors further suggested that more complex formulas taking into account the effects of other dietary factors, as well as fatty acids, are probably required. We must also bear in mind that diet is only one part of the equation and other factors

cannot be discarded such as lifestyle (e.g. smoking habits, exercise), hypertension, diabetes mellitus, age, obesity and genetic background, just to name a few (IOM, 2005).

1.7.5 Proteins

Proteins are macromolecules consisting of different amino acids arranged in a linear chain and folded into a globular form. All amino acids possess common structural features, including an α -carbon to which an amino group, a carboxyl group, and a variable side chain are bonded (Figure 1.18). The amino acids in a polymer chain are joined together by the peptide bonds between the carboxyl and amino groups of adjacent amino acid residues (deMan, 1997).

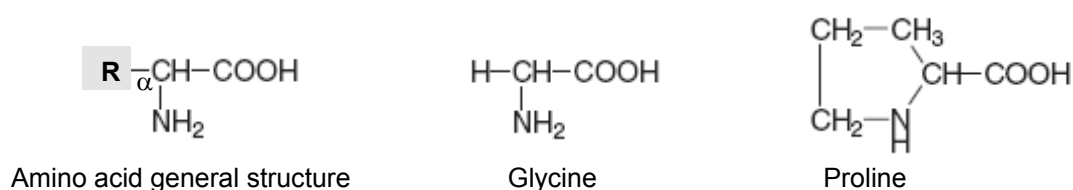


Figure 1.18 From left to right, general amino acid structure showing the α -carbon, the amino group and the variable side chain (R), glycine, where R is an hydrogen and, proline which differs from the basic structure as it contains an unusual ring to the N-end amine group, (adapted from IOM, 2005).

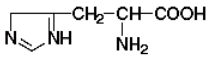
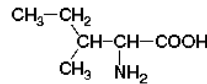
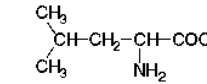
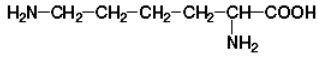
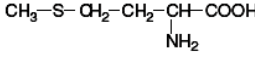
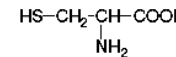
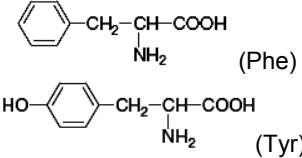
Proteins are the major structural components of all cells in the body, and also function as enzymes, in membranes, as transport carriers, and as hormones. Their component amino acids serve as precursors for nucleic acids, hormones, vitamins, and other important molecules (IOM, 2005). The most important aspect of a protein from a nutritional point of view is its ability to provide the nitrogen and amino acid requirements for growth, maintenance, and repair. In practice, protein quality is principally determined by two factors: digestibility and the relative content and metabolic availability of the individual essential amino acids of the protein in question (IOM, 2005).

Of the 20 amino acids, only about half are essential for human nutrition. According to the original definition by Borman *et al.* (1946), essential or indispensable amino acids are those that cannot be synthesized by the organism out of materials ordinarily available to the cells at a speed commensurate with the demands for normal growth, and consequently, must be provided in the diet. The indispensable amino acids are leucine, isoleucine, valine, lysine, threonine, tryptophan, methionine, phenylalanine and histidine (FAO/WHO/UNU, 2007).

The contribution of all indispensable amino acids to the total nitrogen content of the food has to be considered in order to assess the overall protein quality of the diet. If the content of a single indispensable amino acid in the diet is less than the individual's requirement, then it will limit the utilization of other amino acids and prevent normal rates of protein synthesis even when the total nitrogen intake level is adequate. Thus, the "limiting amino acid" will determine the nutritional value of the total nitrogen or protein in the diet. The concept of limiting amino acid has led to score amino acids, whereby the indispensable amino acid composition of the specific protein source is compared with that of a reference amino acid composition profile (IOM, 2005).

Protein from animal sources usually provides all nine indispensable amino acids, and for this reason is referred to as “complete proteins”, whereas proteins from plants, grains, nuts, seeds, and vegetables tend to be deficient in one or more of the indispensable amino acids and are called “incomplete proteins” (IOM, 2005). About 20 % of the muscle tissue of crustaceans is protein (*C. sapidus*, 19.6 %; *N. norvegicus*, 20.4 - 21.2 %; *Aristeu antennatus*, 21.4 - 22.0; Rosa and Nunes, 2003a; Küçükgülmez and Çelik, 2008). From a nutritional point of view the dominant amino acids in marine crustacean species are the non essential glutamic acid, followed by aspartic acid and glycine, whereas the most important essential acids are lysine and leucine (Rosa and Nunes, 2003, Küçükgülmez and Çelik, 2008). But the ratio of essential to non essential amino acids is high, usually above 0.80 and the amino acid score is also high for most amino acids (Table 1.4). The limiting amino acids in shellfish and fish meals are usually the sulphur-containing methionine and cysteine (Rosa and Nunes, 2003a; Chen *et al.*, 2007).

Table 1.4 Amino acid chemical structure, adult requirements (mg/g protein) and amino acid score of *Eriocheir sinensis* (Chen *et al.*, 2007) and *Maja* spp. (Marques *et al.*, in press).

Amino acid	Structure	protein mg/ g	Score	
			<i>E. sinensis</i>	<i>Maja</i> sp.
Histidine (His)		15	-	133
Isoleucine (Iso)		30	143	87
Leucine (Leu)		59	77	119
Lysine (Lys)		45	56	144
Methionine (Met)		16	-	131
Cysteine (Cys)		6	-	-
(Met) + (Cys)		22	105	-
Phenylalanine (Phe)* + Tyrosine (Tyr)		38	205	197

Scores were obtained according to the estimates of the indispensable amino acid requirements of adults considering a mean nitrogen requirement of 105 mg nitrogen kg⁻¹ per day (0.66 g protein kg⁻¹ per day; FAO/WHO/ONU, 2007). *Phenylalanine is an essential amino acid, while tyrosine is a metabolic product of phenylalanine catabolism, and thus is dependent on phenylalanine to supply the needs for both amino acids (chemical structure source: IOM, 2005).

Amino acids also contribute to the taste of food: glycine, alanine, serine and, threonine taste sweet, while arginine, leucine, valine, methionine, phenylalanine, histidine and, isoleucine give bitter taste. The sweetness of fresh prawn and crab is contributed by the abundance of free glycine in their muscle. The high content of free arginine in crustaceans enriches the sweet taste with complexity

and fullness and yields a seafood-like flavour. Taurine, proline and, alanine are among the major free amino acids in crustaceans (Fuke, 1994; Sikorski, 2000).

1.7.6 Protein in health and in disease

Protein, as other nutrients, must be balanced; its intake must be sufficient but not exceeded. Protein deficiency has adverse effects in all organs (IOM, 2005). In infants and young children, it has been shown to have harmful effects on the brain function. Furthermore, protein deficiency has been shown to have adverse effects on the immune system, resulting in a higher risk of infections. It also affects gut mucosal function and permeability, which, in turn, influences absorption and makes possible gut bacterial invasion resulting in septicaemia. Protein deficiency has also been shown to adversely affect kidney function, particularly on glomerular and tubular function (IOM, 2005).

Nonetheless, a number of adverse effects have also been reported at the very high intakes due to supplement diet use. In addition, some naturally occurring proteins are allergenic to sensitive individuals; for example, the glycoprotein fractions of foods have been implicated in allergic responses. However, relatively few protein foods cause most allergic reactions: milk, eggs, peanuts, and soy in children; and fish, shellfish, peanuts, and tree nuts in adults. High protein intakes have been implicated in chronic diseases such as osteoporosis, renal stones, renal insufficiency, cancer, coronary artery disease, and obesity (IOM, 2005).

1.7.7 The importance of shellfish as a source of taurine in the diet

Fish and especially invertebrates (e.g. molluscs and crustaceans), are high in taurine (2-aminoethanesulfonic acid; Elvevoll, 2008). It was reported that taurine comprised 23 % of total free amino acids. Taurine is not a true amino acid as it lacks a carboxyl group, but it does contain a sulfonate group and may be called an amino sulfonic acid (Figure 1.19) though it is never incorporated into proteins (Elvevoll, 2008). The capacity for taurine biosynthesis varies remarkably among species and, since humans only have limited ability for biosynthesis of taurine by the liver and reabsorption by the kidneys, taurine needs to be acquired with diet (Elvevoll, 2008). Taurine has been considered as a conditionally essential amino sulfonic acid having antioxidant properties. A variety of functions have been ascribed to taurine, ranging from bile acid synthesis to neurotransmission (Shao and Hathcock, 2008).

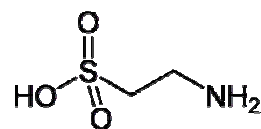


Figure 1.19 Structure of taurine (source: Lipidomics Gateway, 2009).

Beneficial effects of dietary taurine have been observed in animal and human studies and suggestions of a reduced cardiovascular disease (CVD) risk through taurine alone or in combination with n-3 PUFA have been put forward (Yamori *et al.*, 2001; Elvevoll, 2008; Shao and Hathcock, 2008).

1.7.8 Vitamins

Seafood flesh, oils, and offal are very rich in vitamins. The amount of fat soluble vitamins in marine foods is dependent on the concentration of fat and therefore values are different between organs and vary both between and within species and on several biological factors (e.g. size, life cycle, etc.). The fat-soluble vitamins are present in especially high concentrations in the liver oils of some species, whereas the body oils, obtained from whole fish contain lower amounts of vitamin A, D and E. The amount of water-soluble vitamins in marine foods is less dependent upon species. Generally a serving portion of seafood may significantly contribute to satisfying the human daily needs for B vitamins. The contents of both groups of vitamins in the final products depend also on the losses which occur due to oxidation and leakage as the result of industrial processing and culinary preparation (Sikorski *et al.*, 2004)

1.7.9 Inorganic elements

Inorganic elements are usually divided in two major groups: macroelements, including sodium (Na), potassium (K), magnesium (Mg), chloride (Cl), sulphur (S) and calcium (Ca); and trace elements, which are usually available below 50 ppm and include manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), boron (Br), strontium (Sr) and selenium (Se), just to name a few (deMan, 1999). Trace elements also include non essential toxic elements such as mercury (Hg), cadmium (Cd), lead (Pb) and arsenic (As). More than 60 inorganic elements can be found in food (deMan, 1999). Almost all elements considered as essential, *i.e.* those provided through the diet and required to maintain normal physiological functions can also be found in seafood (Oehlenschläger, 1997; Goldhaber, 2003).

Most seafood mineral content is composed by macro elements, crustaceans having high levels of Cl, Na and K in the muscle (Oehlenschläger, 1997; Gökoğlu *et al.*, 2003; Mohapatra *et al.*, 2007). As far as trace elements are concerned crustaceans muscle has a particular high content of Cu, Zn and Se (Gökoğlu *et al.*, 2003; Mohapatra *et al.*, 2007). Elemental composition is known to vary between species and tissues of the same species. For instance, Cd accumulated by aquatic crustaceans is usually bound to metallothionein in the cytosol of hepatopancreas (Rainbow, 2002). Other trace elements particularly abundant in hepatopancreas of *C. maenas* and *N. puber* are Mn, Fe, Cu, Br and Sr (Teixeira *et al.*, 2008).

1.7.10 Inorganic elements in health and disease

Even though most elements found in seafood are necessary in several human metabolic processes they must be taken from the diet in balanced doses, as low intakes results in nutritional deficiencies but on the other hand high intakes results in toxicity (Goldhaber, 2003).

For instance, iron is an essential element for humans as it is a constituent of haemoglobin, myoglobin and a number of enzymes. Iron deficiency results in anemia. However, the accidental ingestion of iron results in acute toxicity and death has occurred from the oral ingestion of iron sulfate at doses ranging from 40 to 1600 mg kg⁻¹ (Goldhaber, 2003). Considering this dichotomy,

risk assessment of essential elements has examined two ends of the toxicity spectrum: that associated with intakes that are too high and the resulting toxicity, and that associated with intakes that are too low and resulting in nutritional deficiencies (Goldhaber, 2003).

The USA Food and Nutrition Board of the Institute of Medicine (IOM) has dealt with nutritional deficiency problems, as well as their toxicity, by setting dietary reference intakes (DRI) which includes the recommended dietary allowance (RDA), the adequate intake (AI) and the tolerable upper intake level (UL) for essential elements (IOM, 2001). Table 1.5 shows the RDA/AI and UL of some essential elements, together with a small description of their functions and adverse effects if consumed in excess.

Table 1.5 Dietary reference intakes for essential elements.

Element	Function	RDA/AI	UL	Adverse effects of excessive consumption
Potassium K (g day ⁻¹)	Major intracellular cation, is required for normal cellular function	4.7*	ND	Hyperkalemia might occur if the capacity of the normal kidney to excrete a K load is exceeded
Sodium Na (g day ⁻¹)	Required to maintain extracellular volume and plasma osmolality	1.5*	2.3	Elevated blood pressure, increased risk factor for CVD and renal diseases
Calcium Ca (mg day ⁻¹)	Blood clotting, muscle contraction, nerve transmission, bone and tooth formation	1.0*	2.5	Kidney stones, hypercalcemia, milk alkali syndrome and renal insufficiency
Iron Fe (mg day ⁻¹)	Component of haemoglobin and numerous enzymes, prevents anemia	18 (♀) 8 (♂)	45	Gastrointestinal distress
Copper Cu (µg day ⁻¹)	Component of enzymes in iron metabolism	900	10000	Gastrointestinal distress, liver damage
Zinc Zn (mg day ⁻¹)	Component of multiple enzymes and proteins, involved in the regulation of gene expression	8 (♀) 11 (♂)	40	Reduced Cu levels
Selenium Se (µg day ⁻¹)	Protection against oxidative stress, regulation of thyroid hormone action, reduction and oxidation of vitamin C and other molecules	55	400	Hair and nails brittleness and loss
Magnesium Mg [†] (mg day ⁻¹)	Cofactor of enzyme systems	320 (♀) 420 (♂)	350	No evidence of adverse effects from consumption of naturally occurring Mg in foods; Mg containing supplements may cause osmotic diarrhea
Manganese Mn (mg day ⁻¹)	Involved in the formation of bone, as well as in enzymes from amino acid, cholesterol and carbohydrate metabolism	1.8* (♀) 2.3* (♂)	11	Elevated blood concentration and neurotoxicity

Abbreviation: (RDA) recommended dietary allowance; (AI) adequate intake; (UL) tolerable upper intake levels. RDA values are in bold type while AI are in ordinary type followed by an asterisk (*). All reference intakes shown are for adult males (♂) and females (♀) aged 19 to 50 years. (ND) not determinable due to lack of data of adverse effects in this age group and concern with regard to lack of ability to handle excess amounts. Source of intake should be from food only to prevent high levels of intake (IOM, 1997; 2001; 2004); [†] female and male adults aged between 31 and 50 years.

RDA is set to meet the daily dietary needs of almost all (97 to 98 %) healthy individuals in a particular life stage (e.g. infants, children, pregnancy and lactation) and gender group. The AI is believed to cover the needs of all individuals in a life stage group, but lack of data prevent being able to specify with confidence the percentage of individuals covered by this intake. The UL is the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population.

In addition to essential elements, non-nutritive toxic elements such as Hg, Cd, Pb and As can also be present in some marine species. Such elements are available in water from natural sources, such as rocks, and as a result of human activities like emissions from industrial processes. These elements are taken up by marine organisms through the diet and gills and many elements tend to accumulate in species from higher trophic levels like fish and shellfish (EFSA, 2004 and 2005). Regular consumption of contaminated seafood can cause severe poisoning. In this respect, the outbreaks of severe Hg poisoning in Minamata (Japan, 1956) and Iraq (early 1970's), and Cd poisoning in Toyama (Japan, 1957) became historical marks that have changed the perception of the development of food poisoning in connection with environmental pollution (Rangan and Barceloux, 2008).

In Minamata Bay, Hg-contaminated effluent was discharged from an acetaldehyde producing factory and methylmercury (Me-Hg) was bio-accumulated by fish and shellfish, and caused Minamata disease in humans by eating contaminated fish. Since 1956 more than 100 people were killed and several thousand became paralyzed around Minamata Bay and the adjacent Yatsushiro Sea. In the early 1970's, a major Me-Hg poisoning catastrophe occurred in Iraq by consumption of seed grain treated with a Me-Hg fungicide. An estimated 10,000 people died and 100,000 were severely and permanently brain damaged (Li *et al.*, 2009).

After the Second World War, Cd released from a mine in the Japanese mountains was transported by the Jinzu River into the plain, where the contaminated water was used for irrigation of rice fields. Cadmium was taken up by rice plant and consumption of contaminated rice was responsible for *itai itai* disease, a bone disease causing fractures and severe pains due to a form of Cd-induced renal osteomalacia (Nordbeg, 2009).

Since these tragic events a vast literature on the toxicology of these contaminants has emerged (Rangan *et al.*, 2008). In addition, guidelines and regulations stipulating maximum concentrations of toxic elements in foods have been set to limit our dietary exposure. In the USA, the FDA has set the action level (AL) for cadmium, lead, methylmercury and arsenic among other toxic elements. AL represents the value at which the FDA takes legal action to remove contaminated products from the market. In the European Union, the European Commission has set maximum levels for Cd, Pb and Hg but not for As (EC No. 1881/2006). To ensure an efficient protection of public health, products containing contaminants exceeding the maximum levels should not be placed on the market either as such, after mixture with other foodstuffs or used as an ingredient in other foods (EC No. 1881/2006).

The Joint Expert Committee on Food Additives of the Food and Agriculture Organization of the United Nations (JECFA) uses the term provisional tolerable weekly intake (PTWI), or provisional tolerable daily intake (PTDI), for contaminants that may accumulate in the body. The weekly designation is used to stress the importance of limiting intake over a period of time for such substances. When using this approach no-observed-effect levels (NOELs) or no-observed-adverse-effect levels (NOAELs) are identified in the critical studies, to which appropriate safety or uncertainty factors are applied (Herrman and Younes, 1999). In table 1.6 the set values for crustaceans are shown.

Table 1.6 Action level (AL, ppm), maximum level (ML, ppm) and provisional tolerable weekly intake (PTWI, mg kg⁻¹ body weight) for Hg, Cd, Pb and As in crustaceans.

Element	AL (ppm)	ML (ppm)	PTWI μg kg ⁻¹ bw	Adverse effects	Observation
Mercury Hg	-	0.5	-	Neurotoxicity. High exposure in uterus has resulted in cerebral palsy or severe mental retardation in the neonate.	Organic Hg is considered to be more toxic than other forms of Hg following ingestion. Methyl-Hg is the predominant form of Hg in fish.
Methyl-Hg	1	-	1.6		
Cadmium Cd	3	0.5	7	Nephrotoxicity, osteoporosis, carcinogenicity and genotoxicity, teratogenicity, and endocrine and reproductive effects.	Long biological half-life in mammals. In humans, steady-state concentrations in the renal cortex are reached after about 40 years.
Lead Pb	1.5	0.5	25	Neurotoxicity, anaemia, renal toxicity and subsequent carcinogenicity and cardiovascular effects; impairment of the reproductive system.	Pb in seafood is inorganic, and is bound to proteins. Bioaccumulation in marine animals is low compared to mercury. Main sites of Pb accumulation in seafood: internal organs, skin and bone, but not in muscle.

The AL refers to Crustacea, while ML refers strictly to crustaceans, excluding brown meat of crab and head and thorax meat of lobster and similar large crustaceans (*Nephropidae* and *Palinuridae*).

As far as crustaceans are concerned, the action level set by the USFDA concerns Crustacea, while the maximum limit set by the European Commission refers strictly to crustaceans, excluding brown meat of crab and head and thorax meat of lobster and similar large crustaceans (*Nephropidae* and *Palinuridae*).

The PTWI set for Methyl-Hg is set to protect the most susceptible life stage, *i.e.* the developing fetuses exposed as a result of the mercury in the mother's body. Therefore the subgroups of special consideration are women who are pregnant or may become pregnant. People at other life stages are likely to be less susceptible, although cardiovascular risk may include other sections of the population.

1.8 Main objectives

In this context, the first aim of this thesis was to describe the national live trade of crustaceans and identify its major critical points and stressors leading to mortality of the most affected species. Once identified the species most affected by mortality, in this case, *C. pagurus*, experiments were designed to characterize and understand the physiological responses of *C. pagurus* to stressors during the trade chain *in situ* and under simulated conditions. This information is essential to improve and develop new transport systems.

Regarding the nutritional quality of homarids and *C. pagurus* the specific aims were as follows:

1. compare the nutritional quality of *Homarus gammarus* and *Homarus americanus*;
2. compare the nutritional quality of two different populations of female and male *Cancer pagurus*. One population comprises crabs from the Scottish coast and the other included crabs from the English Channel;
3. characterize the nutritional quality of female and male *Cancer pagurus* captured off the Scottish coast during the four year seasons.

Chapter 2

Crustaceans' live trade



Pulling the lobster traps
Sue Lynn Cotton (private collection)

Sub-chapter 2.1

Trade of live crustaceans in Portugal

Barrento, S., Marques, A., Pedro, S., Vaz-Pires, P., and Nunes, M. L. 2008. The trade of live crustaceans in Portugal: space for technological improvements. *ICES Journal of Marine Science*, 65: 551-559.



In Portuguese coastal areas, crabs and lobsters maintained alive until prepared for the table are commercially and economically very important. The trade in live crustaceans, mostly imported animals, is an interlinked and complex chain, from fishing, collection, holding facilities, and transportation, to the end-consumer, the various facilities playing a key role. Along the chain, animals can be affected by several stressors, inducing high mortality with consequent economic loss, and contributing to unsustainable exploitation of the resource. A survey was developed to characterize storage, transportation, and handling issues affecting various crustaceans at Portuguese holding facilities. In all, 22 facilities were identified and categorized by activity and water supply system. Despite the wide variation in their infrastructure, there were no major differences in mortality rate of crustaceans between importers, wholesalers, and exclusively retailers. At all facilities, *Necora puber*, *Cancer pagurus*, and *Carcinus maenas* had higher rates of mortality and shorter duration of captivity than *Maja* spp., *Homarus* spp., *Panulirus regius*, or *Palinurus* spp. Overall, the main problems identified were technical issues related to careless handling, high animal density, and the varying physiological needs of each species.

Keywords: crabs, handling, holding facilities, lobsters, transportation.

Introduction

Portugal is the eighth biggest seafood consumer worldwide, with an average per capita consumption of 57.1 kg annually, well above the average world per capita consumption of 16.4 kg (data from 2001 to 2003; Laurenti, 2004). Of the sought-after seafood products, crustaceans are central to Portuguese consumption and an important nutritional source for human consumption, providing all the essential amino acids, trace elements, and polyunsaturated fatty acids, precursors of biologically active molecules and crucial in preventing human cardiovascular and inflammatory disease (Skonberg and Perkins, 2002; Rosa and Nunes, 2003a). In the past decade, the consumption of crustaceans in Portugal has increased by 59 %, corresponding to ~400 g *per capita* in 2005 along (DGPA, 2006; EUROSTAT, 2007). Consumption of seafood varies seasonally, increasing during summer and at the New Year.

In Portuguese coastal areas, there is an ancient tradition of keeping certain crustacean species alive in holding facilities until needed for preparation, as a guarantee of freshness. The national trade of live crustaceans is commercially and economically very important, totalling almost 3000 t and ~ 11 million euros (data from 2005; EUROSTAT, 2007). Crustaceans normally traded alive in Portugal are the edible crab (*Cancer pagurus*; 2325 t), Norway lobster (*Nephrops norvegicus*; 356 t), homarids (*Homarus gammarus* and *Homarus americanus*; 105 t), other crabs (spider crab, *Maja* spp.; green crab, *Carcinus maenas*; velvet crab, *Necora puber*; total 121 t), and spiny lobsters (mostly *Palinurus elephas*, *Palinurus mauritanicus*, and *Panulirus regius*; 70 t; data from 2005; EUROSTAT, 2007). The Portuguese crustacean fleet catches mainly *N. norvegicus* (318 t), *C. maenas* (219 t), *Maja* spp. (67 t), *N. puber* (43 t), *P. elephas* (12 t), *P. mauritanicus* (11 t), *C. pagurus* (9 t), and *H. gammarus* (4 t; data from 2006; EUROSTAT, 2007), but because of the shortage of national product, most live crustaceans are imported, to satisfy consumer demand. In fact, imports of live crustaceans accounted for 88 % of national trade (EUROSTAT, 2007; INE, 2007). Most live animals are imported from other EU countries, especially France, the UK, and Spain (*C. pagurus*, *H. gammarus*, *N. puber*, *Maja* spp., *C. maenas*, and *P. elephas*), and small quantities also from the USA and Canada (*H. americanus*; 13 t), and from African countries (spiny lobsters; 49 t; DGPA, 2006; Holmyard and Franz, 2006; EUROSTAT, 2007).

The trade in imported and national live crustaceans is an interlinked, long, and complex chain from catch, through transportation, holding facilities, to consumption. Throughout this long chain, crustaceans are subjected to several stressors, such as periods of exposure to air, hypoxia, handling, interaction with other individuals, seawater physicochemical variation (e.g. temperature, ammonia, pH, salinity, nitrite, nitrate), and periods of starvation (Winkler, 1987; Whiteley and Taylor, 1992; Jussila *et al.*, 1997; Paterson and Patrick, 1997; Taylor and Waldron, 1997; Taylor *et al.*, 1997; Bergmann *et al.*, 2001; Danford and Uglow, 2001a, b; Fotedar *et al.*, 2001, 2006; Mercier *et al.*, 2006; Ridgway *et al.*, 2006; Woll, 2006). The effects of stress on live crustaceans tend to be cumulative, being reflected in increased rates of mortality at holding facilities (up to 66 %; Burton, 2001), and up to 9.5 % economic loss to those involved in the trade (data from 2003; FISHSTAT, 2007). Consequently, considerable quantities of crustaceans are wasted annually, representing

important losses of valuable food and likely contributing to unsustainable exploitation of the resource. The trade in live crustaceans is clearly highly dependent on importation, centralized in the holding facilities, and carries a considerable risk to what is obviously a valuable and profitable food source for the Portuguese, among others. Therefore, it is crucial that handling conditions at the most critical steps of the trade chain be evaluated. In this context, we carried out a thorough survey to characterize Portuguese holding facilities and handling conditions for each species, with the aim of developing strategies to decrease crustacean mortality.

Material and methods

Surveys

In all, 31 holding facilities for live crustaceans were identified in Portugal, using information collected from (i) the Portuguese General Directorate of Fisheries and Aquaculture (DGPA), the government entity responsible for licensing the facilities; (ii) the Portuguese Association of Traders of Fishery Products (ACOPE); and (iii) the Portuguese national telephone directory. According to the DGPA, only 11 of these companies were officially licensed in 2006 as holding facilities for live crustaceans. We developed a survey and personal interviews were made to managers of these holding facilities; participation was voluntary and, 22 facilities (including some that were officially licensed) across the country agreed to participate in it, representing 80 % of Portugal's total volume of trade (EUROSTAT, 2007). Holding facilities were categorized by activity type: (i) six importer-exporters (out of seven Portuguese companies identified as such; the main importer of spiny lobsters in Portugal declined to participate); (ii) nine wholesalers (which are also licensed as retailers); and (iii) seven that were exclusively retailers. Holding facilities were also categorized by their type of water supply system: open, semi-open, and closed. All surveys were carried out at the holding facilities, and the interviewer was able to observe the prevailing conditions. The three topics addressed in the surveys were: national trade chain, holding facility infrastructure, and species trading/storage conditions (Table 2.1.1). All species traded alive in Portugal were considered in the interviews: *C. pagurus*, *Maja spp.*, *N. puber*, *C. maenas*, *H. gammarus*, *H. americanus*, *P. elephas*, *P. mauritanicus*, and *P. regius*. *Nephrops norvegicus*, although captured off Portugal, is rarely traded alive in national markets, because it is almost exclusively exported to Spain, so it was not considered in our study. For the remaining crustaceans, density, mortality rate, and captivity duration at a holding facility were presented as annual means.

Statistical analysis

Differences among species, the nature of the holding facilities, and water supply systems were investigated with analysis of variance (ANOVA) and Tukey's multiple comparisons. When necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements, followed by non-parametric ANOVA (Kruskal–Wallis test), if transformed data could not meet these assumptions. All statistical analyses were tested at a 0.05 level of probability with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Table 2.1.1 Summary list of all topics addressed in the surveys.

National trade chain
1. Characterization
Holding facility infrastructure
1. Activity (importer, wholesaler, or retailer)
2. Water supply system (open, semi-open, or closed)
3. Seawater origin (estuary, Atlantic ocean, lagoon, or synthetic)
4. Tank number and volume
5. Devices to ensure seawater quality (skimmer, sand, sponges, activated coal, ultraviolet light, bio-balls, chillers, and aeration)
6. Analysis of water quality parameters (temperature, salinity, pH, oxygen, ammonia, nitrite, or nitrate)
7. Feeding conditions
Species trading/storage conditions
1. Species origin (country)
2. Quantities per load
3. National and international transportation (road, air, or sea)
4. Transportation handling conditions
5. Stocking density
6. Maximum duration in captivity
7. Duration between loads
8. Handling conditions at the holding facilities
9. Mortality rate

Results

National trade chain

Most of the crustaceans imported come from foreign fishing vessels that offload their catches directly to be transported immediately to holding facilities in Portugal (Figure 2.1.1).

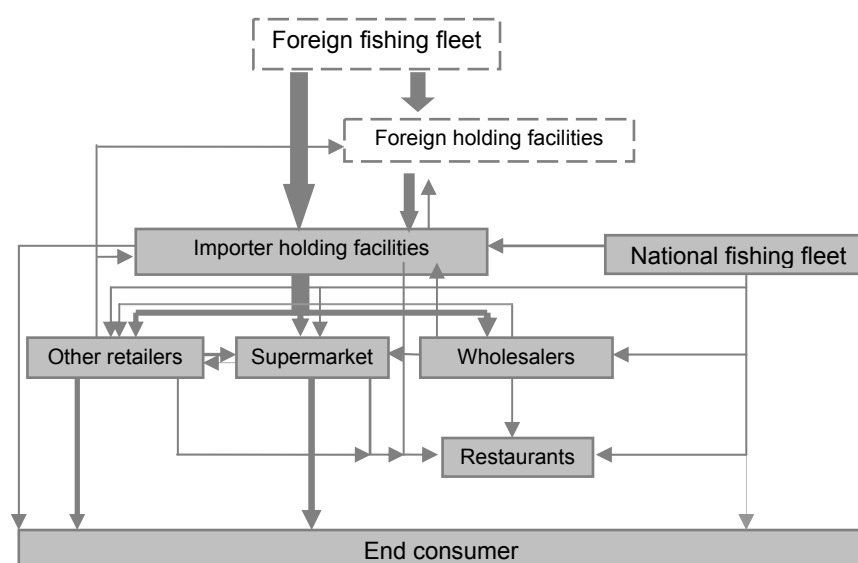


Figure 2.1.1 Schematic representation of the live crustacean trade chain in Portugal. Arrows represent the trade route between the entities, their width being greater for routes with higher trade importance. Dashed lines represent the foreign trade chain.

On arrival, part of the catch is immediately conveyed to national traders, and the balance is stored in tanks. The major national traders are wholesalers/retailers and, to a lesser extent, restaurants, supermarkets, local seafood markets, and end-consumers. Small quantities of some species are exported either by the importation companies or through other holding facilities. The catch of Portuguese fishing vessels is usually sold at official auctions direct to wholesalers and retailers, although a small portion is unofficially sold directly to restaurants or to end-consumers.

Infrastructure

Most of the holding facilities are 10 km from the sea and close to large cities such as Lisbon (Figure 2.1.2). In terms of their water supply systems, closed systems are used by 45 %, open systems by 32 %, and semi-open systems by 23 %. Importers mostly use semi-open systems, wholesalers mainly semi-open or open systems, and retailers mainly closed systems (Table 2.1.2). The seawater used in these systems is mainly natural (95 %) and obtained either by pumping it directly from the nearby open sea (75 % of that pumped directly), estuary (17 %), or lagoon (8 %) in open and semi-open systems, or is transported by tank from the nearby open sea (56 % of the water transported by tank), estuary (33 %), or lagoon (11 %) in closed systems.

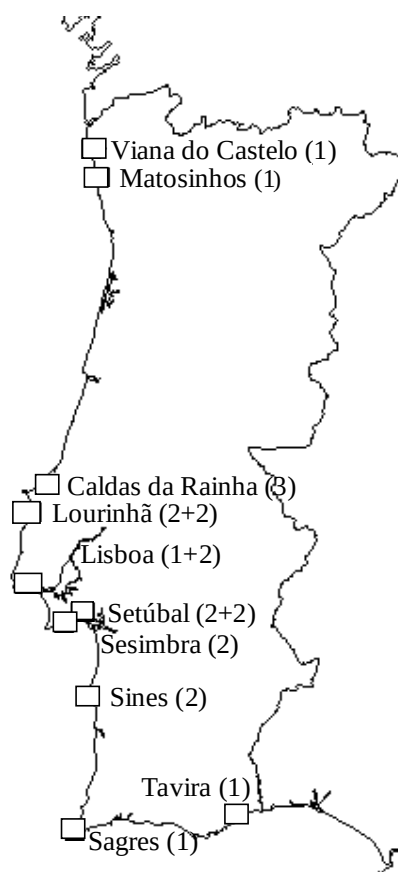


Figure 2.1.2 Map of the Portuguese coast, with the locations of the holding facilities surveyed. Each square corresponds to the location of importers and/or non-importers wholesalers or exclusively retailers. The numbers of facilities, or the numbers of non-importers plus importers, are in parenthesis

The capacity and the number of tanks used for open and semi-open systems are usually more than in closed systems (Table 2.1.2). All closed systems and 80 % of the semi-open systems have chillers to cool seawater (to 10-13 °C), but none of the open-system facilities we saw possessed chillers. Consequently, the water temperature in open and semi-open systems is more variable, dropping regularly to 10 °C in winter and rising to 20 °C in summer. To maintain water quality in the tanks closed and semi-open systems usually have coal, sponges, or protein-skimmers, whereas open systems use sand (Table 2.1.2). In closed systems, more water quality parameters are analysed regularly (salinity, temperature, ammonia, nitrites, pH, and nitrates) than in semi-open or open systems (generally just temperature and salinity; Table 2.1.2). Feeding is common in semi-open and open systems, but rare in closed systems. The practice of feeding is restricted in

Portugal to *Maja* spp., homarids, and spiny lobsters, which are fed exclusively with bivalves such as mussels and clams.

Table 2.1.2 Characterization of the holding facilities.

Parameter	Closed	Semi-open	Open
Number of facilities	10	5	7
Trading category (%)			
Importers	10	60	28
Wholesalers	20	40	44
Retailers	70	0	28
Tank characteristics			
Number	9 ± 5 (4; 20)	20 ± 18 (7; 47)	11 ± 3 (6; 15)
Volume ('000 L)	22 ± 22 (2; 60)	90 ± 45 (30; 130)	80 ± 37 (20; 120)
Devices to maintain water quality (%)			
None	0	0	86
Chillers	100	80	0
Sand	10	20	14
Sponges	50	20	0
Skimmer	30	20	0
Coal	60	20	0
Bioballs	60	0	0
Ultraviolet	20	0	0
Water-quality parameters analysed (%)			
None	30	20	29
Temperature	50	80	71
Salinity	70	60	29
pH	20	0	29
Ammonia	40	0	14
Nitrate	30	0	0
Nitrite	40	0	14
Dissolved oxygen	0	0	14
Animal feeding (%)			
None	80	40	29
Bivalve	20	60	71

Percentages were calculated with data from all surveyed facilities. Results of tank number and volume are presented using the following structure: average ± standard deviation (minimum; maximum).

Transportation and handling

Crustaceans imported from the EU are usually transported by tanks carried in refrigerated lorries (or in tankers), whereas imports from elsewhere are mainly by air. Of the Portuguese importers, five have their own lorries, and one hires specialized transport companies. The loads of tankers and lorries mostly come from Scottish fishing ports or directly from Scottish fishing vessels, but sometimes also from French holding facilities. Usually, each lorry has ten tanks filled with seawater at the animal's landing port or holding facility. Seawater is aerated, but not renewed or filtered during transportation (transport duration from Scotland to Portugal is ~72 h, and from France to Portugal, ~24 h). Each tank contains just one species at a maximum animal density of 1 kg L⁻¹

(though densities are less for *H. gammarus* and *P. elephas*). The transport by air of live crustaceans, mostly *H. americanus*, *P. mauritanicus*, and *P. regius*, is generally in cardboard boxes (in refrigerated and moist conditions), with separation between individual animals. Small refrigerated trucks are generally used to transport the animals between the airport and the holding facilities. Locally caught crustaceans are usually transported to holding facilities in wooden boxes or plastic buckets by small refrigerated trucks. On arrival at a holding facility, part of the load (local and imported) is immediately distributed to wholesalers and retailers throughout the country by refrigerated lorry, and the balance is stored in tanks at the holding facility. Depending on the species, three different procedures are used for offloading: (i) *C. pagurus* and *Maja* spp. are discharged into plastic boxes (30 kg capacity), weighed and thrown into tanks; (ii) homarids and spiny lobsters are unloaded individually, weighed, and placed carefully in the tanks; and (iii) *N. puber* (which are usually transported in 9 kg plastic or wooden boxes) are unloaded, dead animals discarded, and live animals transferred to tanks. In all facilities, dead animals are removed 3 - 5 h after arrival and twice per day thereafter.

Storage

Cancer pagurus, *Maja* spp., and *H. gammarus* are stored by all the holding facilities we surveyed, but *N. puber*, *P. elephas*, *H. americanus*, *P. regius*, *P. mauritanicus*, and *C. maenas* by 91 %; 64 %; 59 %; 45 %; 36 %; and 14 % of the facilities, respectively. Storage conditions varied between holding facilities (e.g. in the numbers of *C. pagurus* stored in closed and open systems (Tables 2.1.3 and 2.1.4). Differences in load size and subsequent mortality were found for the nine crustacean species, depending on the water supply system and the type of activity at the holding facilities (Tables 2.1.3 and 2.1.4). Rates of mortality were significantly higher for *N. puber*, *C. pagurus*, and *C. maenas*, and lower for *P. elephas*, *Maja* spp., *H. gammarus*, and *H. americanus*. In all water supply systems and activity types, significantly more *C. pagurus* were discharged per transportation than the other species.

Table 2.1.3 Estimates of the rates of mortality (%), quantity per load (kg), animal density in tanks (g L⁻¹) and captivity duration (days) per species in holding facilities categorized by water supply system.

System	Species	n	Mortality	Quantity	Density	Captivity duration
Closed system	<i>C. pagurus</i>	9	23 ± 20 (0; 60)ab	1051 ± 2071 (40; 6500)a	162 ± 177 (20; 571)a	19 ± 16 (4; 56)ab
	<i>Maja</i> spp.	9	6 ± 8 (0; 20)b	42 ± 33 (10; 100)b	53 ± 71 (6; 198)ab	28 ± 23 (7; 84)ab
	<i>N. puber</i>	7	32 ± 15 (15; 50)a	142 ± 153 (5; 400)ab	64 ± 39 (18; 112)a	9 ± 7 (0; 14)bc
	<i>C. maenas</i>	2	50 ± 0 (50; 50)a	60 ± 57 (20; 100)ab	20 ± 0 (20; 20)b	45 ± 64 (0; 90)abc
	<i>H. gammarus</i>	9	4 ± 5 (0; 10)b	124 ± 175 (7; 500)ab	59 ± 69 (4; 229)ab	61 ± 43 (14; 112)a
	<i>H. americanus</i>	5	5 ± 11 (0; 25)ab	112 ± 83 (20; 200)ab	79 ± 100 (23; 229)a	70 ± 67 (0; 168)ab
	<i>P. elephas</i>	7	3 ± 4 (0; 10)b	144 ± 275 (11; 700)ab	45 ± 76 (7; 200)ab	96 ± 119 (14; 336)a
	<i>P. mauritanicus</i>	4	10 ± 9 (5; 20)ab	52 ± 44 (15; 100)ab	17 ± 3 (14; 20)b	53 ± 37 (11; 84)ab
	<i>P. regius</i>	3	18 ± 28 (0; 50)ab	32 ± 14 (15; 40)b	14 ± 3 (11; 17)b	40 ± 40 (7; 84)ab
Semi-open system	<i>C. pagurus</i>	4	22 ± 15 (3; 40)a	6500 ± 3317 (2000; 10000)a	322 ± 163 (163; 467)a	11 ± 7 (7; 21)b
	<i>Maja</i> spp.	4	5 ± 5 (1; 10)ab	208 ± 203 (30; 500)b	20 ± 11 (7; 29)c	28 ± 0 (28; 28)a
	<i>N. puber</i>	4	30 ± 26 (15; 60)a	140 ± 87 (40; 250)b	21 ± 9 (8; 28)c	8 ± 4 (4; 14)b
	<i>C. maenas</i>	1	1	90	9	7
	<i>H. gammarus</i>	4	6 ± 8 (0; 15)ab	275 ± 166 (150; 500)b	35 ± 11 (24; 50)bc	26 ± 22 (7; 56)ab
	<i>H. americanus</i>	2	0 ± 0 (0; 0)b	160 ± 198 (20; 300)b	61 ± 80 (5; 118)b	28 ± 40 (0; 56)ab
	<i>P. elephas</i>	3	5 ± 5 (0; 10)ab	300 ± 283 (100; 500)b	13 ± 4 (10; 15)c	42 ± 37 (14; 84)a
	<i>P. mauritanicus</i>	2	3 ± 4 (0; 5)ab	3100 ± 4101 (200; 6000)ab	206 ± 263 (20; 392)a	25 ± 5 (21; 28)a
	<i>P. regius</i>	3	18 ± 28 (0; 50)ab	308 ± 414 (15; 600)b	119 ± 164 (4; 235)ab	21 ± 12 (7; 28)ab
Open system	<i>C. pagurus</i>	6	14 ± 5 (10; 20)a	2508 ± 3733 (150; 10000)a	63 ± 57 (8; 143)a	22 ± 31 (7; 84)ab
	<i>Maja</i> spp.	6	1 ± 2 (0; 5)b	164 ± 122 (50; 350)b	26 ± 27 (4; 74)ab	50 ± 23 (21; 84)a
	<i>N. puber</i>	6	44 ± 19 (20; 70)a	128 ± 125 (40; 350)b	10 ± 14 (2; 30)b	5 ± 5 (0; 14)b
	<i>C. maenas</i>	0	-	-	-	-
	<i>H. gammarus</i>	6	0 ± 0 (0; 0)b	120 ± 188 (25; 500)b	20 ± 27 (1; 71)ab	77 ± 62 (28; 168)a
	<i>H. americanus</i>	3	0 ± 0 (0; 0)b	143 ± 140 (30; 300)b	30 ± 29 (9; 64)ab	88 ± 114 (7; 168)ab
	<i>P. elephas</i>	3	0 ± 0 (0; 0)b	247 ± 307 (40; 600)b	21 ± 18 (5; 40)ab	98 ± 20 (84; 112)a
	<i>P. mauritanicus</i>	0	-	-	-	-
	<i>P. regius</i>	3	5 ± 7 (0; 15)b	500 ± 436 (200; 1000)ab	29 ± 4 (25; 32)a	21 ± 8 (14; 28)ab

Abbreviations: (n) number of facilities that trade each species. Results are presented showing average ± standard deviation (minimum; maximum). In each column per system, different letters indicate significant differences (p<0.05).

Table 2.1.4 Estimates of the rates of mortality (%), quantity per load (kg), animal density in tanks (g L⁻¹) and captivity duration (days) per species in holding facilities categorized by activity type.

Trading category	Species	n	Mortality	Quantity	Density	Captivity duration
Importers	<i>C. pagurus</i>	4	14 ± 10 (3; 25)a	8375 ± 1887 (6500; 10000)a	303 ± 134 (143; 457)a	12 ± 7 (7; 21)b
	<i>Maja</i> spp.	4	2 ± 2 (1; 3)b	120 ± 36 (80; 150)b	24 ± 8 (11; 29)c	28 ± 0 (28; 28)a
	<i>N. puber</i>	4	16 ± 1 (15; 16)a	260 ± 159 (40; 400)b	55 ± 53 (8; 112)c	9 ± 4 (7; 14)b
	<i>C. maenas</i>	1	1	90	16	7
	<i>H. gammarus</i>	4	3 ± 5 (0; 10)c	413 ± 175 (150; 500)b	65 ± 25 (29; 88)c	26 ± 27 (7; 56)ab
	<i>H. americanus</i>	2	0 ± 0 (0; 0)c	180 ± 170 (60; 300)b	118 ± 118 (118; 118)b	28 ± 40 (0; 56)ab
	<i>P. elephas</i>	4	4 ± 5 (0; 10)b	213 ± 250 (40; 500)b	24 ± 14 (15; 40)c	33 ± 21 (14; 56)ab
	<i>P. mauritanicus</i>	2	3 ± 4 (0; 5)b	3100 ± 4101 (200; 6000)ab	214 ± 252 (35; 392)ab	25 ± 5 (21; 28)ab
	<i>P. regius</i>	3	18 ± 28 (0; 50)a	320 ± 396 (40; 600)b	123 ± 158 (11; 235)ab	14 ± 12 (7; 28)ab
Wholesalers	<i>C. pagurus</i>	7	16 ± 12 (0; 40)ab	1907 ± 2358 (150; 7000)a	146 ± 163 (28; 467)a	10 ± 6 (4; 21)ab
	<i>Maja</i> spp.	7	3 ± 4 (0; 10)b	191 ± 190 (15; 500)ab	26 ± 27 (5; 74)ab	35 ± 21 (7; 56)a
	<i>N. puber</i>	6	39 ± 24 (16; 70)a	100 ± 67 (40; 200)b	15 ± 12 (3; 28)b	7 ± 5 (0; 14)b
	<i>C. maenas</i>	1	50	20	-	0
	<i>H. gammarus</i>	7	5 ± 6 (0; 15)b	98 ± 100 (20; 300)b	16 ± 12 (3; 35)ab	42 ± 38 (14; 112)a
	<i>H. americanus</i>	4	0 ± 0 (0; 0)b	125 ± 122 (20; 300)b	25 ± 27 (5; 64)ab	7 ± 7 (0; 14)ab
	<i>P. elephas</i>	4	4 ± 5 (0; 10)b	27 ± 20 (11; 50)b	11 ± 7 (7; 18)b	35 ± 33 (14; 84)ab
	<i>P. mauritanicus</i>	1	5	40	14	28
	<i>P. regius</i>	5	15 ± 20 (0; 50)ab	311 ± 403 (15; 1000)ab	21 ± 12 (4; 32)ab	25 ± 7 (14; 28)ab
Retailers	<i>C. pagurus</i>	8	26 ± 20 (5; 60)ab	458 ± 528 (40; 1500)a	131 ± 185 (8; 571)a	29 ± 28 (7; 84)ab
	<i>Maja</i> spp.	8	6 ± 8 (0; 20)bc	47 ± 26 (10; 80)b	53 ± 71 (4; 91)a	30 ± 23 (7; 84)ab
	<i>N. puber</i>	7	38 ± 13 (15; 50)a	97 ± 103 (5; 300)ab	44 ± 34 (2; 91)a	9 ± 7 (0; 14)bc
	<i>C. maenas</i>	1	50	100	20	90
	<i>H. gammarus</i>	8	2 ± 4 (0; 10)bc	75 ± 111 (7; 300)ab	48 ± 75 (1; 229)a	79 ± 48 (14; 168)a
	<i>H. americanus</i>	4	6 ± 13 (0; 25)bc	113 ± 101 (20; 200)ab	77 ± 101 (18; 229)a	140 ± 48 (84; 168)a
	<i>P. elephas</i>	5	1 ± 2 (0; 5)c	186 ± 290 (15; 700)ab	52 ± 83 (5; 200)a	146 ± 118 (28; 336)a
	<i>P. mauritanicus</i>	3	13 ± 11 (5; 20) bc	58 ± 60 (15; 100)ab	19 ± 2 (17; 20)a	61 ± 40 (14; 84) ab
	<i>Panulirus regius</i>	1	0	15	17	12

Abbreviations: (n) number of facilities that trade each species. Results are presented showing average ± standard deviation (minimum; maximum). In each column per trading category different letters indicate significant differences (p<0.05).

Storage was at significantly greater density in closed systems holding *C. pagurus*, *N. puber* and *H. americanus*, in semi-open systems holding *C. pagurus* and *P. mauritanicus*, in open systems holding *C. pagurus* and *P. regius*, at importers holding *C. pagurus* and *H. americanus*, and at wholesalers holding *C. pagurus*. The duration of captivity was usually longer for *Maja* spp., homarids and spiny lobsters, and shorter for *C. pagurus*, *N. puber* and *C. maenas*. Significant differences in the duration of captivity were found for *H. gammarus* and *P. elephas* held in closed systems, for *Maja* spp., *P. elephas* and *P. mauritanicus* held in semi-open systems, for *Maja* spp., *H. gammarus* and *P. elephas*, held in open systems, for *Maja* spp. at importers' facilities, for *Maja* spp. and *H. gammarus* at wholesalers, and for both species of homarids and *P. elephas* at retailers. Differences in the storage conditions of each species were also analysed in terms of type of water supply system and activity (Table 2.1.5).

Table 2.1.5 ANOVA p-values related to the differences in storage conditions of each species between the three water-supply systems (closed, semi-open, and open), and among the three activity types (importers, wholesalers, and retailers).

Species	n	Mortality	Quantity	Density	Captivity duration
Water supply system					
<i>C. pagurus</i>	19	0.588	0.023	0.047	0.687
<i>Maja</i> spp.	19	0.412	0.013	0.626	0.125
<i>N. puber</i>	17	0.482	0.982	0.024	0.513
<i>H. gammarus</i>	19	0.505	0.337	0.359	0.279
<i>H. americanus</i>	10	0.662	0.880	0.739	0.714
<i>P. elephas</i>	13	0.483	0.761	0.761	0.722
<i>P. mauritanicus</i>	6	0.346	0.253	0.266	0.369
<i>P. regius</i>	9	0.644	0.145	0.325	0.528
Activity type					
<i>C. pagurus</i>	19	0.338	0.000	0.425	0.133
<i>Maja</i> spp.	19	0.972	0.047	0.818	0.796
<i>N. puber</i>	17	0.435	0.509	0.641	0.813
<i>H. gammarus</i>	19	0.711	0.001	0.003	0.028
<i>H. americanus</i>	10	0.528	0.813	0.101	0.014
<i>P. elephas</i>	13	0.506	0.821	0.322	0.139
<i>P. mauritanicus</i>	6	0.223	0.776	0.132	0.222
<i>P. regius</i>	9	0.798	0.979	0.336	0.318

The results for green crab (*Carcinus maenas*) are not presented because data were too few to perform the analyses.

The analyses revealed no statistically significant differences in mortality rates, though retailers usually had higher rates of mortality of *C. pagurus*, *Maja* spp., *C. maenas*, *H. americanus*, and *P. mauritanicus* (Tables 2.1.4 and 2.1.5). Generally, semi-open systems and importers carried more animals per load of all species (quantity), though the differences were only significant for *C. pagurus*, *Maja* spp., and *H. gammarus*. Similarly, semi-open systems and importers usually maintained crustaceans at higher density, but differences were only significant for *C. pagurus* and *H. gammarus*. The densities naturally varied with the quantities carried by each lorry or tanker, always being higher on the day of receipt at the facility, then decreasing as the product was sold,

before the next load was received. In terms of duration of storage, open systems and retailers generally kept the crustaceans for longer in the tanks, and semi-open systems for shorter periods, though only significantly shorter for homarids. The length of time between loads varied among species, season (higher in summer and at the new year) and facility. Generally, the interval between loads was weekly (*C. pagurus*, *N. puber*, and *C. maenas*), two-weekly for homarids, and monthly for *Maja* spp. *Palinurus mauritanicus* whereas *P. regius* is typically traded whenever quantities of *P. elephas* are insufficient to satisfy market demand (mainly in summer). The periods between loads are usually more irregular and longer at wholesalers and retailers.

Discussion

Infrastructure

Several studies have documented the trade chain of live crustaceans in the UK (Beard and MacGregor, 1991; Burton, 2001), France (Chartois *et al.*, 1994), Australia (Bremner *et al.*, 1997; Spanoghe and Bourne, 1997), India (Vijayakumaran and Radhakrishnan, 1997), the USA (Estrella, 2002), and Portugal (Araújo, 2006). However, to date, the type of holding facility has only been documented once, for France (Chartois *et al.*, 1994). To the best of our knowledge, this work is the first attempt to quantify the conditions employed at the holding facilities to maintain crustaceans alive while awaiting sale. The Portuguese trade of live crustaceans has some unique characteristics, in which the holding facilities play a central role. The facilities themselves are located mainly near landing ports and water supplies, to reduce costs related to transportation of natural seawater. Of the holding facilities we surveyed, importers and wholesalers generally had larger infrastructure than exclusive retailers in terms of storing the large numbers of live animals sought by the national market, particularly *C. pagurus*, *H. gammarus*, and *P. mauritanicus*. Importers and wholesalers generally employ cost-effective open or semi-open systems, with the purpose of reducing the costs related to treating large volumes of seawater. Such systems typically employ basic or no filtration systems, and control fewer water-quality parameters than closed systems (Salisbury, 2002). In contrast, retailers use mainly closed systems, because of the smaller facilities and volumes of seawater required. The management of closed systems is complex and laborious, requiring regular surveillance and maintenance of the physicochemical parameters associated with water quality that are critical for crustaceans (temperature, salinity, oxygen, pH, ammonia, nitrite, and nitrate; Taylor *et al.*, 1997). The larger volumes of seawater required for open and semi-open systems make the control of seawater temperature with chillers very expensive and difficult. However, owing to the importance of maintaining optimal conditions for crustaceans, 50 % of importers, 57 % of wholesalers, and 78 % of retailers have such a device available. Differences in seawater temperature along the trade chain pose an additional problem to the entrepreneurs. In particular, species from temperate regions are captured at low temperature (<10 °C). Importers transport these animals at low temperature (average 10-15 °C) to maintain low metabolic rates (e.g. of oxygen consumption and excretion) and to preclude stress and mortality (Chartois *et al.*, 1994; Spanoghe and Bourne, 1997; Taylor *et al.*, 1997). However, especially in summer, tank temperatures in open and semi-open systems without chillers increase above the optimal level (up

to 20 °C), thus contributing to higher rates of mortality. Salinity can also be problematic during winter because of precipitation, especially in open systems located close to estuaries or lagoons, because some crustacean species have a low tolerance to salinity variation (Chartois *et al.*, 1994; Taylor *et al.*, 1997).

Storage

Despite the great differences we saw in the infrastructure of the holding facilities, no major differences were found in the rates of mortality of all crustaceans between importers, wholesalers, and retailers. Generally, *N. puber*, *C. pagurus*, and *C. maenas* had greater rates of mortality and shorter duration of storage at the holding facilities than *Maja* spp., homarids, and spiny lobsters. The mortality of crustaceans is a direct consequence of cumulative stress along the trade chain, depending of course on the initial condition of the animal (soft shell, pale, empty, sick, and weak animals have little chance of survival to the table), the animal's physiological demands, the handling conditions, and technical issues related to storage (Chartois *et al.*, 1994; Gomez-Jimenez *et al.*, 2001).

Data obtained during our survey indicate that each species faces different handling conditions during capture, transportation, and storage (e.g. the duration of transport, the unloading conditions, animal density, the extent of exposure to air, temperature variations, water quality, time in captivity), which can influence the levels of stress and hence the rates of mortality. Each species' load quantity and economic value seems to influence the handling conditions. Crustaceans of low value per kilogramme in Portugal (e.g., *C. pagurus*, € 5 -12; *C. maenas*, € 5 - 7; *N. puber*, € 14; *Maja* spp., € 16) are generally handled less carefully than high value per kilogramme species such as *P. elephas* (€ 59 - 91), *H. gammarus* (€ 40 - 47), *H. americanus* (€ 20 - 28), *P. mauritanicus* (€ 61), and *P. regius* (€ 48). Note that these values are for 2007, obtained from a local Portuguese retailer.

Soon after capture of *C. pagurus*, the tendons in the dactyls of the chelae are cut to avoid injury/damage by the claws attributable to stress along the trade chain. This procedure causes needless wounds to animals that diminish their chances of survival, owing to the potential entrance of pathogens (e.g. *Aerococcus viridans*, *Vibrio* spp.) and loss of haemolymph (essential for gas transport and nutrition reserves; Ellender *et al.*, 1992; Chartois *et al.*, 1994). In terms of transportation, several tonnes of *C. pagurus* are loaded into lorry tanks, at high densities. Consequently, the crabs at the bottom are compressed by others above them and by handlers, and seawater accumulates significant quantities of excreted product (Chartois *et al.*, 1994). On arrival at an importer's holding facility, the *C. pagurus* must be unloaded as fast as possible, meaning that careless discharge procedures are common (animals thrown into tanks, exposed to air, sunlight, and high temperature, and limbs lost). Finally, at the holding facilities, *C. pagurus* are maintained at high density (maximum density 300 kg m⁻³, even though the recommended stocking density for *C. pagurus* is 120 kg m⁻³; Chartois *et al.*, 1994).

Despite the lesser quantities of *N. puber* and *C. maenas* traded, mortalities of those species can reach 50 % during the first day of captivity. Despite the tendons in the dactyls of both species' chelae not being cut after capture, the animals have very low tolerance to captivity, likely because of their fast rate of metabolism, aggressive behaviour, and their limited capacity for acclimatization to temperature variation (Ingle, 1997; Cuculescu *et al.*, 1998; Hopkin *et al.*, 2006). The conditions during transport of those two species might also influence the rates of mortality, because individual animals are compressed and legs lost, and the death of one animal can promote the mortality of others (Hearn, 2002). Generally, the rate of mortality of *Maja* spp. at holding facilities was low. Perhaps this was due to the different handling conditions of *Maja* spp. after capture (the tendons in the dactyls of the chelae are not cut), and to its rather greater physiological tolerance to variation in seawater temperature (8-19 °C; Le Foll, 1993).

A different handling procedure is used for high-value species such as *H. gammarus* and *H. americanus*, to preclude cannibalism and physical damage following capture: the chelae are immobilized with elastic bandages. *Palinurus elephas* and both species of homarid had the lowest rates of mortality we found, likely because of the ultra-careful handling procedures (e.g. low density, animals loaded individually), and their high physiological tolerance to variations in seawater temperature in nature (*H. gammarus*, from 7 to 19 °C; *H. americanus*, from -1 to 24 °C; *P. elephas*, from 9 to 20 °C; Chartois *et al.*, 1994). However, despite the careful handling of *P. mauritanicus* and *P. regius*, the rate of mortality of those species at the holding facilities was higher than that of *P. elephas*. This could be related to the species having different physiological demands. The optimal temperature range for *P. regius* is from 14 to 29 °C thus mortality is heavy at temperatures below 12 °C (Chartois *et al.*, 1994). The optimum holding temperature for this species can differ from the temperatures found at national holding facilities without chillers (10 °C in winter).

During our survey, several traders stressed the seasonal nature of mortality trends, mainly of *C. pagurus*, *C. maenas*, *N. puber*, and *P. regius*. However, it was not possible for us to quantify the true seasonal variation in rates of mortality. Mortality of crabs was certainly highest during summer, likely because of the strong temperature variations along the trade chain, and lowest during winter. Seasonal mortality patterns for *P. regius* are the opposite of those for crabs, because this lobster requires higher temperatures to survive (14 - 29 °C).

Conclusions

Overall, the main problems faced by Portuguese holding facilities are technical, related to the complex and time-consuming trade chain, storage conditions, handling procedures, animal densities, and the different economic value and physiological demand by species. Of course, possible solutions can be proposed to lessen the rate of mortality of live crustaceans during the trade chain.

Following capture, all animals should be examined carefully (discarding individuals with soft shell, pale-exoskeleton, diseases, and notoriously weak), bandages should be used to immobilize the

chela, instead of cutting the tendon (plastic strips are currently used to immobilize claws in Australasian crab fisheries), air exposure and sunlight can be avoided, and on-board tanks with constant air flow can be used. Further, as the seawater temperature in some harbours is higher and the water more polluted than where the animals were originally caught, water flow to the tanks on board catching vessels should be stopped before entering port, though aeration should be maintained. Also at this stage, an anaesthetic agent could be used to decrease metabolism and stress. The same anaesthetic concentration could be used in the water of the lorries and tankers used for international and national transportation. Anaesthetics such as AQUI-S® are widely used in transporting ornamental aquatic animals, and for the culture of finfish and wild crustaceans. Cost-effective filtration systems and chillers could be installed on the lorries and tankers to maintain water quality at optimum levels, so allowing transportation in lesser volumes of seawater. Additionally, animal loading and unloading procedures need to be improved, to preclude mechanical and physicochemical stress to the animals, air or sunshine exposure, and temperature variations. In holding facilities, water quality parameters should be regularly monitored and maintained at optimum levels for each species, by implementing efficient and cost-effective filtration systems and chillers. The maintenance of temperature throughout the trade chain is fundamental to precluding mortalities.

Finally, rigorous codes of conduct and cautious handling conditions need to be implemented along the trade chain (similar to those described by FAO/WHO, 1979; Beard and MacGregor, 1991; APEC, 1999; Bennison, 2000; Woll, 2006).

To conclude, numerous improvements are required for the trade chain, to enhance animal welfare, to maintain high nutritional quality and safety of the animals, and to lessen the rates of mortality, economic losses, and ultimately the waste of an important, but finite, resource. To promote innovation and technological development, synergies between small and medium-size enterprises, scientific research institutes and industry are crucial tools in ensuring the ongoing viability of these important European products and the sector.

Chapter 3

C. pagurus physiological responses to transport



Two crabs, 1889
Vincent van Gogh (The National Gallery, London, UK)

Sub-chapter 3.1

Live shipment of *C. pagurus* from England to Portugal

Barrento, S., Marques, A., Vaz-Pires, P., and Nunes, M. L. 2010. Live shipment of immersed crabs *Cancer pagurus* from England to Portugal and recovery in stocking tanks: stress parameter characterization. ICES Journal of Marine Science, 67: 000-000.



Cancer pagurus is commercially one of the most important crustaceans exploited in the UK and Ireland but the main markets are in Southern Europe, to where live edible crabs are transported. In this study, potential stressors during the live trade chain from England to Portugal were identified and related to changes in haemolymph parameters. Before their live transport, 60 crabs were tagged, their vigour was assessed, and their haemolymph was sampled; 30 crabs were placed in the bottom of a vivier-lorry tank and the balance at the top (1 kg crab L⁻¹ in a total of 700 kg). The sampling procedure was repeated after immersed live transport (58 h), and during subsequent recovery (6, 24, 48, 72, and 96 h) in seawater at Portuguese storage facilities. Haemolymph parameters included D-glucose, L-lactate, pH, and haemocyanine. Cumulative mortalities at the end of the experiment (96 h in recovery tanks) of bottom and top crabs were 8.9 and 10.7 %, respectively. Vigour assessment predicted crab mortality well. The main stressors identified were poor handling, air exposure during unloading and, deficient transport conditions. D-Glucose and L-lactate increased during transport with acidification of the haemolymph. Concentration of L-lactate reached control levels after 24 h of recovery, but haemolymph remained acidic and hyperglycaemic even after 96 h. The transport conditions promoted anaerobiosis, so alternatives need to be considered.

Keywords: crustaceans, handling, live transport, stress.

Introduction

The European edible crab (*Cancer pagurus*) is much sought by Mediterranean countries, mainly France, Spain, Portugal, and Italy (Sub-chapter 2.1). In Portugal, it is the most marketed live crustacean (2400 t), and because national production is insufficient to supply consumer demands, crabs must be imported from production countries mainly the UK (31 000 t), Ireland (11 500 t), and France (5700 t; data from 2007; Eurostat, 2009). Live transport of *C. pagurus* is commonly used to satisfy demand and to maximize returns from the fishery, but the journey to the end of the marketing chain is usually long (~2 to 3 days) and involves several post-harvest operations (Uglow *et al.*, 1986; Sub-chapter 2.1). Soon after capture, the tendons in the dactyls of the claws are cut (nicked) to avoid injuries to handlers and other crabs. However, this practice promotes haemolymph loss and infection, which weakens the crabs (Chartois *et al.*, 1994). Crabs are also transported at high densities in tanks (1 kg L⁻¹ seawater) and crabs at the bottom are compressed by the others and by handlers. During transport, no filtration systems are used, so the seawater accumulates significant quantities of excreted products. The unloading process is usually careless and promotes temperature changes, air and sun exposure (Uglow *et al.*, 1986; Chartois *et al.*, 1994; Paterson and Spanoghe, 1997; Sub-chapter 2.1). Such stressors have been associated with losses attributable to heavy mortality during transport (1-50 %), but mainly during recovery at the importer's premises (15-70 %; Uglow *et al.*, 1986; Sub-chapter 2.1).

There is a stress response when a regulated physiological system is pushed beyond normal bounds by one or more external factors or stressors acting upon it (Barton and Iwama, 1991). Under such circumstances, the stress can be either readily reversible or irreversible, the latter when crabs are weakened to the point at which homeostasis is impaired leading to death, and the former through changes that are within the homeostatic capability of animals at least when the original stressor is removed (Taylor *et al.*, 1997).

Stress responses may be evaluated subjectively (e.g. behaviour, vigour) in a non-invasive process, or objectively, when expressed quantitatively by measuring changes in the physiological variables that can either implicate non-invasive approaches, such as the measure of the oxygen rate, or moderate invasive techniques of haemolymph sampling to measure its chemistry (pH, L-lactate, D-glucose, ammonia, total proteins, haemocyanine, ions, and hormones; Taylor *et al.*, 1997). Objectively, stress responses can also include more-invasive techniques such as the quantification of muscle metabolites and the crustacean hyperglycaemic hormone by eye-stalk removal and histology (Webster, 1996; Ridgway *et al.*, 2006). Many studies have been carried out to evaluate the effect of capture, air exposure, and simulated live shipment on the physiology of spiny lobsters (Jussila *et al.*, 1997; Paterson and Spanoghe, 1997; Paterson *et al.*, 1997; Spanoghe and Bourne, 1997; Taylor and Waldron, 1997; Fotadar *et al.*, 2006), the American lobster, *Homarus americanus* (Chang *et al.*, 1998; Lorenzon *et al.*, 2007), Norway lobster, *Nephrops norvegicus* (Harris and Andrews, 2005a, b; Bernasconi and Uglow, 2008), and in crabs such as the edible crab, *C. pagurus* (Webster, 1996; Lorenzon *et al.*, 2008), green crab, *Carcinus maenas* and velvet crab, *Necora puber* (Durand and Regnault, 1998), and spider crab *Maja squinado* (Durand *et al.*, 2000).

Such studies allow the identification and comprehension of potential stressors during the trade chain, which is essential for developing improved procedures leading to less mortality and a better quality of live animals. Additionally, such studies are important for developing the criteria capable of recognizing stressors and reversing the effects along the market chain (Gómez-Jiménez *et al.*, 2001).

In this context, this study aimed to identify the main stressors in the distribution chain from the fishing vessel in England to a typical import facility in Portugal and to ascertain changes in the haemolymph chemistry (pH, D-glucose, L-lactate, and haemocyanine) and crab vigour, physical injury, and mortality after transportation and storage. To our knowledge, no other studies have assessed crab vigour and related it to changes in haemolymph parameters. Such information is important to aid implementation of preventive approaches in the trade of live crustaceans, such as codes of practice, which will lead ultimately to improvements in overall intrinsic quality, survival, and welfare of the crabs and profitability in international markets.

Material and methods

Experimental design

The general procedure is depicted in Figure 3.1.1. Edible crabs were harvested by a commercial fishing vessel operating traps in the English Channel at a depth of 70 m in late April 2009. The fishing trip took 8 days and 7 t of edible crab were captured and maintained on board in tanks supplied with running seawater at ambient temperature; 30 kg of the catch died on board. Part of the catch (6.5 t) was sold to a Portuguese importer that purchases edible crab every 2 weeks from the same fishing vessel and distributes them across Portugal.

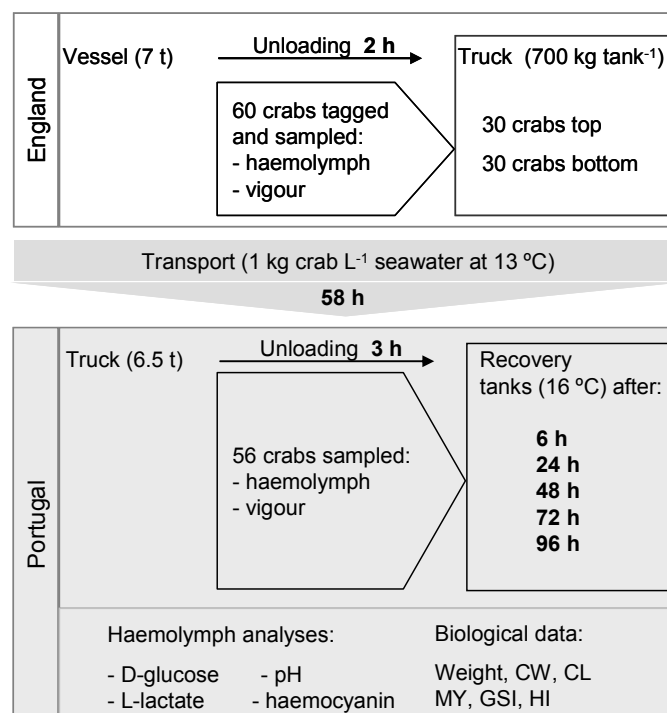


Figure 3.1.1 Diagram summarizing the experimental procedure. Abbreviations: (CW) carapace width; (CL) carapace length; (MY) claw muscle yield; (GSI) gonadosomatic index; (HI) hepatossomatic index.

On arrival of the catching vessel at the port of Weymouth, England (20:00 h local time), seawater was emptied from the fishing vessel tanks and the 6.5 t of crabs unloaded directly to tanks in a vivier lorry operated by the Portuguese import company (tank dimension: 1.1 m wide, 1.4 m high, 1.5 m long). The tanks had been pre-filled with seawater at 18 °C from a local holding facility with an open system, and then refrigerated to 13 °C. Unloading was carried out with plastic buckets taking 40 kg of crabs at a time; it took 2 h to unload all the crabs.

Those crabs awaiting loading to the truck were kept exposed to air in the plastic buckets at air temperature (~19 °C). Meanwhile, 60 crabs were tagged, and haemolymph samples were taken during the unloading process (before transport, BT). Care was taken to avoid extra stress due to sampling, so tagging consisted of a numbered plastic brace being wrapped around one claw and a marked number pencilled onto the carapace (Figure 3.1.2); tagging took ~30 s *per* crab, and it was non-invasive. Haemolymph was sampled in no more than 50 s (~1 mL) from the fifth walking leg with 1.2 mL paediatric syringes (Sarstedt, S-monovet) and 20 Gx 1(1/2)' hypodermic needles (Sarstedt, S-monovet needle). Haemolymph samples were stored immediately in cryogenic microtubes (Nalgene, 1.5 mL) in liquid nitrogen to preclude clotting and physiological reaction. In all, 30 tagged crabs were placed in the bottom of a tank on the vivier lorry immediately after haemolymph sampling, and the balance of tagged crabs placed at the top of the same tank after 2 h of being exposed to air in a bucket. Each tank carried ~700 kg of crab at a density of 1 kg L⁻¹ seawater. Animals were selected randomly and were all intermoult hardshelled of both sexes.



Figure 3.1.2 The plastic tag wrapped around the claw and the pencil-numbered mark on the carapace of the edible crabs (Photos by Sara Barrento ©).

Transport from England to Portugal lasted 58 h and during this period, four tags were lost. On arrival to the import facilities and subsequently at the stocking tanks, seawater parameters (pH, temperature and dissolved oxygen) were measured with a Multi 350i compact precision hand-held meter (WTW, Wissenschaftlich-Technische Werkstätten, Weilheim). Before measurement, calibration was performed with stock solutions. The surviving 56 tagged crabs were sampled for haemolymph, and damage was recorded (*i.e.* missing limbs and claws).

The unloading process from the vivier lorry to storage tanks took 3 h, and during this period (after transport, AF) crabs were exposed to air, haemolymph was sampled and vigour checked. In the following 96 h crabs were maintained in the storage tanks and haemolymph was sampled periodically at 6, 24, 48, 72, and 96 h. The haemolymph parameters of ten crabs maintained for 2

weeks in the stocking tanks were considered as control. Crabs were not fed at all during the experiment, and their vigour was always checked at the time of bleeding. Vigour was assessed by reference to behaviour, such as eye-stalk response, and the strength of claws and walking legs. A scale of vigour was adapted from Spanoghe and Bourne (1997), and five classes of health status were defined (Table 3.1.1).

Table 3.1.1 Vigour scale of crabs, adapted from Spanoghe and Bourne (1997).

Somatic response	0 (dead)	1 (moribund)	2 (weak)	3 (healthy)	4 (very healthy)
Eyestalk response	No	Yes	Yes	Yes	Yes
Claws and legs weightless	Yes	Yes	No	No	No
Slow claw and leg movements	No	No	Yes	No	No
Evident claw and leg movements	No	No	No	Yes	Yes
Strong claws with quick movements (aggressive); claws and legs strongly huddled to the abdomen (defensive)	No	No	No	No	Yes

Data collection

Total weight, carapace width (CW), and carapace length (CL) of the crabs were recorded at the end of the experiment (n=56); 30 of them, including dead crabs, were dissected and their tissues collected to determine claw muscle yield (MY), hepatossomatic index (HI), and gonadossomatic index (GSI). The values of MY, GSI, and HI were calculated as follows: [tissue wet weight (g)/ body wet weight (g) × 100].

Live crabs were euthanized before being dissected, by piercing the two nerve centres by a stainless steel rod, the crabs having been kept under refrigerated conditions (~4 °C) for 1 h to decrease their metabolism and to stun them before being euthanized. The rod was inserted through one of the eyes and through the vent. Ovarian maturity stage was also identified using a scale adapted from Edwards (1979). Females were classified as stage 1 (S1) when no ovarian tissue was found (immature), as stage 2 (S2) when initial ovary development was characterized by a small spot of light orange near the posterior part of the carapace, as stage 3 (S3) when the developing ovaries extended into the carapace and were orange in colour, and as stage 4 (S4) when the ovaries were ripe, filling the carapace with bright red ovarian material.

For all haemolymph parameters studied, 30 haemolymph samples were analysed in duplicate for each period: before transport (0 h), after transport (58 h), and during recovery (6, 24, 48, 72, and 96 h after arrival).

D-Glucose

Haemolymph was deproteinated according to the procedure of Paterson *et al.* (1997). Thawed-vortexed haemolymph was deproteinated by mixing with an equal volume of cold 0.6 M perchloric acid (Panreac), and the inactivated proteins were precipitated by centrifugation for 10 min at 40 000g (Sigma 3k30, rotor no. 12154; 24 × 1.5 - 2.2 mL; Osterode am Harz). The supernatant was

neutralized with 3 M potassium hydroxide (Sigma), and the precipitated potassium perchlorate formed was separated by centrifuging at 40 000g for 10 min. The supernatant was collected in microtubes, and D-glucose concentration was measured using a D-glucose assay kit (Sigma GAGO-20). Briefly, samples of haemolymph (150 µl) were incubated for 30 min at 37 °C with 300 µl of the assay reagent in disposable cuvettes. In this step, D-glucose is oxidized to gluconic acid and hydrogen peroxide by D-glucose oxidase. Hydrogen peroxide reacts with o-ianisidine in the presence of peroxidase to form a coloured product. The reaction was stopped by the addition of 300 µl of 12 N sulphuric acid (Riedel-deHaën, 95-97 %), which reacts with the oxidized o-dianisidine to form a more stable pink product. The intensity of the colour measured at 540 nm (using a spectrophotometer Unicam UV-Vis spectrometry Helios Alpha) is proportional to the original D-glucose concentration. D-Glucose concentration was calculated from a calibration curve using standards of known concentration (Sigma D-glucose standard solution G6918).

L-Lactate

Haemolymph was deproteinated as above, and the L-lactate concentration measured using L-lactate reagent Trinity Biotech (no. 735-10). Samples of haemolymph (5 µl) were added to the reconstituted L-lactate reagent (500 µl), then incubated for 10 min at room temperature. In this step, lactic acid is converted to pyruvate and hydrogen peroxide by the enzyme L-lactate oxidise. The enzyme peroxidase catalyses the oxidative condensation of the chromogenic precursors to form a coloured dye with a maximum absorbance of 540 nm that was read on a spectrophotometer (Unicam UV-Vis spectrometry Helios Alpha). L-Lactate concentration was calculated from a calibration curve prepared with standards of known concentration (Trinity L-lactate standards set no. 735-11).

Haemocyanine

Haemocyanine was determined spectrophotometrically (Unicam UV-Vis spectrometry Helios Alpha) in 10-mm quartz cuvettes using 50 µl of thawed haemolymph diluted in 2 mL of distilled water. Absorbance was measured at 335 nm, and haemocyanine concentration (mg mL^{-1}) was calculated from the molar extinction coefficient $E^{\text{mg mL}^{-1}}_{(1\text{cm})} = 0.0283$, as previously described by Harris and Andrews (2005).

pH

Thawed-vortexed haemolymph samples free of clots were kept at 13 °C (haemolymph from transport crabs) or 16 °C (haemolymph from recovery crabs). Samples were measured with a pH meter (Hanna Instruments, HI 221) using a pre-calibrated micro-electrode (Hanna Instruments, HI 1083P) and pH buffers 4.01, 7.01, and 10.00 (WTW, Wissenschaftlich- technische Werkstätten) as standards.

Statistics

Results were expressed as mean value $\pm$ standard deviation (sd). To satisfy ANOVA assumptions, the data were transformed, and then subjected to multiple comparison tests (Tukey honestly significant differences) to identify differences between groups. If transformed data could not meet ANOVA assumptions, non-parametric analysis of variance (Kruskal-Wallis) was performed, followed by a non-parametric multiple comparisons test (Mann-Whitney). Whenever two groups were compared, a Student's t-test was applied instead of ANOVA. If data could not meet Student's t-assumptions, the correspondent non-parametric test (Mann-Whitney) was used. Data on recovery duration and vigour were subjected to correlation analyses, using the Pearson (r) correlation coefficient. All statistical analyses were tested at a level of probability of 0.05 with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa OK74104, USA).

Results and discussion

Seawater quality

After 58 h transport, the seawater from the vivier tanks was very low in dissolved oxygen 2.8 ± 0.8 mg L⁻¹ (Table 3.1.2), which corresponds to the lower limit of 3.0 mg L⁻¹ recommended for the edible crab (Chartois *et al.*, 1994). Moreover, the pH value of 7.41 was low compared with that of the natural environment, varying from 7.8 to 8.2 (Chartois *et al.*, 1994). At the stocking tanks, the seawater dissolved oxygen, temperature, and pH were similar to those experienced by the crabs in the wild.

Table 3.1.2 Seawater temperature, dissolved oxygen and pH in the vivier tanks after 58 h of transport, in the holding tanks before loading the crabs (empty), and during recovery.

Tank	Temperature (°C)	Oxygen (mg L ⁻¹)	pH
Transport	10.9 ± 0.4	2.8 ± 0.8	7.41 ± 0.05
Holding (empty)	12.6 ± 0.0	9.1 ± 0.5	7.72 ± 0.04
Holding (recovery)	14.6 ± 0.1	7.1 ± 0.8	7.73 ± 0.02

Biological data and vigour

The biometric data of sampled crabs and the maturity stages of females are listed in Table 3.1.3. Most females were at maturation stage S2 (57.1 %) with a GSI of 1.3 ± 0.4 % or S1 (35.7 %) with a GSI of 0.4 ± 0.3 %, and just one female was recorded as S3 with a GSI of 2.4 %. There were no ripe females (S4).

Between capture and landing at Weymouth, 0.43 % mortality (30 kg out of 7,000 kg) was registered, whereas during transport to Portugal, mortality rose to 0.92 % (60 kg out of 6,500 kg), although this value increased further to 20 % after 4 days in the recovery tanks. Similar values of mortality were recorded at the end of the experiment, totalling 19.6 %, of which 10.7 % were crabs transported at the top and 8.9 % crabs transported at the bottom of the tanks.

Table 3.1.3 Biometric data of male and female crabs and ovarian maturation stages.

Parameter	Female (n = 14)	Male (n = 16)
Weight (g)	675.4 ± 223.7	784.9 ± 184.0
CW (mm)	156.7 ± 16.4	163.5 ± 16.2
CL (mm)	97.5 ± 9.5	100.1 ± 6.1
MY (%)	9.9 ± 1.9	13.1 ± 1.9
HI (%)	12.7 ± 3.8	13.2 ± 3.9
GSI (%)	1.0 ± 0.7	0.9 ± 0.8
S1 (%)	35.7	–
S2 (%)	57.1	–
S3 (%)	7.1	–
S4 (%)	0.0	–

Abbreviations: (CW) carapace width; (CL) carapace length; (MY) claw muscle yield; (HI) hepatosomatic index; (GSI) gonadosomatic index, (S1–S4) female ovarian maturation stages 1 to 4.

The pattern of vigour in the recovery tanks suggested that among the crabs transported at the bottom, mortality occurred within the first 48 h (13.3 %), whereas crabs transported at the top only started to die after 48 h (7.7 %; Figure 3.1.3).

These values are lower than the 30 % reported by Lorenzon *et al.* (2008) and much lower than those reported by Uglow *et al.* (1986), who monitored a consignment of 90 % female *C. pagurus* which, on arrival at their destination, had suffered mortality of ~50 %, increasing to 70 % after 2 days at the importer's premises.

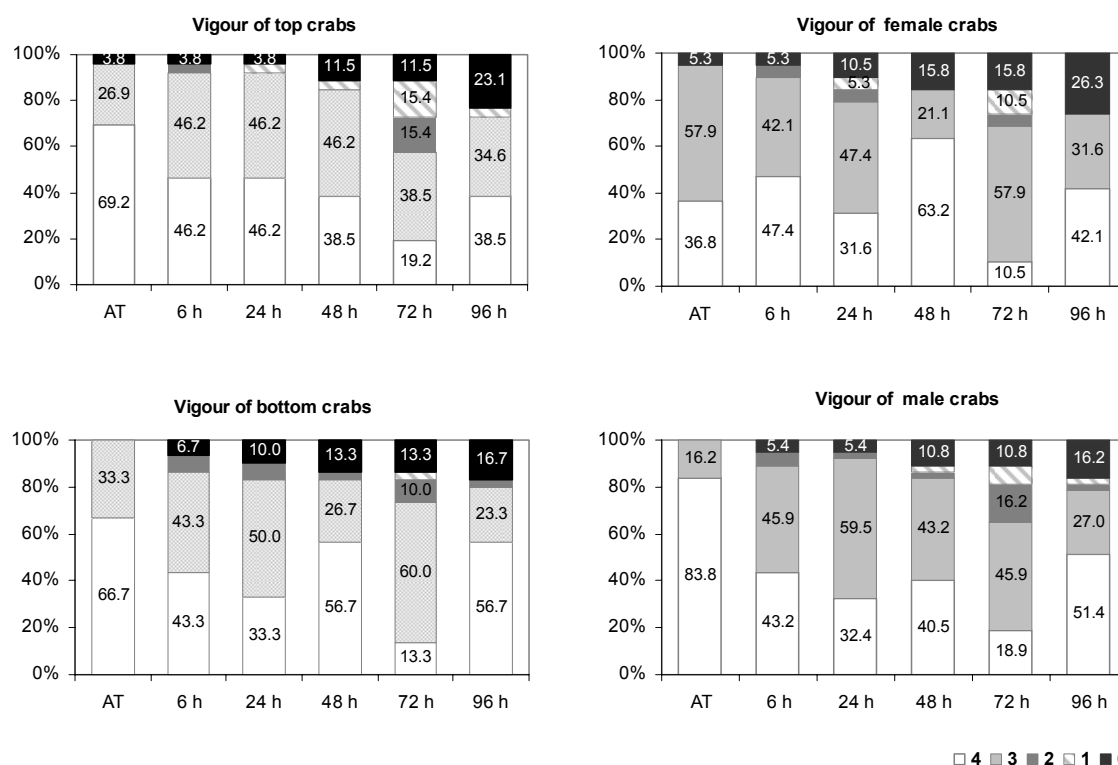


Figure 3.1.3 Vigour levels of crabs transported at the top (n=26) and bottom (n=30) of the tanks and of male (n=37) and female (n=19) after transport (AT) and at the recovery tanks after 6, 24, 48, 72, and 96 h. Vigour-level index: (0) dead crabs (cumulative); (1) moribund crabs; (2) weak crabs; (3) healthy crabs; (4) very healthy crabs.

Ugnow *et al.* (1986) suggested that the most likely cause of the high mortality rate was a condition factor, probably related to the breeding cycle, because 60 % of female crabs were at the point of spawning or immediately post-spawning. In the present study, the comparatively lower rate of mortality might be attributable to the fact that there were less females and that most of them were either at the beginning of ovary development or immature, but also because this study was carried out in late April, and mortality is known to be heavier in summer (Ugnow *et al.*, 1986).

After 58 h of transport, most crabs were still very healthy (vigour level 4) or healthy (vigour level 3; Figure 3.1.3). During the first 48 h of recovery in the stocking tanks, vigour levels stayed at stages 4 and 3, although between 48 and 72 h, the percentage of weak (vigour level 2) and moribund crabs (vigour level 1) increased mostly in crabs at the top of the tanks, resulting in 11.6 % mortality in the next 24 h while the comparative figure for crabs at the bottom of the tanks was just 3.4 %.

Overall, most crabs classified as moribund died (67 %), compared with just 8 % of crabs classified as weak. This suggests that moribund (vigour level 1) crabs were weakened to a point at which homeostasis was impaired, leading to death, whereas most weak crabs (vigour level 2) were still able to cope with the stress levels to which they were subjected and recovered. Comparing in terms of vigour levels of females and males (Figure 3.1.3), males seem to have been more vigorous after transport (vigour level 4; 83.8 %) than females (vigour level 4; 36.8 %). The level of vigour apparently influences the recovery process, especially after 48 h, and ultimately 26.3 % of the females died compared with just 16.2 % of the males.

Physical injuries were also monitored after transport and during recovery (Table 3.1.4). Missing limbs and claws are the symptoms of stress that can further weaken the crabs (Juanes and Smith, 1995). Moreover, lost limbs contribute towards economic loss, particularly in males, because their selling price is usually higher than females because of their larger claws (Sub-chapter 2.1).

After transport, crabs at the bottom of the tanks had more missing legs (ML1) than crabs at the top of the tanks, and male crabs at the bottom had more missing claws than female crabs (Table 3.1.4). These results suggest that males lose their claws more often than females, possibly related to the more aggressive behaviour typical of males (Edwards, 1979). These findings should, however, be investigated further, especially considering that larger males are graded on board and transported in separate tanks in the vivier trucks.

Table 3.1.4 Physical injuries (%) of female and male crabs.

Tank position	Sex	ML1	ML2	MC1	MC2	L2C1	L1C2
Bottom	Female	25 (13)	0 (13)	0	0	0	0
Bottom	Male	18 (23)	0	9 (14)	0	5	0
Top	Female	18	9 (18)	0	0	18	0
Top	Male	13	0	0 (7)	0 (7)	0	0 (7)

Abbreviations: (ML1) missing one leg; (ML2) missing two legs; (MC1) missing one claw; (MC2) missing two claws; (L2C1) missing two legs and one claw; (L1C2) missing one leg and two claws. Data report to the period immediately after 58 h of transport at the top (females, n=11; males, n=15) and bottom (females, n=8; males, n=22) of the tank, and at the end of the recovery period (values in parentheses).

Haemolymph parameters

There were no differences in haemolymph parameters between males and females. On the other hand differences between crabs at the top and the bottom of the tanks were found and it is clear that the extra hour that crabs at the top were exposed to air before transport influenced L-lactate, pH, and haemocyanine (Table 3.1.5). Crabs at the top of the tank had greater concentrations of L-lactate and haemocyanine, but a lower pH than crabs at the bottom.

D-glucose

Haemolymph D-glucose is the major fuel for adenosine triphosphate (ATP) production in decapod crustaceans, which provides the chemical energy necessary to maintain metabolic processes. Therefore, the levels of D-glucose should reflect in part the potential activity of the crabs (Briffa and Elwood, 2001). The concentration pattern obtained in the present study, with hyperglycaemia developing during transport (Figure 3.1.4), is similar to that obtained by Lorenzon *et al.* (2008). Hyperglycaemia has been reported in decapod crustaceans as a result of endocrine-mediated mobilization of D-glucose from stored reserves causing various types of stress, including handling, hypoxia, and anoxia (Taylor and Spicer, 1987; Spicer *et al.*, 1990; Danford *et al.*, 2001; Lund *et al.*, 2009). D-Glucose concentration showed a significant negative correlation with animal vigour ($r = -0.31$; $p < 0.01$), meaning that vigour level 4 (very healthy) corresponded to lower D-glucose concentrations, whereas weak and moribund crabs were hyperglycaemic. There was also a clear negative correlation with recovery duration ($r = -0.38$; $p < 0.01$). The slight increase in D-glucose during the first 24 h of recovery period might be related to further stress on the crabs during unloading to the recovery tanks (Figure 3.1.4). That process can last up to 3 h as crabs are stacked without water in the tanks of the vivier truck. Usually one handler is inside the tank standing above the crabs and grading them. However, in this study, the unloading process from the boat to the vivier truck clearly did not influence D-glucose concentration. The lack of statistical differences might be due to variability in the haemolymph D-glucose concentration. The level of D-glucose is controlled by reactions to external stimuli and the physiological status of individual crabs (Matsumasa and Murai, 2005), so differences might be masked by individual diversity. Lorenzon *et al.* (2008) reported that edible crab transported during 36 h in seawater returned to basal levels of D-glucose after 96 h of recovery, so the fact that the edible crabs in the current experiment did not reach such levels might be a consequence of the duration of stress to which they were exposed (58 h).

L-lactate

L-Lactate concentration varied widely between crabs, particularly after 6 h of recovery. Nonetheless, there was a significant increase during the 58 h of transport (from 2.19 to 5.86 mM), and values stayed high (5.25 mM) during the subsequent 6 h of recovery (Figure 3.1.4). After 24 h of recovery in the tanks, no statistical differences were found between the experimental and control groups (2.02 and 1.40 mM, respectively). The L-lactate concentration in the control group was similar to *in situ* measurements (1.6 mM) carried out on *C. pagurus* by Watt *et al.* (1999).

Table 3.1.5 Haemolymph parameters (D-glucose, L-lactate, haemocyanine, and pH) of crabs before transport (BT), after 58 h of transport (AT), and during recovery (after 6, 24, 48, 72, and 96 h; n = 30).

Parameters	Tank position	BT	AT	Recovery				
				6 h	24 h	48 h	72 h	96 h
D-glucose (mM)	Top	0.27 ± 0.08a	1.73 ± 0.92a	1.49 ± 0.51a	2.31 ± 0.68a	1.14 ± 0.64a	1.02 ± 0.32a	1.00 ± 0.56a
	Bottom	0.25 ± 0.10a	1.13 ± 0.74a	1.57 ± 0.77a	1.91 ± 0.76a	1.15 ± 0.37a	0.91 ± 0.26a	0.89 ± 0.33a
	p	0.39	0.05	0.99	0.07	0.41	0.21	0.80
L-lactate (mM)	Top	2.80 ± 1.20a	6.09 ± 1.31a	5.83 ± 4.18a	2.23 ± 2.07a	0.60 ± 0.31b	1.57 ± 1.21a	1.30 ± 1.94a
	Bottom	1.92 ± 0.75b	5.77 ± 1.23a	5.02 ± 2.53a	1.93 ± 1.47a	1.13 ± 0.89a	0.83 ± 0.39a	0.83 ± 0.66a
	p	0.00	0.46	0.94	0.83	0.01	0.14	0.27
pH	Top	7.09 ± 0.11b	7.28 ± 0.06b	7.33 ± 0.12a	7.27 ± 0.10a	7.25 ± 0.08a	7.34 ± 0.16a	7.29 ± 0.11a
	Bottom	7.19 ± 0.11a	7.33 ± 0.06a	7.30 ± 0.13a	7.23 ± 0.14a	7.22 ± 0.11a	7.25 ± 0.10a	7.21 ± 0.10a
	p	0.00	0.01	0.65	0.70	0.35	0.11	0.10
Haemocyanine (mg mL ⁻¹)	Top	51 ± 19a	52 ± 17a	48 ± 19a	51 ± 21a	46 ± 23a	40 ± 17a	42 ± 23a
	Bottom	37 ± 18b	49 ± 16a	50 ± 16a	45 ± 16a	41 ± 13a	43 ± 17a	37 ± 13a
	p	0.00	0.76	0.74	0.48	0.57	0.67	0.59

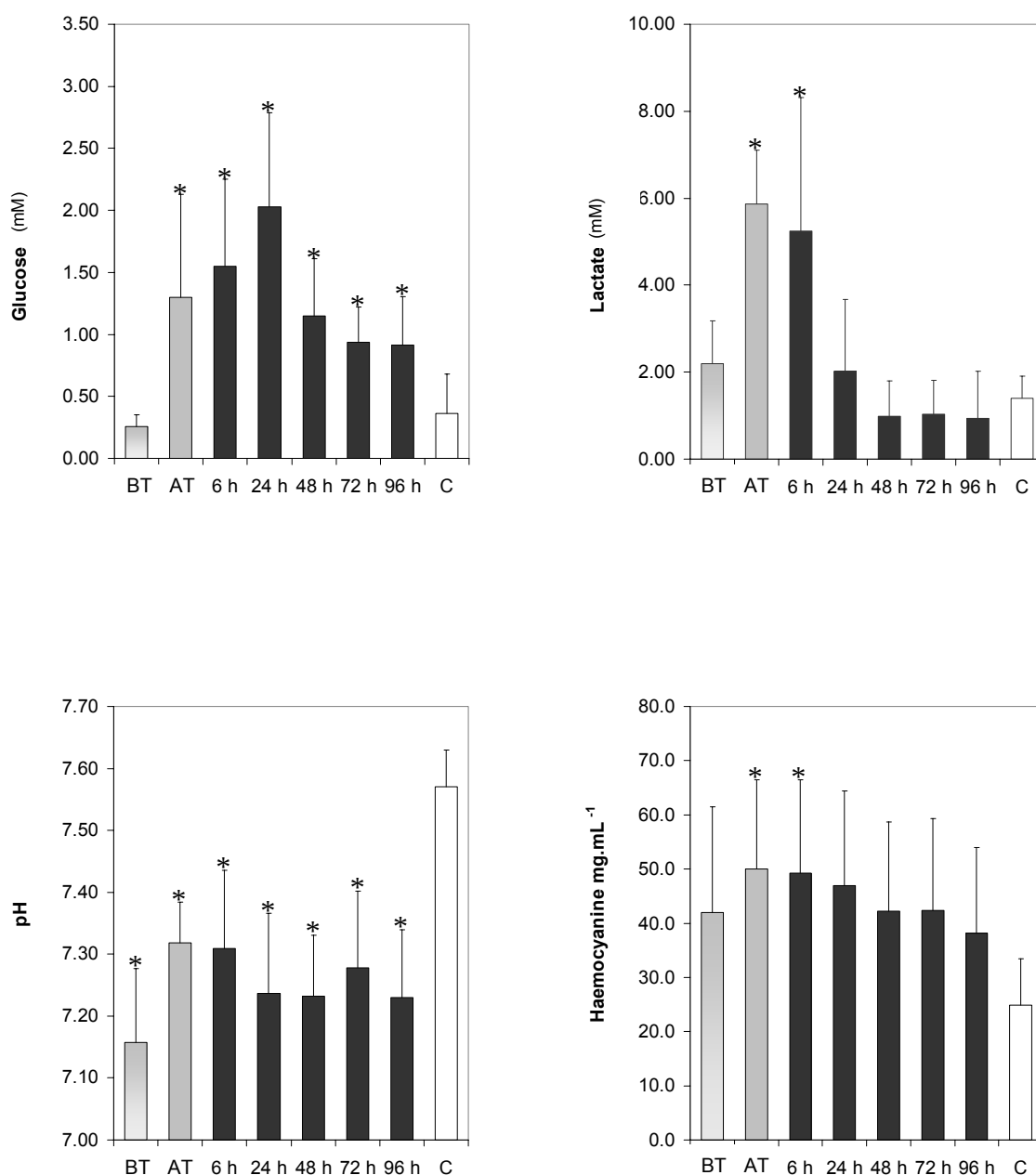


Figure 3.1.4 D-Glucose, L-lactate, pH, and haemocyanine variations in the haemolymph of crabs (n= 30), before transport (BT, light grey bar), after 58 h of transport (AT, solid grey bar), and during recovery after 6, 24, 48, 72, and 96 h (black bars), and in the control group (C, white bar). Values are presented as average $\pm$ standard deviation. The asterisk means that the result was significantly different from that of the control mean ($p < 0.05$).

Therefore, only after 24 h do the values revert to basal levels. L-Lactate is the major end product of anaerobic metabolism in crustaceans (Spicer *et al.*, 1990), so an increase in its level in the haemolymph of *C. pagurus* is indicative of an insufficient supply of oxygen to tissues and an inability to excrete this metabolic end-product.

Hence, the crabs were transported under hypoxia and acidic conditions most probably as a result of increased carbon dioxide and initial L-lactate excretion. Haemolymph pH stayed acidic during the entire experiment, never reaching control values, which might explain the mortalities even after L-lactate had re-attained resting levels (Table 3.1.5; Figure 3.1.4). Even pH values in the control

group (7.57) were lower than basal levels (7.75) measured *in situ* in the same species by Watt *et al.* (1999).

Haemocyanine concentrations were very wide between individual crabs, but statistically higher values were observed after transport and in the subsequent 6 h of recovery compared with the control group (Figure 3.1.4). Similar values were reported by Uglow *et al.* (1986), ranging from 20.42 to 63.62 mg L⁻¹. Haemocyanine is the most important protein in haemolymph (75-100 % of the total haemolymph protein) and is responsible for oxygen transport and serves as a food reserve. Haemocyanine can be disabled by several stressors, such as extreme oxygen deprivation, but compensatory synthesis of additional haemocyanine in response to hypoxia has been reported (Hagerman and Baden, 1990). In this context, the observed increase could have been a direct response to the hypoxia experienced during transport. The variability among the crabs indicates that the initial nutrition condition was different, which may further have influenced the ability of the crabs to cope with stressful conditions during transport and subsequent recovery without feeding (Uglow *et al.*, 1986). Haemocyanine levels are particularly important if animals are retained for several weeks at holding facilities without feeding, because haemocyanine can be catabolized as a food source, along with hepatopancreas and muscle. In open-water systems subject to fluctuation in temperature, haemocyanine and tissue catabolism might be a real problem during summer when the seawater temperature usually rises, promoting a further increase in metabolism.

Conclusions

The unloading process of non-immersed crabs, poor water quality (*e.g.* low oxygen and low pH), and high animal densities during transport seemed to be the most important stressors on the crabs. After transport and during recovery, physical damage was reported, for instance limb loss and an impairment of aerobic metabolism with a shift to anaerobic metabolism. Consequently, D-glucose concentration increased as a source of fuel necessary for anaerobic metabolism, resulting in the production and accumulation of L-lactate. There was also acidification of haemolymph, which might explain crab mortality even after L-lactate had re-attained control levels.

The results of this study have revealed that crabs transported at the bottom of tanks react differently from crabs transported at the top of the tanks. Throughout the loading process from the boat to the vivier truck, crabs at the top were put in seawater tanks an hour after the crabs at the bottom. During this extra hour, the crabs were kept in buckets without seawater, so influencing initial haemolymph parameters and mortality before transport, after transport, and after 48 h of recovery. Additionally, crabs at the bottom of the tanks had more lost limbs and claws than crabs at the top, especially males.

All these results demonstrate that transport conditions need to be improved significantly. Moreover, the transport of immersed crabs requires a better quality of seawater through efficient filtration systems specifically designed for vivier trucks and a supply of pure oxygen to the seawater. Alternatively, transport without seawater could be tested and optimized, so avoiding the extra cost of transporting the seawater. This is already common practice for trading species of homarid and

spiny lobster (Sub-chapter 2.1), and the industry is also clearly interested in transporting *C. pagurus* exposed to air, as previously suggested by Danford *et al.* (2001) and Lorenzon *et al.* (2008). Minimum handling could be implemented by designing mechanical unloading processes. Still, such improvements need to be cost effective and tested rigorously. Additionally, it is basic to grade crabs before transport, and this study showed that the vigour levels of crabs, though a subjective observation, can allow a good prediction of crab survival. Therefore, vigour levels can be important in selecting edible crabs strong enough to cope with the stress of transport.

Sub-chapter 3.2

C. pagurus simulated transport

Barrento, S., Marques, A., Vaz-Pires, P., and Nunes, M. L. *Cancer pagurus* (Linnaeus, 1758) physiological responses to simulated live transport: influence of temperature, air exposure and AQUI-S® (submitted to ICES Journal of Marine Science, November 2009).



Cancer pagurus is a commercially important crab mostly appreciated in Southern Europe countries, including Portugal to where it is usually live transported from the UK and France. Once in Portugal, crabs are redistributed across the country in small refrigerated vivier lorries in air or immersed conditions, while some are sent by air to the Azores archipelago with mortality reaching 40 to 60 %. In order to optimize transport conditions and survival, simulated live transport of immersed and air exposed crabs sedated or not with an anaesthetic, AQUI-S® was tested at different temperatures. It was found that crabs experienced stress during the experiment (increased L-lactate, D-glucose and lowered pH), but with different magnitudes according to temperature, treatment and transport duration, resulting in 100 % mortalities at 16 °C in immersed seawater and in the treatment group exposed to air with AQUI-S®. Results indicate that long duration transport in semi-dry conditions is viable at low temperatures (8 °C), while immersed transport is viable at 12 °C. AQUI-S® was not an efficient solution at low temperatures in semi-dry conditions, but for short duration transport in immersed conditions at 16 °C it was the only treatment without mortality.

Keywords: AQUI-S®, crustaceans, edible crab, stress, vigour, temperature, air exposure.

Introduction

The European edible crab, *Cancer pagurus*, is one of the most important commercial species in England, Wales and Ireland (Tully *et al.*, 2006). However, this species is most appreciated in southern European countries, such as France, Spain, Italy and Portugal where the demand for live edible crab is high, but the production is almost inexistent, e.g. Portugal (Sub-chapter 2.1). The journey is performed in vivier lorries from fishing countries to Portugal taking about 2 to 3 days and live crabs are transported in immersed conditions with transport mortalities of 1 % (Sub-chapter 3.1). Once in Portugal crabs are either placed in plastic boxes exposed to air or immersed in tanks to be redistributed across the country in small refrigerated vivier trucks (12 °C), which further increases mortalities at the wholesalers and retailers (up to 40 and 60 % respectively; Sub-chapter 2.1). Some crabs are sent by air to the Azores archipelago in Styrofoam sealed boxes (20 kg each; approximate transport duration 12-24 h) with coolers, but mortalities are also high (40 %). It is therefore highly recommended to improve national transport systems for edible crabs.

The transport of homarids and palinurid lobsters exposed to air has proven to be efficient, showing low mortalities (Paterson and Spanoghe, 1997). This type of transport can be an interesting approach in the live distribution of *C. pagurus* as it might elicit lower mortalities than those reported by traders. However, to achieve better survival rates several considerations must be taken into account; for instance *Cancer pagurus* occupies subtidal habitats and, therefore, rarely experiences air exposure except during live marketing. Furthermore, environmental temperatures are usually of low amplitudes (from 4 to 16 °C) compared with other species like *Homarus americanus* (from -1 to 24 °C) (Chartois *et al.*, 1994; Lorenzon *et al.*, 2007). Several studies reported that the gills collapse during emersion of subtidal species, resulting in respiratory acidosis, internal hypoxia and anaerobic metabolism (Durand and Regnault, 1998). Therefore, the transport conditions (e.g. temperature, moisture, duration) must be optimized to the specific needs of *C. pagurus*. Several studies have been conducted on the effect of emersion to physiological responses of crustaceans, like lobsters (Whiteley and Taylor, 1992; Jussila *et al.*, 1997; Paterson and Spanoghe, 1997; Fotedar *et al.*, 2006; Lorenzon *et al.*, 2007; Márques-Ríos *et al.*, 2007) and crabs (Otwell and Webb, 1977; Durand and Regnault, 1998; Danford *et al.*, 2001). However, as far as the European edible crab is concerned only two studies were carried out so far by Uglow *et al.* (1986) and Lorenzon *et al.* (2008). In the former, the effect of air exposure and starvation were studied and it was concluded that both conditions were adverse to *C. pagurus*. While the later study evaluated a consignment of crabs transported from France to Italy during 36 h in semi-dry conditions and it was concluded that air transport of *C. pagurus* appears to have a positive impact on animal condition and welfare.

Anaesthetics are being tested in crustacean fisheries, following their successful application in finfish aquaculture industry, not only to avoid mortalities but also for welfare reasons (Seccombe, 2001). Methods of paralyzing crabs can benefit commercial procedures but it is important to ensure that the quality of the product is not harmed by effects such as autotomy, which also negates the use of toxic chemicals (Gardner, 1997). AQUI-S[®] is a commercial fish anaesthetic with isoeugenol

as active ingredient that is approved for food-safe use in New Zealand, Australia, Chile, Korea, Costa Rica and Honduras (Bowker *et al.*, 2006). In the European Union and USA, AQUI-S[®] is undergoing the approval process for use on seafood. This anaesthetic has the advantage of zero withdrawal period before animals can be slaughtered. Though the mode of action of isoeugenol is not yet fully understood, it has proven to be effective on freshwater prawns (*Macrobrachium rosenbergii*), but only at concentrations 5 to 10 times higher (100 to 200 mg L⁻¹) than those used on finfish (20 mg L⁻¹; Coyle *et al.*, 2005; Meyer *et al.*, 2008). AQUI-S[®] efficacy has never been tested so far in the edible crab.

In this context, this study aimed to understand *C. pagurus* physiological changes during different transport systems, namely in immersed and semi-dry conditions, using AQUI-S[®] as an anaesthetic at different transport temperatures. An increased understanding of the physiological changes imposed to crabs during transport may lead to improvements in commercial handling methods with better survival rates, less waste and more efficient and sustainable utilization of this valuable resource.

Material and methods

Ethical Statement

Though crustaceans are not protected in the scientific procedures act, all live animals utilized in the experiments have been treated with proper care minimizing discomfort and distress. Also, the number of animals was kept to the minimum necessary to obtain scientific results, considering that the gain in knowledge and long term benefit to the subject species is high.

Collection and treatment of animals

A total of 95 intermoult crabs with nicked claws (procedure used by fishermen to inactivate claws) (average weight 667.3 ± 93.2 g; width 134.1 ± 29.6 mm; length 115.9 ± 29.1 mm) were sampled in this study. Crabs were obtained in summer from a local importer, where they were maintained in a well aerated seawater re-circulating system at 12 °C without feeding for 6 days to recover from capture and transport prior to the experiment. Refrigerated truck transport was simulated during 48 h in cooling chambers at three temperatures (8, 12 and 16 °C) with rescaled plastic tanks measuring 16.5 cm in depth, 28.0 cm width and 38.0 cm length. Crabs were exposed to four treatments at each temperature: a) immersed in aerated seawater (WT; 1 kg crabs L⁻¹); b) pre-anaesthetized and immersed in aerated seawater with dissolved AQUI-S[®] (New Zealand, Ltd.; 40 mg L⁻¹; WQS; 1 kg crab L⁻¹); c) exposed to air and covered with seawater-soaked cloth (AIR); and d) pre-anaesthetized, exposed to air and covered with dissolved AQUI-S[®] (40 mg L⁻¹) soaked cloth (AQS). Crabs exposed to air were placed in boxes with a drainage system to avoid direct contact with excretion fluids. Crabs' pre anaesthetisation involved immersion in seawater at 12 °C with dissolved AQUI-S[®] (300 mg L⁻¹) for 10 to 15 minutes until absence of claw aggressiveness reflex and reaction handling (AQUI-S[®] NZ, 2009). Animals were monitored by haemolymph bleeding and vigour determination, at 0 (immediately before being put in tanks), 3, 8, 24 and 48 h. Care was taken to avoid extra stress attributable to sampling as haemolymph was sampled in no more than

50s. Vigour was visually estimated and scaled according to behavioural observations, such as eye-stalk response, strength of claws and walking legs (with the modified scale of Sub-chapter 3.1; Table 3.2.1). Animals used for simulated transport were chosen similarly to industry common practices, *i.e.* selection of crabs without weakness signs (vigour 3).

Haemolymph was sampled (1 mL) from the fifth walking leg with 1.2 mL pediatric syringes (Sarstedt, S-monovet) and 20 Gx 1(1/2'') hypodermic needles (Sarstedt, S-monovet needle), and transferred to microtubes where pH was immediately measured. Blood parameters of 10 undisturbed animals maintained for eight days without feeding in the stocking tanks of the importer were analyzed and considered as the control group. Several seawater quality parameters (pH, temperature, and dissolved oxygen) were measured in the beginning and end of the simulated live transport of immersed crabs with a Multi 350i compact precision handheld meter (WTW, Wissenschaftlich-Technische Werkstätten). Before measurements, calibrations were performed with calibration solutions. An aquarium test kit was used to measure ammonia (Nutrafin, Ref. A-7820) and nitrites (Nutrafin, Ref. A-7825).

Table 3.2.1 Vigour scale of European edible crab, adapted from sub-chapter 3.1.

Somatic response	0 (dead)	1 (moribund)	2 (weak)	3 (Strong)
Eyestalk response	No	Yes	Yes	Yes
Claws and legs weightless	Yes	Yes	No	No
Slow claw and leg movements	No	No	Yes	No
Evident claw and leg movements	No	No	No	Yes
Strong claws with quick movements (aggressive); claws and legs strongly huddled to the abdomen (defensive)	No	No	No	Yes/No

Determination of haemolymph parameters

D-glucose

Haemolymph was deproteinated according to the procedure of Paterson *et al.* (1997). D-glucose concentration was measured using a D-glucose assay kit (Sigma GAGO-20), according to the procedure previously described in chapter 3.1.

L-lactate

L-lactate concentration of deproteinated haemolymph was measured using L-lactate reagent Trinity Biotech (no. 735-10), according to the procedure previously described in chapter 3.1.

Haemocyanine

Haemocyanine was determined spectrophotometrically (Unicam UV-Vis spectrometry Helios Alpha) in 10-mm quartz cuvettes using 50 µl of thawed haemolymph diluted in 2 ml of distilled water. Absorbance was measured at 335 nm, and haemocyanine concentration (mg mL⁻¹) was calculated from the molar extinction coefficient $E^{\text{mg mL}^{-1}}_{(1\text{cm})} = 0.0283$, as previously described by Harris and Andrews (2005).

pH

Thawed vortexed haemolymph samples free of clots were kept at 8, 12 or 16 °C depending on the temperature treatment. Samples were measured with a pH meter (Hanna Instruments, HI 221) using a pre-calibrated micro-electrode (Hanna Instruments, HI 1083P) and pH buffers 4.01, 7.01, and 10.00 (WTW, Wissenschaftlich-Technische Werkstätten) as standards.

Statistics

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (sd). In order to satisfy ANOVA assumptions data were transformed, followed by multiple comparison tests (Tukey HSD) to identify differences between groups. If transformed data could not meet ANOVA assumptions, non-parametric analysis of variance (Kruskal-Wallis) was performed, followed by non-parametric multiple comparisons test (Mann-Whitney). Whenever two groups were compared, Student's t-test was applied instead of ANOVA, followed by the correspondent nonparametric test (Mann-Whitney) if data could not meet Student's t assumptions. Transport duration, vigour and temperature were subjected to correlation analyses, using the Pearson r correlation coefficient. All statistical analyses were tested at the 0.05 level of probability with the software STATISTICA™ 6.1 (Statsoft, Inc., Tulsa, OK74104, USA).

Results

Water quality

During transport in immersed conditions seawater quality revealed a strong decrease in oxygen and pH, but an increase in ammonia and nitrite levels (Table 3.2.2). After 48h of simulated transport the lowest dissolved oxygen concentration was obtained at 8 °C with AQUI-S® and 16 °C without AQUI-S®. In contrast, the highest oxygen and lowest nitrite values were obtained at 8 °C in seawater without the anaesthetic, while in treatments with AQUI-S® the highest oxygen content was obtained at 12 °C. Seawater became more acidic at 8 and 16 °C with AQUI-S®.

Table 3.2.2 Seawater pH, dissolved oxygen (mg L^{-1}), ammonia and nitrites concentration (mg L^{-1}) before simulated live transport (0 h) and after 48 h.

Treatment	Temperature	O ₂		pH		Ammonia		Nitrites	
		0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h
Water	8 °C	9.8	4.8	8.1	7.5	0.0	0.6	0.0	1.6
	12 °C	9.5	2.8	8.0	7.5	0.0	0.6	0.0	3.3
	16 °C	9.7	1.9	8.0	7.6	0.0	1.2	0.0	3.3
AQUI-S®	8 °C	9.8	1.9	8.1	6.7	0.0	1.2	0.0	3.3
	12 °C	9.7	3.2	8.1	7.6	0.0	0.6	0.0	3.3
	16 °C	9.5	2.9	8.0	7.0	0.0	1.2	0.0	3.3

Vigour

The effects of temperature and duration of simulated live transport on *C. pagurus* vigour are shown in figure 3.2.1.

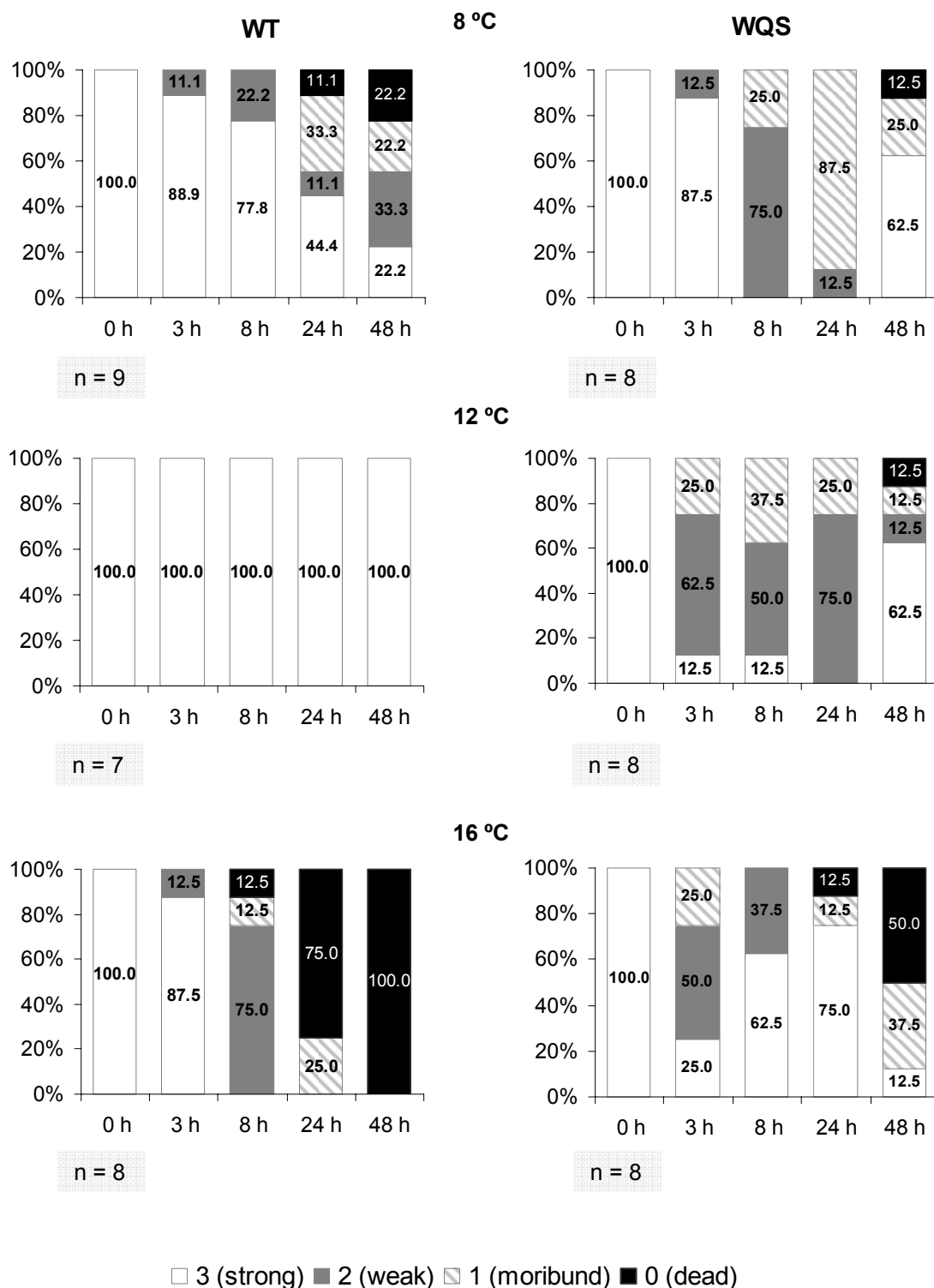


Figure 3.2.1 Vigour of crabs during experimental conditions. Abbreviations: (WT) immersed crabs in seawater; (WQS) immersed crabs with AQUI-S®; (AIR) air exposed crabs in semi-dry conditions; (AQS) air exposed crabs in semi-dry conditions pre treated with AQUI-S®; vigour-level index: (0) dead crabs (cumulative); (1) moribund crabs; (2) weak crabs; (3) strong crabs.

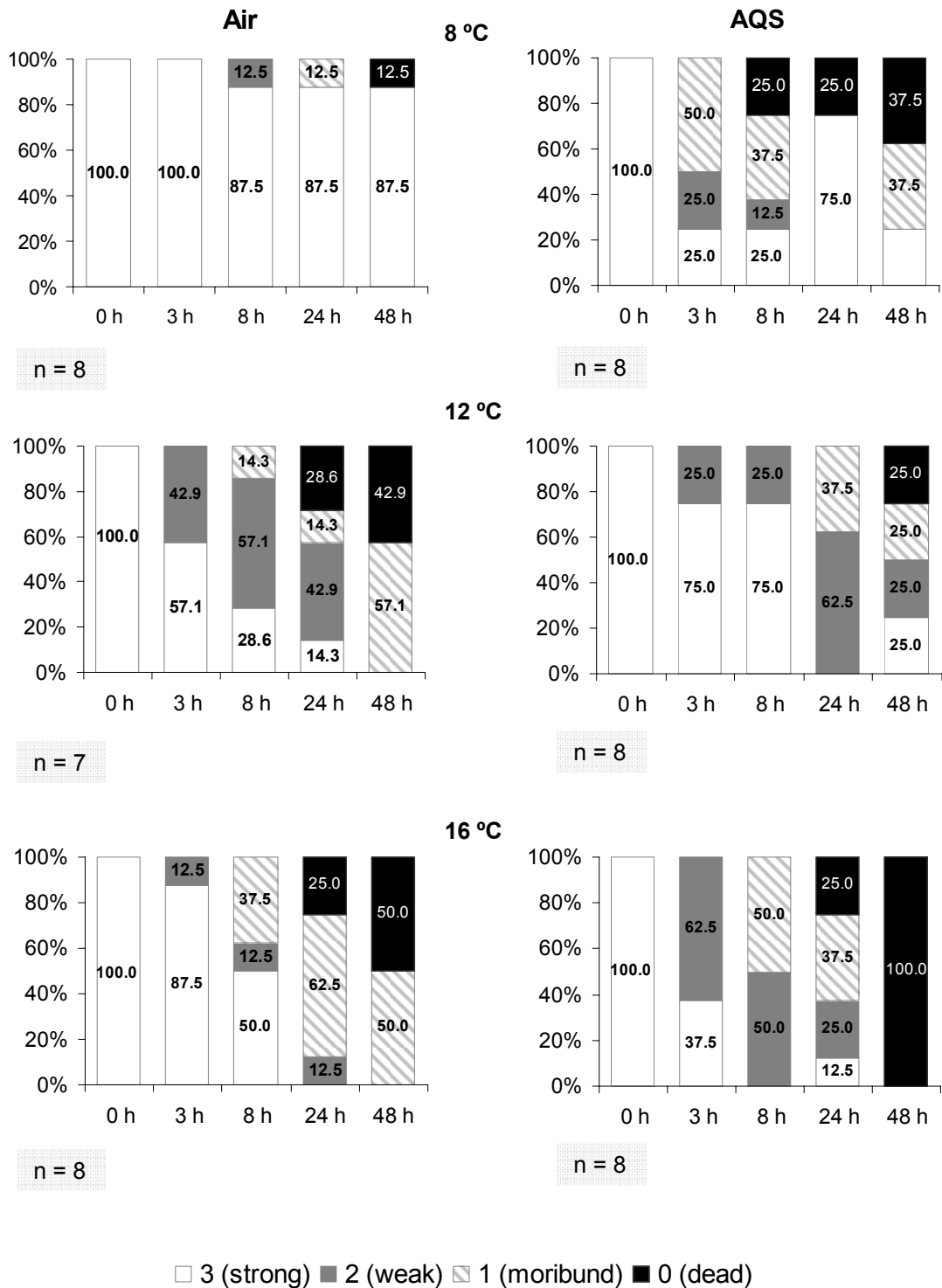


Figure 3.2.1 (cont). Vigour of crabs during experimental conditions. Abbreviations: (WT) immersed crabs in seawater; (WQS) immersed crabs with AQUIS[®]; (AIR) air exposed crabs in semi-dry conditions; (AQS) air exposed crabs in semi-dry conditions pre treated with AQUIS[®]; vigour-level index: (0) dead crabs (cumulative); (1) moribund crabs; (2) weak crabs; (3) strong crabs.

In general, vigour was lower at higher temperatures and prolonged transport duration. *C. pagurus* was unable to withstand prolonged simulated live transport (48 h) at 16 °C in WT and AQS treatments. Whereas in WT treatment, mortalities occurred after 8 h and at 24 h most crabs had already died (75 %), while in AQS mortalities only started to occur after 24 h. At 16 °C, even though both WQS and AIR treatments ended up at 48 h with 50 % mortality; at 24 h AIR had twice the mortality than WQS.

The only treatment without any mortality after 48 h was WT at 12 °C. The treatment showing higher mortalities and lower vigour at this temperature occurred in AIR, with 42.9 % mortality and 57.1 % moribund crabs after 48 h of simulated transport. At 8 °C, the lowest mortalities were observed in AIR and WQS (12.5 %), while AQS crabs experienced the highest mortalities (37.5 %) at this temperature.

Blood parameters

L-lactate

The parameters measured in the haemolymph of crabs not submitted to any treatment, that is the control group, revealed low levels of L-lactate (0.29 ± 0.06 mM). The concentration in crabs' haemolymph at the three tested temperatures was always above the control levels (Table 3.2.3).

Significant differences in the L-lactate concentration were observed between temperatures (Table 3.2.3) and among treatments at the same temperature (Figure 3.2.2). WT crabs at 8 and 16 °C had more L-lactate in the haemolymph than crabs at 12 °C (Table 3.2.3). These differences were detected after 8 h in crabs at 16 °C but only after 24 h in crabs at 8 °C. Among WQS crabs, those at 12 °C started with the lowest L-lactate levels and kept low values compared to crabs at 16 °C. AIR crabs at 8 °C showed the lowest L-lactate values between 8 and 48 h than in the remaining temperatures. A similar trend was observed in the AQS group, though with comparatively higher values.

Significant differences between treatments at 8 °C occurred from 8 h onwards, with AIR treatment having lower L-lactate values, as well as WQS at 48 h (Figure 3.2.2). At 12 °C, significantly lower L-lactate values were detected in WT and WQS treatments compared to air exposed crabs independently of using anaesthetic (AIR and AQS), which was detected from 8 h onwards. Crabs kept at 16 °C in WQS treatment had statistically lower L-lactate levels between 8 and 24 h than AQS.

pH

Haemolymph pH of crabs from all treatments is presented in Table 3.2.3. All crabs had lower pH in the beginning of the experiment compared to the control group (7.64 ± 0.03). Regardless of temperature, WQS crabs had pH levels similar to the control group after 24 h. Particularly, crabs at 8 °C resumed to control values after 8h and kept these values until the end of the experiment. WT (12 °C) and AIR (8 °C) crabs also returned to control levels after 24 h. WT crabs at 16 °C, had the most acidic pH of all groups in the beginning of the experiment (0 h) and did not survive the

experiment (Table 3.2.3). Also AQS crabs at 16 °C had the most acidic pH at 24 h and did not survive till the end of the experiment.

Table 3.2.3 L-lactate (mM) and pH in *C. pagurus* haemolymph measured for each treatment at 8, 12 and 16 °C during 48 h. Values are presented as average followed by standard deviation.

L-lactate	T°C	0 h	3 h	8 h	24 h	48 h
WT	8	4.54±1.50a ***	8.88±3.43a ***	11.57±2.83ab ***	12.66±5.83a ***	15.78±5.76a ***
	12	5.06±2.16a ***	5.42±0.91a ***	4.80±1.50b ***	2.48±0.26b ***	7.30±5.12b ***
	16	5.51±1.62a ***	8.84±2.05a ***	14.62±3.64a ***	21.19±0.05a ***	-
WQS	8	3.70±0.50ab ***	9.85±1.31ab ***	12.10±1.65a ***	12.58±5.03a ***	6.49±4.07b ***
	12	2.90±0.70b ***	5.20±1.58b ***	6.36±2.19b ***	8.36±4.62a ***	6.58±4.70b *
	16	4.38±0.81a ***	15.74±3.11a ***	9.97±3.51ab *	10.82±5.18a ***	29.88±5.76a ***
AIR	8	4.40±0.62a ***	8.63±1.05a ***	7.91±1.48b ***	2.93±1.75b **	1.75±0.28b ***
	12	5.00±0.85a ***	8.36±1.80a ***	13.17±2.65a ***	19.71±3.99a ***	35.95±16.32a ***
	16	3.53±0.95a ***	9.07±2.05a ***	14.04±3.15a ***	16.12±11.31a **	41.92±8.92a ***
AQS	8	2.83±1.22a ***	7.88±3.53a ***	10.80±3.60b ***	10.78±3.57b ***	14.96±3.35b ***
	12	4.17±0.66a ***	8.45±0.92a ***	15.12±5.00 ab ***	22.34±4.98a ***	30.31±6.62a ***
	16	4.51±2.01a ***	10.01±2.34a ***	16.48±1.07a ***	24.07±2.92a ***	-
pH	T°C	0 h	3 h	8 h	24 h	48 h
WT	8	7.23±0.03a ***	7.22±0.07b ***	7.16±0.08b ***	7.27±0.16b ***	7.30±0.13a ***
	12	7.29±0.04a ***	7.41±0.07a ***	7.42±0.12a ***	7.65±0.09a ***	7.36±0.06a ***
	16	7.12±0.09b ***	7.14±0.06b ***	7.14±0.14b ***	6.91±0.12b ***	-
WQS	8	7.28±0.08a ***	7.49±0.10a **	7.59±0.10a ***	7.60±0.14a ***	7.65±0.16a ***
	12	7.26±0.08a ***	7.28±0.27a **	7.43±0.25ab ***	7.55±0.13 a ***	7.48±0.11ab **
	16	7.21±0.08a ***	7.31±0.09a ***	7.28±0.14b ***	7.67±0.09a ***	7.23±0.02b ***
AIR	8	7.25±0.05a ***	7.49±0.09a ***	7.44±0.11a ***	7.63 ± 0.08a ***	7.63±0.08a ***
	12	7.21±0.12a ***	7.26±0.20b ***	7.15±0.12b ***	7.22±0.04b ***	7.03±0.09b ***
	16	7.23±0.11a ***	7.30±0.11b ***	7.31±0.06ab ***	7.35± 0.24b **	6.67±0.13c ***
AQS	8	7.28±0.07a ***	7.18±0.20a ***	7.15±0.17a ***	7.32±0.17a ***	7.47±0.23a ***
	12	7.30±0.06a ***	7.31±0.04a ***	7.28±0.03a ***	7.30±0.04a ***	7.10±0.06b **
	16	7.23±0.06a ***	7.24±0.08a ***	7.20±0.05a ***	7.03±0.12b ***	-

For each treatment different letters in each column represent significant differences between temperatures (p values shown); Statistical differences to control values are represented as follows: *** p<0.001; ** p<0.01; * p<0.05. Abbreviations: (WT) immersed crabs in seawater; (WQS) immersed crabs with AQUI-S®; (AIR) air exposed crabs in semi-dry conditions; (AQS) air exposed crabs in semi-dry conditions pre treated with AQUI-S®.

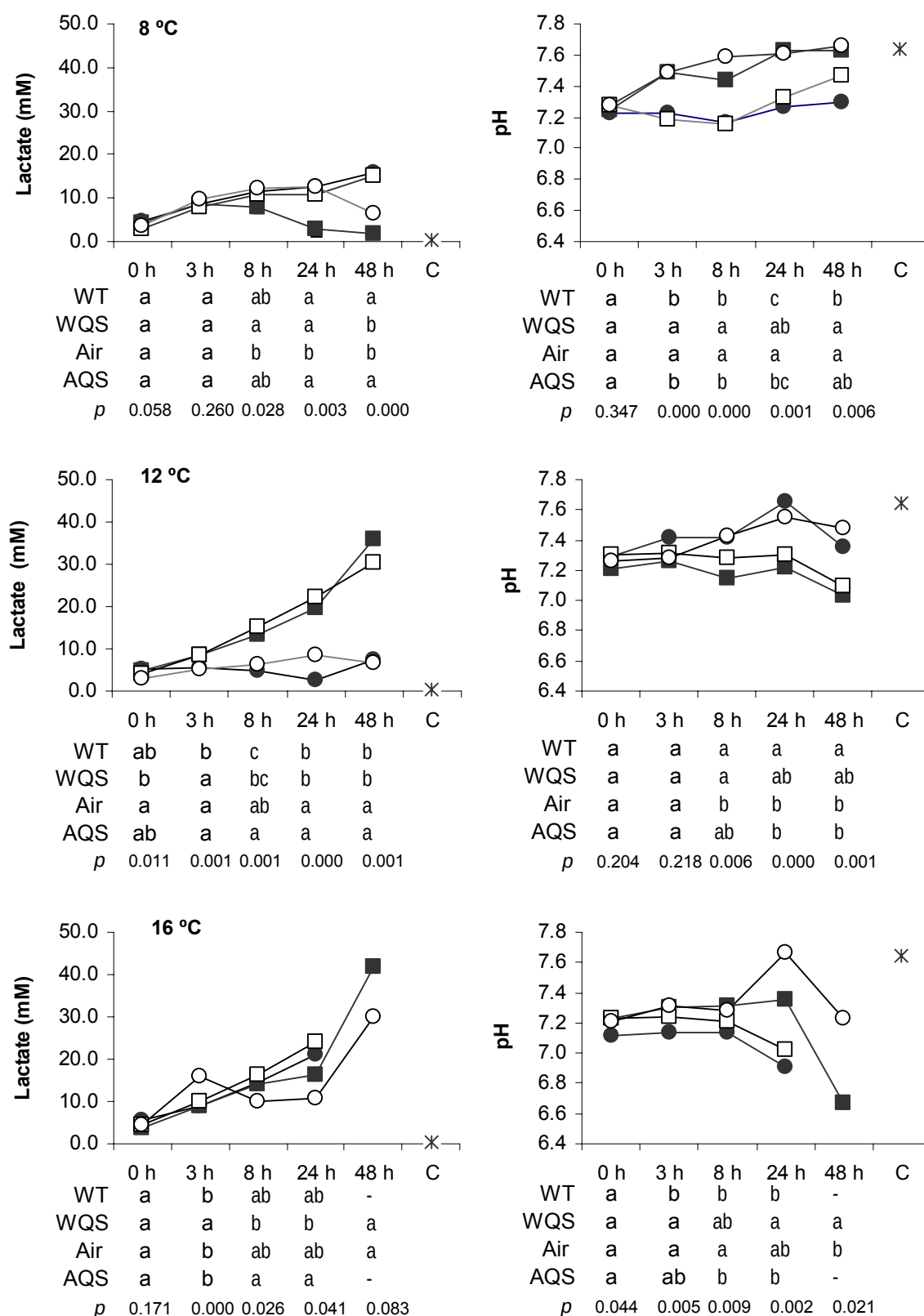


Figure 3.2.2 Changes in L-lactate concentration (mM) and pH values of *C. pagurus* during the experiments at 8, 12 and 16 °C in crabs exposed to air in semi-dry conditions (■; AIR), immersed in seawater (●; WT), immersed in seawater with AQUI-S® (○; WQS), and exposed to air in semi-dry conditions with AQUI-S® (□; AQS). The p value is shown for each column and different letters for each column represent significant changes between treatments. Control values shown as C (*).

Major differences were observed in the AIR treatment with crabs at 8 °C having higher pH than crabs at 12 and 16 °C since 3 h and until the end of the experiment.

Significant differences between groups at the same temperature were also detected (Figure 3.2.2). In fact, at 8 °C WT and AQS crabs had the most acidic haemolymph since 3 h. At 12 °C there was a clear separation between WT and AIR (since 8 h) and AQS (since 24 h), with WT crabs showing higher pH values. At 16 °C, WT crabs and AQS crabs had the most acidic haemolymph values from 3 and 8 h onwards, respectively, and these animals did not survive.

D-glucose

Haemolymph of all crabs was mostly hyperglycaemic throughout the experiments compared to the control group (0.19 ± 0.05 mM), except at 16 °C in WT (24 h) and at 12 °C in AQS (48 h; Table 3.2.4). Few significant differences were detected in glucose concentration between temperatures and between treatments at the same temperature and, D-glucose variability during the experiments did not reveal any clear pattern.

Table 3.2.4 D-glucose (mM) and haemocyanine (mg mL^{-1}) in *C. pagurus* haemolymph measured for each treatment at 8, 12 and 16 °C during 48 h. Values are presented as average followed by standard deviation.

D-glucose (mM)	T°C	0 h	3 h	8 h	24 h	48 h
WT	8	0.87±0.59a **	0.95±0.64a **	1.70±0.45a ***	0.77±0.64a ***	1.10±0.87a *
	12	0.65±0.24a ***	0.64±0.31a **	1.02±0.41b ***	0.63±0.16a ***	0.35±0.09a **
	16	1.81±0.82a ***	0.76±0.15a ***	1.48±0.53 ab ***	0.39±0.33a	-
WQS	8	0.10±0.06b ***	0.69±0.56a *	1.07±0.56a ***	1.12±0.52a ***	0.88±0.73a *
	12	0.06±0.03b ***	0.99±0.44a ***	0.87±0.40a ***	1.30±0.50a ***	0.43±0.31a *
	16	2.05±0.58a ***	1.23±0.32a ***	1.20±0.17a ***	1.46±0.37a ***	1.23±0.35a ***
AIR	8	1.24±0.45a ***	1.00±0.47a ***	1.08±0.43a ***	0.47±0.26a *	0.82±0.74a *
	12	0.88±0.55a **	0.84±0.67a *	0.94±0.52a ***	0.59±0.41a *	0.97±0.19a ***
	16	1.59±0.90a ***	0.70±0.49a *	1.15±0.53a ***	1.20±0.84a **	1.11±0.62a **
AQS	8	0.89±0.34b ***	1.33±0.54a ***	1.48±0.74a ***	1.39±0.63a ***	1.23±0.44a ***
	12	1.18±0.66a **	1.35±0.57a ***	1.46±0.48a ***	1.13±0.56a ***	0.62±0.60a
	16	1.97±0.58a ***	1.39±0.57a ***	1.71±0.28a ***	1.65±0.42a ***	-

For each treatment different letters in each column represent significant differences between temperatures (p values shown); Statistical differences to control values are represented as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Abbreviations: (WT) immersed crabs in seawater; (WQS) immersed crabs with AQUIS[®]; (AIR) air exposed crabs in semi-dry conditions; (AQS) air exposed crabs in semi-dry conditions pre treated with AQUIS[®].

Table 3.2.4 (cont.)

Haemocyanine	T°C	0 h	3 h	8 h	24 h	48 h
WT	8	37.5±15.2a	37.6±13.3a	38.0±14.4ab	38.0±17.2 ^a	48.8±21.0a
	12	45±6.8a ***	43.6±4.4a ***	48.0±5.6a ***	46.7±7.7a ***	48.8±6.6a ***
	16	36.9±9.8a *	40.6±12.8a *	23.9±9.3b	48.2±15.3a **	-
WQS	8	41.4±12.4a *	54.9±13.3a ***	49.1±11.0a ***	51.6±14.4a ***	55.4±13.5a ***
	12	37.0±9.3a *	42.0±9.5a **	41.0±9.1ab ***	43.0±8.8ab **	43.4±8.7a **
	16	37.8±15.4a	42.4±8.8a **	25.1±10.3b	28.6±4.0b	46.5±6.6a *
AIR	8	30.9±10.5a	39.7±10.7a *	37.1±11.8a	34.9±11.3a	31.3±18.1a
	12	36.0±10.2a	35.7±11.0a	36.6±9.8a	36.2±12.5a	35.0±22.0a
	16	39.7±14.0a *	45.7±16.9a *	22.3±13.9 a	28.5±12.9a	51.8±11.0 a *
AQS	8	28.4±18.5a	31.0±16.0a	29.2±15.5ab	31.6±16.3a	39.1±18.6a
	12	40.2±12.1a *	40.5±10.2a **	39.8±8.7a **	47.9±9.3a ***	51.0±10.7a ***
	16	43.4±11.7a **	46.0±14.6a **	19.7±7.4b *	36.2±10.2a	-

For each treatment different letters in each column represent significant differences between temperatures (p values shown); Statistical differences to control values are represented as follows: *** p<0.001; ** p<0.01; * p<0.05. Abbreviations: (WT) immersed crabs in seawater; (WQS) immersed crabs with AQUI-S[®]; (AIR) air exposed crabs in semi-dry conditions; (AQS) air exposed crabs in semi-dry conditions pre treated with AQUI-S[®].

Haemocyanine

In general, haemolymph haemocyanine showed great variability in all treatments (Table 3.2.4). Haemocyanine concentration of WT, AQS and AIR crabs was similar to the control group (28.2 ± 5.0 mg l⁻¹) at 8 °C, while generally higher haemocyanine values were obtained at 12 and 16 °C (except AIR at 12 °C and WQS at 16 °C). No significant differences in haemocyanine concentration of crabs were detected between treatments at the same temperatures.

Correlations

Correlations between each haemolymph parameter and transport temperature, duration and vigour of crabs are shown in Table 3.2.5. Considering that vigour in crabs treated with AQUI-S[®] can be misleading, correlations were not analysed in treatments with AQUI-S[®].

Significant positive correlations were detected between temperature and L-lactate (AIR and AQS crabs), also with D-glucose (WQS and AQS), whereas negative correlations were seen with pH (WQS and AIR) and haemocyanine (WQS).

Table 3.2.5 Pearson's correlation coefficient of haemolymph parameters of each treatment in relation to temperature, transport duration and vigour.

Parameter	Treatments	T °C	Transport duration			Vigour		
			8°C	12°C	16°C	8°C	12°C	16°C
L-lactate	WT	r = -0.05 ns	r = 0.68 ***	r = -0.22 ns	r = 0.88 ***	r = -0.66 ns	r = 0.26 ns	r = -0.57 **
	WQS	r = 0.15 ns	r = 0.21 ns	r = 0.41 *	r = 0.58 **	-	-	-
	AIR	r = 0.43 ***	r = -0.59 **	r = 0.96 ***	r = 0.79 ***	r = 0.32 ns	r = -0.67 ***	r = -0.43 *
	AQS	r = 0.24 *	r = 0.81 ***	r = 0.94 ***	r = 0.96 ***	-	-	-
pH	WT	r = -0.15 ns	r = 0.13 ns	r = 0.43 *	r = -0.04 ns	r = -0.48 **	r = 0.13 ns	r = 0.06 ns
	WQS	r = -0.37 ***	r = 0.68 *	r = 0.49 **	r = 0.44 *	-	-	-
	AIR	r = -0.46 *	r = 0.81 *	r = -0.33 ns	r = -0.06 ns	r = 0.30 ns	r = 0.50 **	r = 0.04 ns
	AQS	r = -0.22 ns	r = 0.20 ns	r = -0.50 **	r = -0.54 **	-	-	-
D-glucose	WT	r = 0.08 ns	r = 0.00 ns	r = -0.32 ns	r = -0.32 ns	r = -0.24 ns	r = -0.38 *	r = 0.26 ns
	WQS	r = 0.40 ***	r = 0.54 **	r = 0.32 ns	r = -0.34 ns	-	-	-
	AIR	r = 0.11 ns	r = -0.47 **	r = -0.01 ns	r = -0.13 ns	r = -0.18 ns	r = -0.22 ns	r = 0.09 ns
	AQS	r = 0.23 *	r = 0.32 ns	r = -0.29 ns	r = -0.09 ns	-	-	-
Hcy	WT	r = -0.01 ns	r = 0.10 ns	r = 0.23 ns	r = -0.18 ns	r = -0.05 ns	r = 0.29 ns	r = 0.31 ns
	WQS	r = -0.45 ***	r = 0.29 ns	r = 0.22 ns	r = -0.11 ns	-	-	-
	AIR	r = 0.04 ns	r = 0.09 ns	r = 0.16 ns	r = -0.08 ns	r = 0.11 ns	r = -0.24 ns	r = 0.21 ns
	AQS	r = 0.13 ns	r = 0.23 ns	r = 0.42 *	r = -0.37 *	-	-	-

Symbols: *** p<0.001, ** p<0.01, * p<0.05, ns, not significant. Abbreviations: Hcy, haemocyanine; (WT) immersed crabs in seawater; (WQS) immersed crabs with AQUI-S[®]; (AIR) air exposed crabs; (AQS) air exposed crabs pre treated with AQUI-S[®]. The correlation between haemolymph parameters and vigour was not analysed for crabs treated with AQUI-S[®].

In general, L-lactate concentration was positively correlated with transport duration, except at 8 °C in AIR (negative correlation) and WQS (no correlation), and at 12 °C in WT (no correlation). Significant negative correlations with transport duration were registered for pH only at 12 and 16 °C in AQS, whereas positive correlations were detected in WQS at all temperatures, at 12 °C in WT, and at 8 °C in AIR. Significant correlations between transport duration and D-glucose were only observed at 8 °C in WQS (positive correlation) and AIR (negative correlation). As far as haemocyanine is concerned, the only positive correlation with transport duration was detected in AQS at 12 °C, while the only negative correlation was registered for the same treatment at 16 °C.

Correlations between vigour and blood parameters revealed that L-lactate was negatively correlated at 16 °C in both treatments (WT and AIR), and at 12 °C in AIR. Regarding pH, WT at 8 °C had a negative correlation with vigour, while AIR had a positive correlation at 12 °C. D-glucose concentration was negatively correlated with vigour in WT. Finally, haemocyanine was not correlated with vigour in any treatment.

Discussion

This experiment is an attempt to simulate the transport systems that are already in use to distribute crabs in Portugal and to test possible alternatives that might improve these systems. Therefore, it is important to bear in mind that the control group is not meant to represent *C. pagurus* basal levels, as crabs have already been exposed to several stressors (mainly capture and import transport). It is rather a control with unfed crabs not subjected to additional stressors. Similar results were reported by Lorenzon *et al.* (2008) for the control group of 15 crabs imported from France (transport duration 36 h) and rested at the premises of the Italian importer company for 96 h feeding *ad libitum* (pH, 7.76; L-lactate, 0.34 mM; D-glucose, 0.39 mM).

In the beginning of the experiments crabs already revealed stress signs, as L-lactate and D-glucose levels were above those of the control group and pH was more acidic. Such stress could be attributed to the handling procedures. It is also important to state that crabs were affected in different ways, as apparently WQS crabs at 12 °C had a lower L-lactate and D-glucose concentration than the remaining crabs. The results indicate that crabs experienced physiological stress during simulated live transport, though with different magnitudes depending on temperature, treatment and transport duration. The haemolymph parameters that provided clear indications of stress in response to simulated transport over a 48 h period were L-lactate and pH.

It is known that the development of anaerobic metabolism leads to the production of L-lactate and acidic pH (Ridgway *et al.*, 2006). Anaerobic metabolism can be elicited by a hypoxic environment and by an increase in metabolic rate of animals due to increasing temperature, which boosts the energy demands of the animal (Durand *et al.*, 2000; Ridgway *et al.*, 2006). The results of seawater quality after 48 h clearly indicate that a hypoxic environment was created in the immerse systems tested, and that pH decreased probably due to L-lactate and CO₂ excretion. The development of anaerobic metabolism and the decrease in seawater quality is an inevitable consequence of high density and low dissolved oxygen. As expected at higher temperatures the consequences were more severe. Even though seawater quality was poorer, an increase in temperature from 8 to 12 °C in immersed crabs (WT and WQS) did not cause an increase in the stress response by the crabs' as measured by pH and lactate levels. On the contrary, WT crabs at 12 °C had no mortalities and had less L-lactate concentration (7.3 mM) than crabs at 8 °C (lactate, 15.8 mM; 22.2 % mortality; Figure 3.1.1). Also, the same temperature increase in WQS treatments did not reveal any clear effect on mortality, lactate concentration and pH.

The effect of raising temperature from 8 to 12 °C had a much larger detrimental effect on the stress parameters of crabs exposed to air in both treatments, but the effect on vigour was greater in AIR

crabs (Table 3.2.3 and 3.2.4; Figure 3.2.1). L-lactate accumulation has been extensively described as being associated with aerial exposure, particularly in subtidal crustaceans, as emersion impairs gill functioning and the efficient release of excretion products, thus leading to further hypoxia or anoxia and anaerobic metabolism (Durand *et al.*, 2000). Previous studies have reported that L-lactate concentration and pH are negatively influenced by air exposure and increased temperature in several crustacean species, such as *Nephrops norvegicus* (Spicer *et al.*, 1990; Ridgway *et al.*, 2006), *Penaeus japonicus* (Chen and Chen, 1998), *Jasus edwardsii* (Morris and Oliver, 1999), and *Homarus americanus* (Lorenzon *et al.*, 2007). On the other hand, low temperatures have been referred to as anaesthesia inducer, which allows animals to be handled without apparent stress (Spanoghe and Bourne, 1997). Therefore, survival rates in crustaceans exposed to air at low temperatures have been reported to improve in *H. americanus* (5-10 °C; Mcleese, 1965), *Callinectes sapidus* (10 °C; Otwell and Webb, 1977), *H. gammarus* (5-10 °C; Whiteley and Taylor, 1992), *Panulirus cygnus* (17-20 °C; Spanoghe and Bourne, 1997), *P. japonicus* (6-12 °C; Chen and Chen, 1998) and *N. norvegicus* (10 °C; Ridgway *et al.*, 2006).

In this study, haemolymph pH of AIR crabs at the lowest temperatures (8 °C) was positively correlated with transport duration, while L-lactate was negatively correlated. A similar trend was reported by Bernasconi and Uglow (2008) for *N. norvegicus* during prolonged emersion (72 h) at 5 °C. Likely, the high survival rate monitored in air exposed crabs at 8 °C is due to the low L-lactate levels and high pH, which has also been shown to be positively correlated in other crustaceans like *P. japonicus* (Chen and Chen, 1998). Of all crabs that survived the 48 h experimental period, the best treatments as far as L-lactate, pH and vigour are concerned were AIR at 8 °C and WT at 12 °C.

It is known that anaesthetics reduce or minimize fish stress by decreasing metabolism (Iversen *et al.*, 2003). It is likely that low temperature and AQUI-S[®] decreased metabolism far too much in AQS crabs, leading to reduction of breathing and gas transfer, hypoxia and respiratory acidosis due to the reduction of blood O₂ tension and a concomitant rise in blood CO₂. This is characteristic of deep anaesthesia, and prolonged maintenance of this stage may result in death (Ackerman *et al.*, 2005). In fact, at 8 °C the highest mortality rate was observed in the AQS treatment (37.5 %); this rate was greater than those reported at 12 °C for WT (0 %), WQS (12.5 %) and AQS (25 %), but similar to the AIR treatment at 12 °C (42.9 %). Therefore, the tested concentration of AQUI-S[®] was not an efficient solution for live transport of crabs at 8 °C in semi-dry conditions but an option at 12 °C. Nevertheless, further studies are still required to test different AQUI-S[®] concentrations that might optimize the use of this anaesthetic under such particular conditions.

Crabs stored at 16 °C were not able to adapt to such high temperature, and consequently, stress responses developed as fast as 3 h either in immersed or exposed to air conditions. The stress responses were cumulative to a point that some crabs were forced beyond their tolerance limits and died (WT and AQS treatments). Nevertheless, AQUI-S[®] had a positive effect on *C. pagurus* survival rate at higher temperatures and blood L-lactate and pH values during simulated transport in AQS (12 °C) and WQS (16 °C) treatments. Probably, the anaesthetic is counteracting the

negative effects of high temperatures, by delaying metabolic and energy demands. Other authors suggested that temperature increase is likely to increase the efficacy in anaesthetics (Olsen *et al.*, 1995; Stehly and Gingerich, 1999). However, at such high temperatures (16 °C) transport duration is detrimental, as confirmed by the positive correlation with L-lactate.

Vigour was a good visual estimate of physiological stress, especially in treatments at higher temperatures and exposed to air, where crabs experience more severe stress and wider variations in physiological haemolymph variables.

Overall, D-glucose and haemocyanine did not change much over the 48 h transport period. It is possible that the initial air exposure was sufficient to increase D-glucose levels to such an extent that no further increase was elicited. In this study, there was only a reduction of D-glucose in crabs at 16 °C in WT treatment after 24 h and at 12 °C in AQS treatment after 48 h. This reduction in D-glucose to control levels might have been due to the elevated metabolic rates that lead to a depletion of tissue glycogen concentrations, and a more premature cessation of glucose release. Once exhausted of glycogen, tissues are unable to re-supply glucose to haemolymph and maintain carbohydrate homeostasis (Ridgway *et al.*, 2006).

On the other hand, haemocyanine is the most important protein in crustacean's haemolymph (75-100 % of total blood proteins), is responsible for oxygen transport and is also a food reserve (Uglow *et al.*, 1986). The haemocyanine concentration in haemolymph of crabs revealed great variation between individuals (from 7.2 to 72.5 mg mL⁻¹). Haemocyanine in *C. pagurus* haemolymph has been previously monitored by Uglow *et al.* (1986) who also reported variable values ranging from 20.42 to 63.62 mg mL⁻¹. Considering that this protein is an important food reserve, the great variability among crabs is probably due to different nutritional conditions, which may further influence their ability to cope with stressful conditions during transport and subsequent recovery without feeding (Uglow *et al.*, 1986).

Conclusions

From a commercial perspective it is important to strive to ensure that it is possible to transport live *C. pagurus* with less mortality than the systems currently employed by the industry. This can be achieved by keeping the animals at low temperatures (8 °C or lower), in humid conditions and ensuring they are not pilled. It is also essential to bear in mind that transport duration strongly affects the crabs' quality and mortality; therefore transport journeys should be as short as possible. Moreover, grading animals prior to transport is fundamental because initial condition influences survival. Whilst it is also feasible to have zero mortality during 48 h transport of immersed crabs at 12 °C, it requires complex logistics, is expensive such that semi-dry transport is more advantageous from a commercial perspective. Therefore, it is highly recommended to develop *in situ* tests in industrial operating conditions.

In this study the advantage of using AQUI-S[®] was mainly observed for short transport duration at 16 °C. More studies are still required to test different concentrations in order to optimize the use of anaesthetics at different temperatures.

The key issue is the handling procedure before transport and the temperatures employed in semi-dry transport. Moreover, it is important to bear in mind that even though mortalities can be lowered during transport, the subsequent procedures after transport can not be neglected and further attention must be paid to avoid temperature changes.

Sub-chapter 3.3

C. pagurus simulated transport and recovery

Barrento, S., Marques, A., Vaz-Pires, P., and Nunes, M. L. Physiological changes during simulated live transport of *Cancer pagurus* and recovery in holding tanks (submitted to *Aquaculture*, October, 2009).



The trade of live edible crab *Cancer pagurus* in Portugal is totally dependent on imports mainly from UK. Therefore, animals face different transport conditions (international long duration immersed transport and national distribution) that promote high mortalities (40 to 60 %) at the final destination due to cumulative stressors. Previous reports showed that semi-dry transport at low temperatures is a feasible system to transport crabs across the country (national distribution). However, mortality is cumulative and to verify the effectiveness of such system mortality must be monitored at the reception facilities. Therefore, the present work aimed to assess the physiological responses of edible crab during simulated live shipment (48 h) of immersed crabs at 12 °C and air exposed crabs at 4 °C and subsequent recovery at 16 °C with and without pre-acclimation period at different temperatures. Both simulated transport systems induced physiological stress, culminating in one dead crab out of 8 (12.5 %) in immersed conditions and only one out of 24 (4 %) in air exposed conditions. Both transport systems elicited anaerobic responses of similar magnitude marked by hyperglycaemia and increased L-lactate concentration in haemolymph. At the end of the recovery period, only acclimated animals resumed to L-lactate and pH control values and the lowest mortality was achieved with crabs acclimated at the lowest temperature (from 6 to 16 °C at 1.5 °C/h) showing half mortalities (12.5 %) than the remaining treatments (25 %). Overall, results showed that crabs can be transported in semi-dry conditions at 4 °C and acclimated from 6 to 16 °C and reach better physiological conditions than immersed crabs at 12 °C.

Keywords: crustacean, crab, transport, recovery, stress, vigour.

Introduction

The effects of emersion on crustacean haemolymph chemistry have been extensively studied and are mainly species dependent (McLeese, 1965; Taylor and Whiteley, 1989; Webster, 1996; Schmitt and Uglow, 1997; Chen and Chen, 1998; Durand *et al.*, 2000). Adaptations to improve oxygen uptake depend on the ecological niches occupied by different species, with intertidal and semi-terrestrial crustaceans more adapted to reduced internal hypoxia during emersion than fully aquatic species that rarely experience air exposure (Durand and Regnault, 1998). One of the main reasons for studying the responses of sub littoral crustaceans to emersion is the commercial importance that some species have for the live market, such as the edible crab *Cancer pagurus*. This species occupies subtidal biotopes where temperature changes from 4 to 16 °C (Chartois *et al.*, 1994; Metzger *et al.*, 2007).

C. pagurus is the most important live traded crustacean in Portugal. However, the national production of this crab is almost inexistent and most crabs must be imported from the UK, where it is one of the most valuable fisheries (Tully *et al.*, 2006). During this journey crabs face different transport conditions: vivier lorries are used in import transport to Portugal with tanks with aerated seawater (1kg crab L⁻¹) at about 12 °C (duration 58 h); while small refrigerated trucks (12 °C) are used for national distribution in plastic boxes exposed to air or immersed. Some specimens are air freight to the Azores islands in Styrofoam sealed boxes (20 kg each) with coolers (Figure 3.3.1A). Handling is careless as crabs are piled and some boxes are inadequate having holes where animals damage their limbs (Figure 3.3.1B). In the end of the journey, many crustacean deposit facilities in Portugal do not have refrigerating systems and mortalities at wholesalers and retailers can be as high as 40 and 60 %, respectively (Sub-chapter 2.1). Previous studies by Lorenzon *et al.* (2008) concluded that semi-dry international transport (36 h duration at 10-13 °C) from France to Italy have positive effects on animal condition and welfare during recovery, but no mortality data was mentioned after transport.

Recent experiments mentioned in sub-chapter 3.2 revealed that mortality during simulated semi-dry transport of 48 h was 42.9 % at 12 °C but only 12.5 % at 8 °C, thus suggesting that mortality could be further decreased if temperature were lowered to the ecological minimum of the species (4 °C) in order to reduce animal metabolism. In this context, it is crucial to determine the best recovery temperature after transport in order to avoid heat shock that might impair homeostasis and lead to poor survival.

The evaluation of stress in crustaceans has been carried out with subjective (e.g. behaviour, vigour) or objective physiological parameters, such as oxygen uptake, blood composition, pH, hormones, ions and haemocytes (Paterson and Spanoghe, 1997; Taylor *et al.*, 1997). Both parameters are useful indicators of the animal's health status. In this context, the present work aim was to assess the physiological changes during simulated national live transport of immersed and air exposed imported crabs (Figure 3.3.1C and D), and subsequent acclimation and recovery at different temperatures in stocking tanks. Several haemolymph parameters were measured

including D-glucose, L-lactate, haemocyanin, pH and vigour. This information is essential to enable wholesalers and retailers to optimise their handling and storage practices along the trade chain.

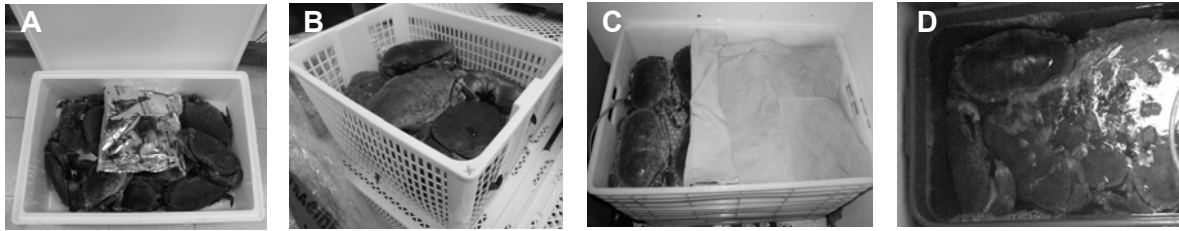


Figure 3.3.1 Boxes currently employed in Portugal to transport edible crab (A and B), semi-dry conditions (C) and immersed conditions (D) used in this study (Photos by Sara Barrento [©]).

Material and methods

Collection and treatment of animals

Thirty two intermoult *Cancer pagurus* (average values $\pm$ standard deviation: weight 717 ± 96 g, width 163 ± 16 mm, length 114 ± 15 mm) were sampled in this study. Crabs with nicked claws (procedure used by fishermen to inactivate claws), were obtained in autumn from a local importer and maintained without feeding in a well aerated recirculating seawater system at 16°C during 6 days prior to the experiment. The experimental design is schematized in Figure 3.3.2.

Refrigerated transport was simulated during 48 h with animals exposed to humid air conditions or immersed in aerated seawater. Twenty four crabs were exposed to air (semi-dry conditions) in a cooling chamber at 4°C . The animals were divided in three polystyrene boxes with a drainage system to avoid direct contact with excretion fluids (8 crabs per box stacked in a single row) with a cloth soaked with seawater (treatments B, C and D, Figure 3.3.1C). The remaining crabs ($n=8$) were put in rescaled polystyrene tanks measuring 16.5 cm depth, 28.0 cm width and 38.0 cm length, immersed with aerated seawater (35 ‰; 1 kg crab L^{-1} seawater) at 12°C (treatment A; Figure 3.3.1D). Animals were monitored by haemolymph bleeding and vigour determination immediately before being put in tanks (0 h) and after 8, 24 and 48 h. Only crabs not showing signs of weakness (vigour 3) were selected for the experiments following industrial procedures.

Vigour was assessed from the evaluation of crab behaviour using a vigour scale with four classes adapted from sub-chapter 3.1 (Table 3.3.1). After 48 h of simulated transport conditions, crabs transported at 12°C in aerated seawater (treatment A) and eight crabs exposed to air in semi-dry conditions (treatment B) were put in a recirculated stocking tank with aerated and filtrated seawater at 16°C without acclimation (highest temperature found in edible crab habitat; Chartois *et al.*, 1994) during 72 h recovery period. Animals were monitored by haemolymph bleeding and vigour determination at 8, 24, 48 and 72 h. The remaining crabs subjected to air exposure had an acclimation period of 6 h in tanks with aerated seawater at 11°C (treatment C) and 6°C (treatment D). Water was renewed every hour and temperature was gradually increased from 11 to 16°C , (approximately 1°C increment per hour - treatment C), and from 6 to 16°C (approximately 1.5°C increments per hour - treatment D). After the acclimation period all crabs were maintained in the

recovery tanks at 16 °C, and monitored by haemolymph bleeding and vigour determination at 0, 8, 24, 48 and 72 h in the recovery tanks.

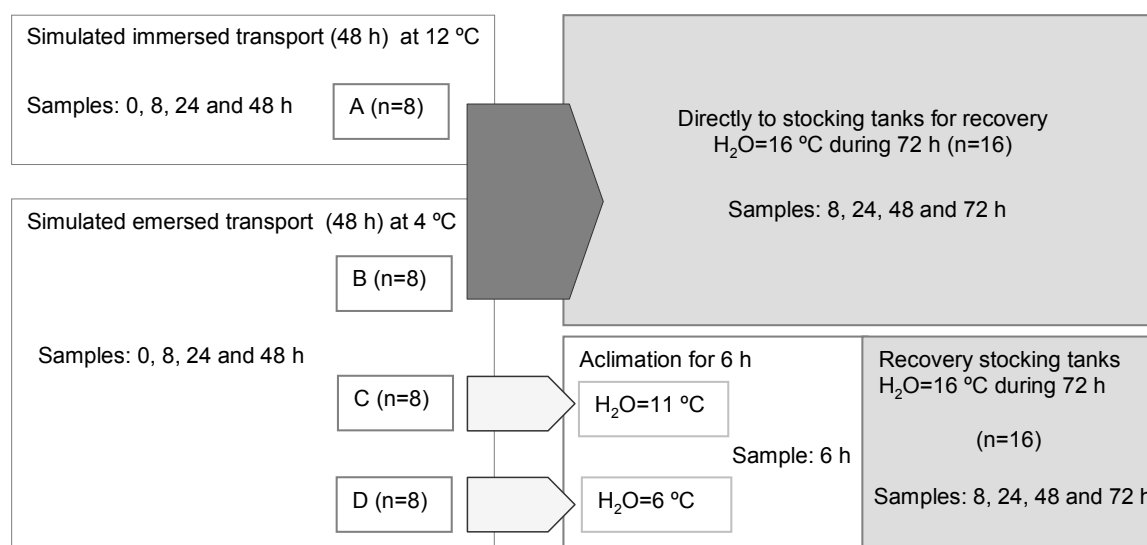


Figure 3.3.2 Diagram summarizing the experimental design of treatments A, B, C and D.

Dead animals were removed from tanks during monitoring. Haemolymph sampling consisted in taking approximately 1 mL from the fifth walking leg with 1.2 mL paediatric syringes (Sarstedt, S-monovet) and 20Gx 1(1/2)" hypodermic needles (Sarstedt, S-monovet needle), transferring haemolymph to micro tubes and rapidly frozen the samples until further analyses (except pH that was measured immediately after sampling). Blood parameters of 10 undisturbed animals maintained for eight days without feeding in the stocking tanks of the importer were analyzed and considered as the control group. Seawater quality parameters (pH, temperature and dissolved oxygen) were measured with a Multi 350i compact precision handheld meter (WTW, Wissenschaftlich-Technische Werkstätten) in the beginning and end of the simulated live transport of immersed crabs, after acclimation and after 72 h storage in stocking tanks. Also, test kits were used to measure ammonia (Nutrafin, Ref. A-7820) and nitrites (Nutrafin, A-7825).

Table 3.3.1 Vigour scale of European edible crab, adapted from sub-chapter 3.1.

Somatic response	0 (dead)	1 (moribund)	2 (weak)	3 (Strong)
Eyestalk response	No	Yes	Yes	Yes
Claws and legs weightless	Yes	Yes	No	No
Slow claw and leg movements	No	No	Yes	No
Evident claw and leg movements	No	No	No	Yes
Strong claws with quick movements (aggressive); claws and legs strongly huddled to the abdomen (defensive)	No	No	No	Yes/No

Haemolymph parameters

D-glucose

Haemolymph was deproteinated according to the procedure of Paterson *et al.* (1997). Briefly, thawed vortexed haemolymph was mixed with an equal volume of cold 0.6 M perchloric acid (Panreac), and the inactivated proteins were precipitated by centrifugation for 10 min at 40 000g (Sigma 3k30, rotor no. 12154; 24 x 1.5-2.2 mL;). The supernatant was neutralized with 3M potassium hydroxide (Sigma), and the precipitated potassium perchlorate formed was separated by centrifuging at 40 000g for 10 min. The supernatant was collected and D-glucose concentration was measured using a D-glucose assay kit (Sigma GAGO-20), by incubating 150 µL supernatant for 30 min at 37 °C with 300 µL of the assay reagent in disposable cuvettes. In this way, D-glucose is oxidized to gluconic acid and hydrogen peroxide by D-glucose oxidase, and the reaction between hydrogen peroxide and o-dianisidine in the presence of peroxidase form a coloured product. The reaction was stopped by the addition of 300 µL of 12 N sulphuric acid (Riedel-deHaën, 95-97 %) that reacts with the oxidized o-dianisidine to form a stable pink product. The intensity of the colour measured at 540 nm (Unicam UV-Vis spectrometry Helios Alpha) is proportional to the D-glucose concentration. D-glucose concentration was calculated from a calibration curve using standards of known concentration (Sigma D-glucose standard solution G6918).

L-lactate

Haemolymph was deproteinated as above, and L-lactate concentration measured using the L-lactate reagent Trinity Biotech (no. 735-10). Briefly, haemolymph samples (5 µL) were mixed with L-lactate reagent (500 µL) and incubated for 10 min at room temperature. In this step, lactic acid is converted to pyruvate and hydrogen peroxide by the enzyme L-lactate oxidase. The peroxidase enzyme catalyses the oxidative condensation of the chromogenic precursors to form a coloured dye with a maximum absorbance of 540 nm that was read on a spectrophotometer (Unicam UV-Vis spectrometry Helios Alpha). L-lactate concentration was calculated from a calibration curve prepared with standards of known concentration (Trinity L-lactate standards set no. 735-11).

Haemocyanine

Haemocyanine was determined spectrophotometrically (Unicam UV-Vis spectrometry Helios Alpha) in 10 mm quartz cuvettes using 50 µL of thawed haemolymph diluted in 2 mL distilled water. Absorbance was measured at 335 nm, and haemocyanine concentration was calculated from the molar extinction coefficient $E^{\text{mg mL}^{-1}}_{(1\text{cm})} = 0.0283$, as previously described by Harris and Andrews (2005).

pH

pH of thawed vortexed haemolymph samples free of clots was measured at 4 and 12 °C (haemolymph from transport crabs) or 16 °C (haemolymph from recovery crabs). Samples were measured with a pH meter (Hanna Instruments, HI 221) using a pre-calibrated micro-electrode

(Hanna Instruments, HI 1083P) and pH buffers 4.01, 7.01, and 10.00 (WTW, Wissenschaftlich-Technische Werkstätten) as standards.

Statistics

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (sd). Whenever necessary, data were transformed in order to satisfy ANOVA assumptions, followed by multiple comparison tests (Tukey HSD). If transformed data could not meet ANOVA assumptions, non-parametric analysis of variance (Kruskal-Wallis) was performed, followed by non-parametric multiple comparisons tests (Mann-Whitney). Whenever two groups were compared, Student's t-test was applied instead of ANOVA, followed by the correspondent nonparametric test (Mann-Whitney) if data could not meet Student's t-test assumptions. Vigour, transport and recovery duration were subjected to correlation analyses, using the Pearson r correlation coefficient. All statistical analyses were tested at 0.05 level of probability with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa, OK74104, USA).

Results

Water quality

During transport in immersed conditions there was a decline in the content of dissolved oxygen from 10.9 to 4.4 mg L⁻¹ and of pH levels, from 8.14 to 7.42, but an increase in nitrites (from 0.3 to 3.3 mg L⁻¹), while ammonia values were maintained at 0.6 mg L⁻¹. In contrast, during the acclimation period of crabs transported in semi-dry conditions, seawater dissolved oxygen was maintained at 8.41-9.44 mg L⁻¹, pH was kept at 8.21-8.42, nitrites were not detectable and ammonia was constant over the 6 h period at 0.6 mg L⁻¹. During the 72 h recovery, seawater dissolved oxygen was kept at 9.1-9.5 mg L⁻¹, pH was 8.01-8.03, nitrites rose from 0 to 0.3 mg L⁻¹, and ammonia was constant, 0.6 mg L⁻¹.

Vigour

C. pagurus vigour during simulated live transport, acclimation and recovery is shown in figure 3.3.3. Two out of 8 crabs immersed in aerated seawater at 12 °C (treatment A) died after 48 h transport period. In contrast, during simulated semi-dry transport only one crab out of 24 exposed to air died, one animal became weak and three moribund. During recovery of animals not subjected to acclimation, one died in each treatment (A and B), and one became moribund in treatment B. Concerning animals subjected to acclimation, one out of the three moribund crabs died during acclimation from 11 to 16 °C (treatment C) and another animal also died in this treatment after 48 h recovery. In contrast, all animals subjected to acclimation from 6 to 16 °C (treatment D) were strong, and at the end of the recovery period only one crab died and one became moribund.

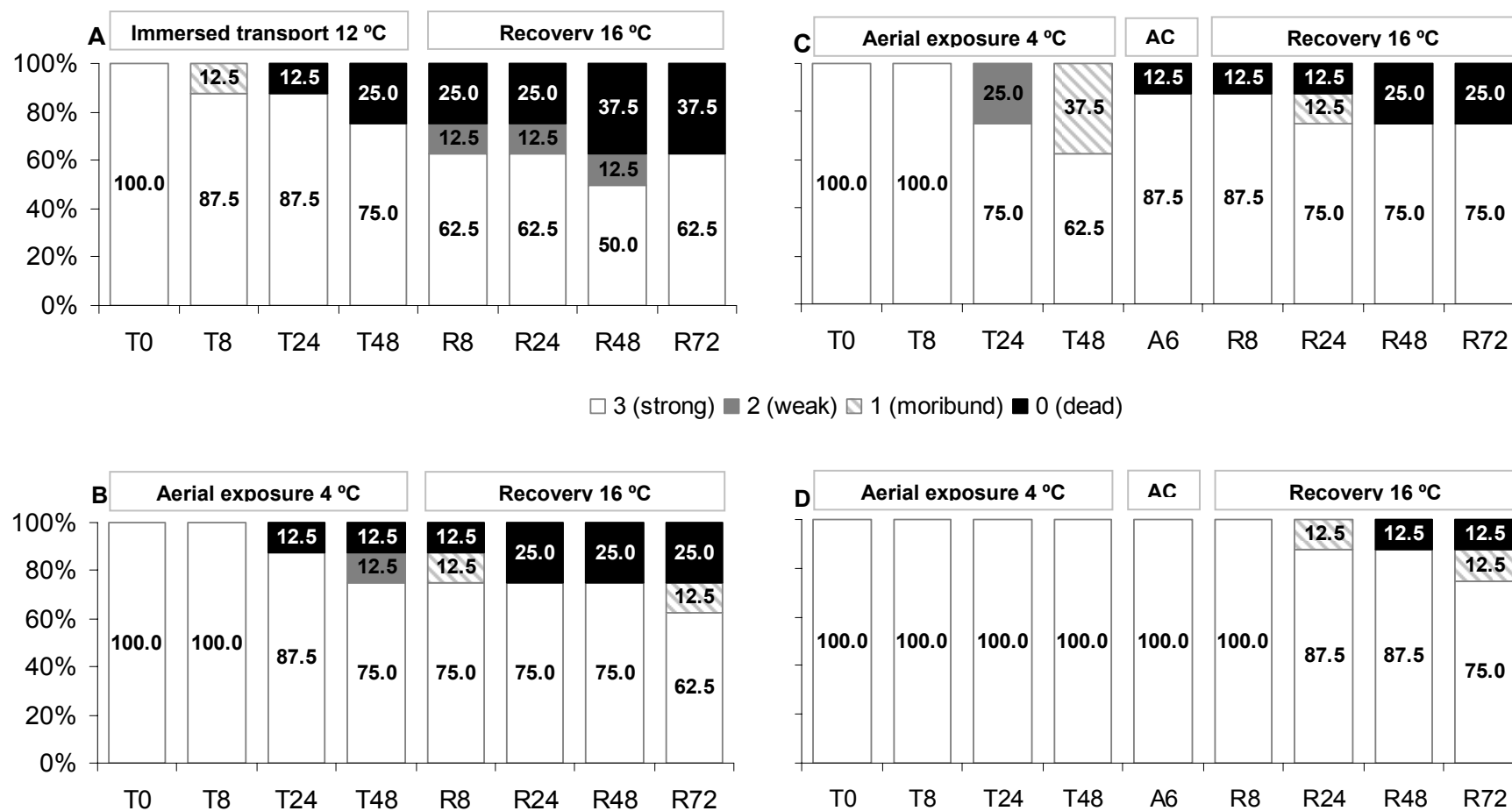


Figure 3.3.3 Vigour of crabs during the experimental conditions. Abbreviations (A) treatment A, crabs transported in seawater at 12 °C during 48 h and immediately put in the stocking tanks to recover at 16 °C; (B) treatment B, crabs transported in semi-dry conditions at 4°C during 48 h and immediately put in stocking tanks to recover at 16 °; (C) treatment C, crabs transported in semi-dry conditions at 4 °C during 48 h, immersed and acclimated during 6 h from 11 to 16 °C and recovered at 16 °C; (D) treatment D, crabs transported in semi-dry conditions at 4 °C during 48 h, immersed and acclimated during 6 h from 6 to 16 °C and recovered at 16 °C. Abbreviations: (T0 - T48) transport at 0, 8, 24 and 48 h; (A6) acclimation after 6 h; (R8 - R72) recovery after 8, 24, 48 and 72 h; (AC) Acclimation.

Blood parameters

L-lactate, D-glucose, haemocyanin and pH values of the control group are presented in table 3.3.2.

Table 3.3.2. Haemolymph parameters of the control group of edible crab.

Haemolymph parameters	Control group
L-lactate (mM)	0.50 ± 0.11
pH	7.60 ± 0.03
D-glucose (mM)	0.24 ± 0.06
Haemocyanine (mg mL ⁻¹)	24.9 ± 8.6

Values are presented as average ± standard deviation.

Generally, air exposed crabs during simulated transport (treatments B, C and D) had similar concentration of L-lactate, D-glucose and haemocyanine (Table 3.3.3), while crabs immersed in seawater at 12 °C showed lower L-lactate and D-glucose concentration but higher pH values after 48 h of simulated live transport, though not always significantly. L-lactate concentrations were always above those of the control group during transport, while D-glucose concentration only resumed to control values in immersed crabs after 48 h.

Values of pH were similar to those of the control group only in immersed crabs at 24 h and treatment B, but higher at 48 h. Haemocyanine concentrations in all transported crabs were similar to those of the control group, but significantly decreased during recovery.

After 6 h acclimation no differences were detected between treatments C and D for all blood parameters, and in both treatments only pH resumed to control values.

Throughout the 72 h of recovery there were no major differences between treatments in the concentration of L-lactate, D-glucose and haemocyanine. But after 72 h, control values were reached for L-lactate (treatments C and D) and pH (treatments B, C and D). In treatment C, L-lactate concentration was lower than the control. Haemocyanine concentrations in all treatments during transport, were similar to those of the control group, but significantly decreased from 48 h and onwards.

Table 3.3.3 Haemolymph parameters (L-lactate, pH, D-glucose and haemocyanine) of edible crab throughout the experiment.

		Transport				Acclimation		Recovery			
		0h	8h	24h	48h	6h	8h	24h	48h	72h	
L-lactate (mM)	A	4.19 ± 2.23 a***	5.45 ± 2.49 a***	4.16 ± 1.61 a***	2.13 ± 0.24 b***	-	0.97 ± 0.36 a***	0.73 ± 0.26 a**	0.75 ± 0.16 a***	0.65 ± 0.20 a*	
	B	3.02 ± 1.23 a***	4.59 ± 1.32 a***	5.14 ± 1.70 a***	4.77 ± 2.91 ab***	-	1.93 ± 0.39 a***	0.93 ± 0.86 a*	0.86 ± 0.24 a***	0.57 ± 0.14 a	
	C	3.76 ± 1.94 a***	6.40 ± 2.72 a***	6.34 ± 3.05 a***	7.22 ± 3.68 a***	4.68 ± 1.16 a***	1.46 ± 1.16 a***	1.05 ± 0.43 a***	0.48 ± 0.15 a	0.27 ± 0.09 a***	
	D	2.49 ± 1.07 a***	5.43 ± 2.51 a***	5.26 ± 2.16 a***	5.57 ± 3.09 a***	4.77 ± 3.53 a***	1.35 ± 1.30 a**	1.15 ± 0.77 a**	0.64 ± 0.21 a*	0.59 ± 0.18 a	
pH	A	7.40 ± 0.04 ab***	7.44 ± 0.10 a***	7.60 ± 0.03 a	7.65 ± 0.03 a**	-	7.54 ± 0.06 a*	7.38 ± 0.06 a***	7.45 ± 0.03 a***	7.53 ± 0.03 a***	
	B	7.46 ± 0.04 a***	7.38 ± 0.04 ab***	7.54 ± 0.10 ab	7.54 ± 0.06 a*	-	7.44 ± 0.13 ab***	7.28 ± 0.18 a***	7.35 ± 0.16 a***	7.60 ± 0.07 a	
	C	7.45 ± 0.03 a***	7.35 ± 0.05 b***	7.39 ± 0.02 c***	7.41 ± 0.05 b***	7.54 ± 0.15 a	7.46 ± 0.06 ab***	7.35 ± 0.04 a***	7.48 ± 0.07 a***	7.55 ± 0.05 a	
	D	7.37 ± 0.05 b***	7.40 ± 0.03 ab***	7.32 ± 0.14 bc***	7.47 ± 0.06 b***	7.56 ± 0.05 a	7.32 ± 0.06 b***	7.30 ± 0.08 a***	7.38 ± 0.11 a***	7.46 ± 0.23 a	
D-glucose (mM)	A	0.50 ± 0.23 a***	0.63 ± 0.25 a***	0.41 ± 0.10 a***	0.26 ± 0.08 b	-	0.27 ± 0.07 a	0.41 ± 0.36 a*	0.44 ± 0.09 a***	0.48 ± 0.09 a***	
	B	0.50 ± 0.22 a***	0.59 ± 0.28 a***	0.60 ± 0.20 a***	0.42 ± 0.22 ab**	-	0.48 ± 0.09 a***	0.44 ± 0.10 a***	0.51 ± 0.20 a***	0.42 ± 0.23 a**	
	C	0.60 ± 0.30 a***	0.52 ± 0.27 a***	0.47 ± 0.26 a***	0.40 ± 0.19 ab**	0.58 ± 0.25 a***	0.23 ± 0.08 a	0.48 ± 0.21 a***	0.49 ± 0.24 a***	0.59 ± 0.32 a***	
	D	0.43 ± 0.25 a**	0.59 ± 0.27 a***	0.46 ± 0.26 a**	0.43 ± 0.10 a***	0.63 ± 0.18 a***	0.38 ± 0.17 a	0.38 ± 0.18 a	0.52 ± 0.16 a***	0.45 ± 0.18 a***	
Haemocyanin (mg mL⁻¹)	A	19.0 ± 5.4 a	20.5 ± 8.3 a	18.0 ± 6.4 a	11.7 ± 2.8 a**	-	10.7 ± 1.4 a**	10.1 ± 2.2 a**	11.5 ± 1.4 a**	9.7 ± 2.4 a**	
	B	20.5 ± 5.7 a	16.5 ± 6.1 a	18.1 ± 7.1 a	12.3 ± 4.3 a**	-	11.6 ± 3.8 a**	9.6 ± 2.1 a**	10.7 ± 3.3 a**	11.5 ± 3.3 a**	
	C	19.6 ± 5.7 a	15.2 ± 5.8 a	17.9 ± 6.3 a	12.0 ± 3.8 a**	13.4 ± 4.2 a**	12.3 ± 1.4 a**	11.2 ± 2.2 a***	11.3 ± 1.4 a**	12.4 ± 2.4 a**	
	D	21.4 ± 4.6 a	18.8 ± 6.0 a	19.5 ± 6.3 a	14.4 ± 4.4 a**	12.5 ± 3.3 a**	11.6 ± 4.3 a**	9.6 ± 3.3 a***	12.1 ± 3.1 a**	10.4 ± 3.3 a***	

Values are presented as average ± standard deviation. For each parameter, different letters in each column represent significant differences ($p < 0.05$). Statistical differences to values of the control group are represented as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Abbreviations: (A) treatment A, crabs transported in seawater at 12 °C during 48 h and immediately put in the stocking tanks to recover at 16 °C; (B) treatment B, crabs transported in semi-dry conditions at 4 °C during 48 h and immediately put in stocking tanks to recover at 16 °C; (C) treatment C, crabs transported in semi-dry conditions at 4 °C during 48 h, immersed and acclimated during 6 h from 11 to 16 °C and recovered at 16 °C; (D) treatment D, crabs transported in semi-dry conditions at 4 °C during 48 h, immersed and acclimated during 6 h from 6 to 16 °C and recovered at 16 °C.

Correlations

Results of correlations are shown in table 3.3.4. L- Lactate was negatively correlated with vigour in all treatments, with transport duration in immersed crabs and with recovery duration in air exposed crabs, whereas L-lactate was positively correlated with the transport duration in air exposed crabs. As far as pH is concerned only positive correlations were found with vigour in air exposed crabs (treatment C), with transport duration in immersed crabs and exposed to air (treatments B and D), and with recovery duration in air exposed crabs (treatments C and D). D-glucose showed negative correlation with transport duration in immersed crabs, while positive correlation was obtained with recovery duration in immersed crabs and air exposed crabs (treatment D). Concerning haemocyanin, only negative correlations were detected with transport duration for all treatments.

Table 3.3.4 Pearson's correlation coefficient between haemolymph parameters of crabs in each treatment (A, B, C and D) and vigour, transport or recovery duration.

Haemolymph parameters	Treatments	Vigour	Transport duration	Recovery duration
L-lactate	A	$r = -0.207$ ***	$r = -0.418$ **	$r = -0.279$ ns
	B	$r = -0.483$ ***	$r = 0.307$ *	$r = -0.638$ ***
	C	$r = -0.286$ ***	$r = 0.359$ **	$r = -0.682$ ***
	D	$r = -0.317$ *	$r = 0.460$ ***	$r = -0.600$ ***
pH	A	$r = -0.041$ ns	$r = 0.913$ ***	$r = -0.038$ ns
	B	$r = 0.224$ ns	$r = 0.540$ **	$r = 0.339$ ns
	C	$r = 0.285$ *	$r = -0.248$ ns	$r = 0.468$ *
	D	$r = 0.076$ ns	$r = 0.435$ *	$r = 0.485$ **
D-glucose	A	$r = -0.057$ ns	$r = -0.388$ **	$r = 0.707$ ***
	B	$r = 0.090$ ns	$r = -0.067$ ns	$r = -0.239$ ns
	C	$r = 0.082$ ns	$r = -0.242$ ns	$r = 0.496$ ns
	D	$r = -0.091$ ns	$r = 0.059$ ns	$r = 0.255$ *
Haemocyanine	A	$r = -0.056$ ns	$r = -0.459$ *	$r = 0.121$ ns
	B	$r = -0.197$ ns	$r = -0.423$ *	$r = -0.051$ ns
	C	$r = 0.224$ ns	$r = -0.388$ *	$r = 0.133$ ns
	D	$r = 0.227$ ns	$r = -0.400$ *	$r = -0.024$ ns

Abbreviations: (A) treatment A, crabs transported in seawater at 12 °C during 48 h and immediately put in the stocking tanks to recover at 16 °C; (B) treatment B, crabs transported in semi-dry conditions at 4 °C during 48 h and immediately put in stocking tanks to recover at 16 °C; (C) treatment C, crabs transported in semi-dry conditions at 4 °C during 48 h, immersed and acclimated during 6 h from 11 to 16 °C and recovered at 16 °C; (D) treatment D, crabs transported in semi-dry conditions at 4 °C during 48 h, immersed and acclimated during 6 h from 6 to 16 °C and recovered at 16 °C. Symbols: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, not significant.

Discussion

The simulated transport systems tested induced physiological stress to *C. pagurus* culminating in mortalities of 12.5 % in the immersed system and 4 % in the semi-dry system. Both systems elicited anaerobic responses marked by hyperglycaemia, increased L-lactate concentration and decreased pH and haemocyanine content in haemolymph. Anaerobiosis is a common physiological mechanism to maintain the energy required by tissues during impaired oxygen supply and has been reported to occur in several crustacean species exposed to air (Uglow *et al.*, 1986; Chen and Chen, 1998; Morris and Oliver, 1999; Ridgway *et al.*, 2006; Lorenzon *et al.*, 2007, 2008). Air and water are very different respiratory environments for crustaceans. Dissolved oxygen is about 30 times higher in air than in an equivalent volume of water at the same oxygen pressure and temperature (Randall *et al.*, 2002). However, gills are not appropriate air-breathing organs, as they usually collapse in air thus reducing the surface area and limiting both oxygen and carbon dioxide exchanges (Durand *et al.*, 2000). Therefore, aquatic crustaceans exposed to air rapidly accumulate carbon dioxide, leading to respiratory acidosis, and are deprived of adequate oxygen supply (Durand *et al.*, 2000).

Usually, such problems are not a concern in seawater, but live transport requires seawater confinement that is not filtrated and the animal density is too high to maintain an adequate aeration based only on compressed air. In the present study, dissolved oxygen was insufficient (below 75 %) and pH was low (7.42) after 48 h. Haemolymph acidosis particularly affected air exposed crabs and was most likely due to respiratory difficulties (hypercapnia) and to L-lactate accumulation (Schmitt and Uglow, 1997). On the contrary, immersed crabs returned to pH values similar to the control after 24 h and the highly statistical positive correlation between pH and transport duration ($r = 0.913$, $p < 0.001$) further supports the pH increase. A similar result was obtained by Lorenzon *et al.* (2008) in *C. pagurus* transported in immersed conditions at 10-13 °C during 36 h. These authors suggested that the high pH was probably related to a higher concentration of ammonia in haemolymph that buffered the potential acidosis caused by the increment of L-lactate.

Another trend observed during transport and recovery was the decrease in haemocyanine concentration compared to the control group. Haemocyanine concentration decreased in the last hours of transport with a negative correlation in the duration of both transport systems and low values remained during recovery. Haemocyanine is a respiratory pigment that binds oxygen and, therefore, increases the O₂ content in the blood (Randall *et al.*, 2002; Powell and Watts, 2006), and also functions as food reserve (Uglow *et al.*, 1986). Variations in oxygen capacity and affinity of haemocyanine have been associated with hypoxic exposure in crustaceans (McMahon, 2001). Furthermore, haemocyanine can be disabled by extreme oxygen deprivation, but compensatory synthesis of additional haemocyanine in response to hypoxia has also been reported in crustaceans (Hagerman and Baden, 1988). Therefore, the decreased haemocyanine concentration observed in this study is unlikely related to changes in the respiratory pigment capacity, but likely due to the food reserve feature. Either way, lowered haemocyanine concentration can difficult the crab recovery process.

Considering blood parameters, differences between both transport systems were only observed after 48 h; for instance L-lactate and D-glucose were lower in immersed crabs, while pH was higher in immersed crabs. These results evidence that crabs physiological response to simulated transport, *i.e.* development of anaerobiosis and decreased haemocyanine, was similar in semi-dry and immersed conditions but with great variation between individuals. Therefore, it seems that edible crab can be transported in semi-dry conditions at 4 °C without becoming more stressed than crabs transported in seawater at 12 °C.

Most crab's mortality is known to occur during recovery at reception facilities. In this experiment different recovery conditions were tested, as some crabs were acclimated while others were immediately immersed and recovered at 16 °C. The response of crabs under these circumstances is essential considering that holding facilities of most importers do not have the necessary infrastructures to lower the temperature below 16 °C.

C. pagurus lives preferably in thermal ranges between 4 and 16 °C depending on the season and geographical location (Chartois *et al.*, 1994; Metzger *et al.*, 2007). At the lower thermal limit for this species the metabolic rate is decreased due to temperature induced dormancy. Therefore, crabs submitted to a sudden increase of 12 °C (*i.e.*, from 4 to 16 °C, treatment B) or of 7 °C (*i.e.*, from 4 to 11 °C, treatment C) would be expected to experience heat shock and show signs of stress. In contrast, crabs submitted to a 2 °C increase followed by a slow increase up to 16 °C (treatment D) would be expected to show a minor stress responses and a quicker recovery. Despite such events were not perceived in haemolymph parameters between these treatments, crabs not subjected to drastic temperature changes (treatment C) had stronger vigour.

It is important to state that acclimation can represent an extra step and more handling. Excess handling can be solved if boxes are designed to be used from the fishing vessel to holding facilities and to be immersed or flushed with seawater to revitalise crabs at the holding facilities. Results also indicate that vigour can be severely affected (increasing mortalities) if a crab is stressed during transport to a point that it becomes moribund, even if the stressor is removed and crabs are revitalized in good seawater quality (treatments B and C). Since capture, crabs face an alien environment that can easily convert a good quality and vigorous animal to moribund crabs, but it is nearly impossible to reverse this process (Ugnow, 1997). Therefore, the selection of strong crabs (vigour 3) is the key step to achieve a good survival rate.

The lack of differences regarding the haemolymph parameters during recovery can be due to other reasons: (1) the acclimation was too fast; (2) the thermal tolerance limits of this species were not exceeded and/or (3) the recovery was more dependent on the magnitude of the inflicted stress during transport than on the recovery temperature amplitude *per se*.

The gradual increase in temperature during acclimation could have been too fast and, therefore, differences between treatments were not perceived. In fact, the time taken to remove L-lactate from subtidal crustaceans' haemolymph is generally protracted, whereas animals that regularly experience hypoxia in their natural habitat may be more adapted to a rapid recovery (Taylor and Spicer, 1987). Additionally, L-lactate disappearance by oxidation and/or gluconeogenesis can be

masked by diffusion into the haemolymph of L-lactate from other tissues (muscle, hepatopancreas and heart tissue) that also accumulate L-lactate, resulting in steady levels during recovery (Schmitt and Uglow, 1997; Frederich and Pörtner, 2000). In this study, L-lactate concentration reached control values at 72 h only in treatments C and D, whereas Lorenzon *et al.* (2008) reported that *C. pagurus* only reached control values after 96 h recovery.

Concerning the thermal tolerance limits, it is known that extended exposure of animals to temperatures above their upper limit or below the lower limit leads to death (Frederich and Pörtner, 2000; Pörtner *et al.*, 2006). In between these limits a window of maximum scope for aerobic activity exists, which indicates the range of optimum performance supporting successful survival. Cuculescu *et al.* (1998) determined the upper critical temperature limit for *C. pagurus* acclimated at 8 °C, which was 21.5 °C in winter caught crabs and 25.1 °C in summer caught crabs, thus concluding that there was a marked seasonal effect. Considering this concept and the ecological tolerance range of *C. pagurus* (4 to 16 °C) it can be hypothesised that crabs were not recovered beyond the window of maximum scope for aerobic activity, and therefore their survival was not threatened by the temperature increase in all treatments.

Another possibility is related to the magnitude of the inflicted stress, which can be reversible or irreversible. Irreversible stress occurs when crabs are weakened to a point that homeostasis is impaired leading to death, while reversible stress consists of changes that lie within the homeostatic capability of animals when the original stressor is removed (Taylor *et al.*, 1997). Crabs that died during acclimation or recovery were probably unable to recover from the inflicted stress during transport and not due to temperature stress after transport. Since in this study both transport systems caused a similar stress response, it can be argued that recovery was also alike, taking into consideration that crabs had a similar initial vigour. This suggests that *C. pagurus* transported in semi-dry conditions can be put directly in stocking tanks to recover at 16 °C without being acclimated, as long as seawater is well aerated and filtered. However, such conclusion can be hasty and should not be regarded as a generalization, especially considering that thermal tolerance of crustaceans is seasonally dependent (Cuculescu *et al.*, 1998; Hopkin *et al.*, 2006). Moreover, crustaceans' critical temperatures are measured considering a progressive increase in temperature (0.2 °C per minute) instead of sudden increases, which is rarely seen in nature (Jiang *et al.*, 2008).

Transport in semi-dry conditions followed by 6 h acclimation from 6 to 16 °C (at 1.5 °C per hour) is certainly an alternative to immersed conditions considering that crabs under these conditions resumed control levels of L-lactate and pH at the end of the recovery period and showed the lowest mortality. Moreover, this transport system promotes a strong reduction in economical and ecological costs by avoiding the transportation of seawater.

This experiment was an attempt to prove that survival of crabs transported in semi-dry conditions even after major stressors were previously inflicted, such as capture, international transport and poor handling, is still possible with minor changes (low temperature, humid conditions, boxes without holes, and stacking crabs in a single row). This system can be further improved if grading is implemented, as well as systems allowing less handling and suitable for transport from shipboard

to the final destination. Such crate system was previously suggested by Uglow (1997), and if implemented it would reduce the number of times that stock is handled in post-harvest treatment, facilitate grading with less handling and it would also be a common link between fishermen and final destination companies thus enabling traceability.

Chapter 4

Nutritional quality of clawed lobsters and edible crab

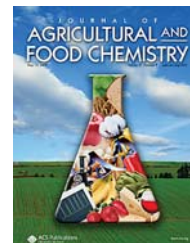


Still life with cat and lobster, 1962
Pablo Picasso (private collection)

Sub-chapter 4.1

Biochemical composition of homarids

Barrento, S., Marques, A., Teixeira, B., Vaz-Pires, P., and Nunes, M. L. 2009. Nutritional quality of the edible tissues of European lobster *Homarus gammarus* and American lobster *Homarus americanus*. Journal of Agricultural and Food Chemistry, 57: 3645-3652.



The clawed lobsters *Homarus gammarus* and *Homarus americanus* are high-priced and appreciated food items in southern Europe. From a nutritional point of view there is still limited information on the chemical composition of edible tissues (muscle, hepatopancreas, and gonads) of both species. Therefore, the aims of the present work were to study the proximate chemical composition, energy, fatty acid and amino acid profiles, and cholesterol content in each edible tissue of both species and to evaluate their nutritional quality. Differences were observed between tissues, sexes, and species. Muscle and gonads were rich in protein, whereas hepatopancreas had high fat, cholesterol, and energy contents. All edible tissues were valuable sources of essential amino acids. Contrary to the common belief that shellfish consumption should be reduced in a low-fat and cholesterol diet, all edible tissues of clawed lobsters have a nutritious value perfectly compatible with nutritious and healthy diets.

Keywords: clawed lobsters, *Homarus americanus*, *H. gammarus*, hepatopancreas, gonads, muscle, biochemistry, nutritional quality.

Introduction

Marine resources are important constituents of the human diet, and their consumption has increased worldwide in recent decades (FAO, 2007). At the same time, seafood has been much publicized for its health-promoting qualities. In general, seafood is a valuable nutrient source, providing all essential amino acids, trace elements, vitamins, and polyunsaturated fatty acids that are precursors of biologically active molecules and crucial in preventing human cardiovascular and inflammatory diseases (Karakoltsidis *et al.*, 1995). Among seafood, crustaceans are regarded as nutritionally valuable sources of proteins and minerals (Gökoğlu and Yerlikaya, 2003; Garofalaki *et al.*, 2006; Elvevoll *et al.*, 2008). Yet, crustaceans have long been assumed to be rich in cholesterol, and their use in low-fat and low-cholesterol diets is sometimes considered to be controversial (Mathew *et al.*, 1999).

Clawed lobsters present an increasing nutritional, commercial, and economical importance, being widely consumed in southern Europe, particularly in Mediterranean countries. The European lobster, *Homarus gammarus*, is a commercially high-priced species that is mainly captured off the northeastern Atlantic coasts of Europe, between southern Norway and Portugal (Bliss, 1990; Ingle, 1997). In 2006, captures of *H. gammarus* in European countries totaled 3,383 t (Eurostat, 2008). Due to the decrease of *H. gammarus* catching figures, American lobsters, *Homarus americanus*, are imported by several European countries. This species is caught off the eastern American coasts of the United States and Canada, supporting an important inshore fishery (Bliss, 1990; Floreto *et al.*, 2000). In 2006, the American lobster captures totaled 94,750 t (Eurostat, 2008). Both lobster species have a marketable higher price when sold alive. Therefore, lobsters not immediately marketed are stored either in large creels or pots moored in sheltered coves and harbours, or in land-based tanks and tidal ponds (Ingle, 1997). However, the European lobster is much more expensive, as much as 3 times the price, and considered to be more flavoured than the American counterpart (Sub-chapter 2.1). In European countries the muscle from claws and abdomen are much appreciated, but viscera (gonads and hepatopancreas) and eggs when present are also very flavorsome. It is well-known that the chemical composition differs between species, tissues, seasons, feeding habits, habitats, and sexes. As far as European and American lobsters are concerned, these parameters were not taken into account in previous studies.

The present work aims to determine the chemical composition (protein, fat, carbohydrate, ash, moisture, fatty acid, amino acid, and cholesterol) of the edible tissues of both female and male *H. gammarus* and *H. americanus* and to evaluate the nutritional quality to human consumption.

Material and methods

Ethical statement

All live animals utilized in the experiments have been treated with proper care, minimizing discomfort and distress and were painlessly killed. Also, the number of animals was kept to the minimum necessary to obtain scientific results, considering that the gain in knowledge and long-term benefit to the subject species is high.

Biological material

Twenty European lobsters *H. gammarus* (10 females and 10 males; origin, Scotland) and 21 American lobsters *H. americanus* (11 females and 10 males; origin, United States and Canada) were acquired in early spring (April 2007) from a retailer in Portugal and transported alive to the laboratory. All animals were intermoult hard-shelled and were kept under refrigerated conditions (~4 °C) during 1 h to decrease their metabolism before being euthanized. The muscle (from claws), hepatopancreas, and gonads from each animal were individually separated and weighed. The edible content of each tissues was calculated as follows: $(\text{tissue weight})/(\text{muscle} + \text{hepatopancreas} + \text{gonads weight}) \times 100$. Samples were pooled when there was insufficient amount of tissue to perform all analyses (e.g. male gonads). Each tissue was subsequently homogenized with a grinder (Retasch Grindomix GM200; 5000 rpm; material: PP cup and stainless steel knives); a portion of the homogenized sample was vacuum packed and stored at -20 °C, whereas the remaining was frozen at -80 °C and subsequently freeze-dried for 48 h at -50 °C and low pressure (approximately 10^{-1} atm). Samples were powdered and stored at -20 °C under controlled moisture conditions (vacuum packed) until further analyses.

Proximate chemical composition and energy content

Moisture, ash, protein, and lipid contents were determined in each specimen's tissue according to the AOAC (2005). Briefly, the moisture content was obtained by drying the sample overnight at 105 °C (laboratory heater, P-Selecta 207), ash was quantified after combustion for 16 h at 550 °C (muffle furnace, Heraeus Hanau, TYP MR170), crude protein content was determined according to the Kjeldahl method, using an automatic distillation and titration unit (VELP Scientifica, UDK152) with a conversion factor of 6.25, and total lipids were determined with the Soxhlet extraction method using ethyl ether (José M. Vaz Pereira S.A.). The energy content was estimated as proteins, 4.27 kcal g⁻¹ of wet weight; lipids, 9.02 kcal g⁻¹ of wet weight (1 kcal = 4184 kJ).

Cholesterol

The quantification of cholesterol content was based on the modified procedure of Naeemi *et al.* (1995). Each sample (300 mg of dry weight) was combined with 260 µL of internal standard solution (5 α -cholestane, Sigma; 5 mg mL⁻¹ cyclohexane, Merck), 3 mL of saturated methanolic potassium hydroxide solution (2 M, Merck), and 3 mL of methanol pro analysis (Merck). Following heating (80 °C; 30 min), samples were cooled and supplied with 250 µL of 1 M magnesium chloride solution (Merck) and 1.5 mL of cyclohexane pro-analysis (Merck). Samples were shaken and centrifuged (1500g; 4 min, centrifuge Sigma 2K15) until phase separation. The moisture content of the upper phase was removed with anhydrous sodium sulfate (Panreac). The cholesterol in the upper phase (2 µL) was separated by gas chromatography (Varian Star 3400 Cx) using helium as carrier gas at a flow rate of 1 mL min⁻¹ in a flame ionization detector and a fused silica capillary CP-Sil 8 CB column (30 m length $\times$ 0.25 mm internal diameter, 0.25 µm film thickness; J&W Scientific). The temperatures of the oven, injector, and detector were 280, 285, and 300 °C, respectively. Cholesterol was identified and quantified by comparison with the calibration curve of a

pure cholesterol standard (Sigma) with 5 α -cholestane (Sigma). Cholesterol/cholestane peak area ratios obtained with the Varian software were plotted with cholesterol concentrations, and a straight line was fitted to data points by linear regression.

Fatty acid analysis

The percent distribution of fatty acids methyl esters (FAME) of nonpolar and polar lipids was based on the experimental procedure of Lepage and Roy, (1986) as modified by Cohen *et al.*, (1988). Each sample (300 mg of dry weight) was dissolved in 5 mL of acetyl chloride/methanol (1:19 v/v; Merck), shaken, and heated (80 °C; 1 h). After cooling, 1 mL of Milli-Q distilled water and 2 mL of n-heptane pro analysis (Merck) were added, and samples were shaken and centrifuged (2000g; 5 min, centrifuge Sigma 2K15) until phase separation. The moisture content of the upper phase was removed with anhydrous sodium sulfate (Panreac). An aliquot (2 μ L) of the upper phase was then injected onto a Varian Star 3800 Cp gas chromatograph, equipped with an autosampler and fitted with a flame ionization detector at 250 °C, for FAME analysis. The separation was carried out with helium as carrier gas at a flow rate of 1 mL min⁻¹ in a capillary column DB-Wax (30 m length $\times$ 0.32 mm internal diameter; 0.25 μ m film thickness; Hewlett-Packard) programmed at 180 °C for 5 min, raised to 220 at 4 °C min⁻¹, and maintained at 220 °C for 25 min, with the injector at 250 °C. FAME were identified by comparing their retention time with those of Sigma standards. Quantitative data were calculated using the peak area ratio (% of total fatty acids) and the Varian software.

Amino acids

To extract total amino acids (protein bound + free), 40-90 mg of sample was placed in 10 mL ampoules with 3 mL of 6 M HCl (Merck) with 0.1 % phenol (Merck), according to the method described by AOAC (2005). Ampoules were vacuum-sealed, and samples were hydrolyzed at 110 °C for 24 h; hydrolysates were filtered (0.45 μ m pore size, Life sciences) and dissolved with Milli-Q distilled water to 20 mL. Samples were frozen at -80 °C and freeze-dried during 48 h at -50 °C under low pressure (approximately 10⁻¹ atm). Samples were dissolved in 5 mL of 0.1 M HCl (Merck) and stored at -80 °C until amino acid separation. After thawing samples were filtered (0.2 μ m pore size Life sciences) and separation was performed with high-performance liquid chromatography (Agilent 1100 HPLC) using precolumn o-phthalaldehyde and 3-mercaptopropionic acid in borate buffer (OPA, Agilent Technologies) and 9-fluorenylmethyl chloroformate in acetonitrile (FMOC; Agilent Technologies) derivatization, a Phenomenex Gemini ODS C18 guard column (4 $\times$ 3 mm), and a Phenomenex Gemini ODS C18 110A column (4.6 $\times$ 150 mm, 5 μ m). The solvents and gradient conditions were those described by Henderson *et al.* (2000). Detection wavelengths were set at UV 338 and 262 nm and fluorescence 340/450 and 266/305 nm. The identity and quantity of the amino acids were assessed by comparison with the retention times and peak areas of standard amino acids (Sigma- Aldrich) using norvaline as internal standard.

Nutritional quality

To measure the propensity of each edible tissue to influence the incidence of coronary heart disease, two indices: the Index of Atherogenicity (IA) and the Index of Thrombogenicity (IT) were calculated according to the Ulbricht and Southgate (1991) equations:

$$IA = [12:0 + (4 \times 14:0) + 16:0] / [(n-6 + n-3) \text{ PUFA} + \text{MUFA}];$$

$$IT = (14:0 + 16:0 + 18:0) / [(0.5 \times \text{MUFA}) + (0.5 \times (n-6) + 3 \times (n-3) + (n-3)/n-6)]$$

Where MUFA means monounsaturated fatty acids and PUFA means polyunsaturated fatty acids. Three ratios related with fatty acid content were also calculated n-3/n-6, n-6/n-3 and PUFA/SFA, in order to allow comparisons with the United Kingdom Department of Health recommendations (UKDH, 1994). Essential amino acid scores (AS) were also computed with respect to reference amino acid requirements for adults (FAO/WHO/UNU, 2007). This score determines the effectiveness with which absorbed dietary nitrogen can meet the indispensable amino acid requirement at the safe level of protein intake (FAO/WHO/UNU, 2007). This is achieved by comparing the content of each essential amino acid in the protein/diet with its content in the requirement pattern: AS (%) = (mg of amino acid in 1 g of test protein/mg of amino acid requirement) $\times$ 100. The ratio of essential amino acids to non-essential amino acids was also calculated.

Statistical analysis

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (sd). Differences in the concentration of nutritional elements between tissues were tested with analysis of variance (ANOVA) followed by multiple-comparison test (Tukey HSD). Whenever necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, differences were analyzed with nonparametric analysis of variance (Kruskal-Wallis) followed by nonparametric multiple-comparison test (Mann-Whitney). All statistical analyses were tested at the 0.05 level of probability with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Results

Proximate chemical composition and energy content

The biometric data of animals sampled in this study are presented in table 4.1.1. The proximate chemical composition of edible tissues of *H. gammarus* and *H. americanus* is shown in table 4.1.2. Significant differences were detected between tissues, sexes, and species. Gonads and muscle were rich in proteins, whereas hepatopancreas had high fat content. On the other hand, gonads and hepatopancreas generally had higher energy and cholesterol contents compared to muscle. Differences between sexes and species were seen in the content of cholesterol (hepatopancreas), moisture (muscle and gonads), and proteins (muscle).

Table 4.1.1 Biometric data of female (F) and male (M), *H. gammarus* and *H. americanus*, including the edible contribution (EC) of muscle, hepatopancreas, and gonads.

Biometric data	<i>H. gammarus</i>		<i>H. americanus</i>	
	F	M	F	M
Body weight (g)	580.1±102.8	607.8±105.0	610.6±32.8	546.8±62.4
Total length (mm)	273.3±17.1	276.5±12.3	266.1±7.2	249.4±11.0
Cephalotorax length (mm)	90.3±5.2	95.0±4.8	89.5±2.4	87.1±4.0
Muscle EC (%)	83.3±1.7	87.8±1.7	82.3±4.4	86.0±1.7
Hepatopancreas EC (%)	12.7±1.5	11.9±1.7	15.0±3.3	13.4±1.7
Gonads EC (%)	4.0±1.7	0.3 ±0.1	2.8±2.0	0.5±0.1
Total meat yield (%)	31.8±5.0	32.4± 4.2	30.2±4.6	28.5±4.6

Fatty acid profile

The fatty acid profiles were different between species and between the edible tissues (Figure 4.1.1). *H. americanus* had proportionally more MUFA, particularly eicosenoic acid (20:1n-9), than *H. gammarus*, but less n-6 fatty acids (Table 4.1.2 and Figure 4.1.1).

For both species, muscle and gonads had a similar pattern dominated by PUFA, followed by MUFA and SFA. In contrast, hepatopancreas had proportionally more MUFA, followed by SFA and PUFA (Table 4.1.2). Muscle had the most homogeneous fatty acid composition with fewer differences between sexes and species compared to gonads and hepatopancreas (Figure 4.1.1). The main saturated fatty acids in all tissues were palmitic (16:0) and stearic (18:0) acids. Palmitic acid was predominant in the hepatopancreas, particularly in males, whereas stearic acid was dominant in the muscle of the European lobster. Among MUFA, oleic acid (18:1n-9) was the prevailing fatty acid mostly in the hepatopancreas of *H. gammarus*. Palmitoleic (16:1n-7) and eicosenoic (20:1n-9) acids also contributed to the high proportion of MUFA in the hepatopancreas and in female gonads.

The main n-3 PUFA in all tissues were eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids. In gonads, docosapentaenoic acid (DPA, 22:5n-3) also contributed to the high proportion of n-3, particularly in males, in contrast to its absence in hepatopancreas. The n-3 PUFA were dominant in muscle (36.2 %) and gonads (37.3 %), compared to hepatopancreas (7.3 %) (Table 4.1.2). The major n-6 PUFA was arachidonic acid (AA, 20:4n-6), mainly in muscle and gonads.

The ratios of n-3 to n-6 and PUFA to SFA were similar in muscle and gonads, but comparatively lower in hepatopancreas (Table 4.1.2). The opposite results were found for ratios of n-6 to n-3 and for IA and IT. Females of both lobster species had generally higher IA and IT in gonads compared to males, whereas IT was lower in hepatopancreas of female lobsters compared to male hepatopancreas.

Table 4.1.2 Proximate chemical composition (%), cholesterol content (mg 100 g⁻¹ wet weight), energy content (Kcal g⁻¹ tissue on a wet weight basis), fatty acids profile (%) and amino acids content (g 100 g⁻¹ of wet weight) in muscle, hepatopancreas, and gonads of female (F) and male (M) lobsters, *H. gammarus* and *H. americanus*.

	Muscle				Hepatopancreas				Gonads			
	<i>H. gammarus</i>		<i>H. americanus</i>		<i>H. gammarus</i>		<i>H. americanus</i>		<i>H. gammarus</i>		<i>H. americanus</i>	
	F	M	F	M	F	M	F	M	F	M	F	M
Moisture	78.1±2.5b	79.2±1.4	79.2±1.7	80.5±1.6a	67.4±8.1	70.7±6.1	58.8±8.9	68.9±5.8	58.9±0.7b		68.5±0.1a	
Ash	1.8±0.2	2.0±0.2	1.8±10.1	1.9±0.1b	1.7±0.2	1.8±0.2	1.5±0.2	1.7±0.2	1.5±0.0		1.5±0.1	
Proteins	18.3±2.0a	17.6±1.2a	17.1±1.0a	15.6±1.4b	12.6±1.7	13.2±0.4	12.3±0.3	12.4±1.0	24.1±1.7		22.1±1.2	
Fat	0.3±0.0	0.5±0.3	0.7±0.3	0.6±0.1	16.6±8.5	11.8±6.6	25.5±12.1	14.9±6.8	3.8±0.8		4.4±0.2	
Cholesterol	36.6±7.6	31.2±6.0	43.2±7.9	37.2±10.5	42.5±8.1b	76.0±14.9	80.1±24.5	103.5±3.1a	114.9		105.4±37.2	
Energy	87.0±11.7	83.0±7.8	82.3±7.1	78.2±7.3	213.1±76.6	172.8±61.4	290.4±99.6	194.9±58.4	185.2		148.3	
SFA	22.7±1.3a	22.6±0.6a	21.6±1.52	20.4±0.7b	26.6±4.4bc	32.1±0.5a	24.4±1.0c	27.2±1.2b	18.7±0.1a	16.0±0.0b	16.8±0.7b	16.7±0.3b
MUFA	28.8±1.7b	28.4±2.0b	31.5±1.3a	30.0±1.1a	47.2±1.9c	55.1±3.8b	58.9±3.2	61.6±2.4a	34.3±0.1b	30.3±0.1c	36.0±0.7a	35.1±0.4ab
PUFA	43.6±2.5	44.8±1.0a	42.0±1.7b	44.6±0.8a	20.1±5.1a	7.0±3.9b	11.2±3.2b	5.8±0.1c	40.8±0.3b	42.9±0.1b	41.0±1.0b	46.3±0.7a
n-3	34.3±1.7	35.0±1.3	35.5±1.4	37.0±0.9	14.2±3.2a	3.9±2.8bc	7.7±2.7b	3.4±0.4c	32.4±0.3c	34.3±0.1b	35.6±1.1b	38.8±0.6a
n-6	8.2±1.0a	8.6±0.6a	5.8±0.8b	6.5±0.5b	4.8±2.0a	2.1±1.0	2.5±0.4	1.6±0.4b	7.9±0.0b	8.3±0.0a	5.4±0.2c	6.8±0.1c
(n-3/n-6)	4.2±0.3b	4.1±0.4b	6.2±0.6a	5.7±0.5a	3.1±0.7a	1.7±0.4b	3.1±0.7a	2.2±0.8a	4.1±0.0b	4.2±0.0b	5.4±0.4a	5.7±0.1a
(n-6/n-3)	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.1	0.6±0.2	0.3±0.1	0.5±0.2	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
PUFA/SFA	1.9±0.2	2.0±0.1	2.0±0.2	2.2±0.1	0.80±0.31a	0.22±0.11b	0.46±0.10a	0.22±0.01b	2.1±0.0c	2.7±0.0a	2.4±0.0b	2.8±0.0a
IA	0.24±0.02	0.23±0.01	0.26±0.04	0.22±0.01	0.40±0.07	0.53±0.08	0.50±0.08	0.47±0.04	0.24±0.00a	0.16±0.00b	0.24±0.01a	0.16±0.00b
IT	0.17±0.01	0.16±0.01	0.15±0.02	0.14±0.01	0.35±0.1b	0.73±0.14a	0.41±0.07b	0.58±0.07a	0.14±0.00a	0.12±0.00b	0.12±0.00b	0.11±0.00b
EAA/NEAA	0.80±0.07	0.78±0.01	0.81±0.01	0.82±0.01	0.81±0.04a	0.82±0.02a	0.74±0.03b	0.75±0.02b	0.96±0.01a	0.73±0.00c	0.78±0.03b	0.65±0.01d
TAA	17.04±2.51	16.45±0.89	16.93±1.76	17.34±1.18	10.99±1.37	9.46±0.04	10.46±1.27	9.69±0.08	24.64±0.63a	10.56±0.39d	17.93±0.09b	10.71±0.02c

In each row different letters indicate significant differences per tissue ($p < 0.05$), whereas values without letters indicate no significant differences. Abbreviations: (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity; (IT) index of thrombogenicity; (EAA) essential amino acids; (NEAA) nonessential amino acids; (TAA) total amino acids.

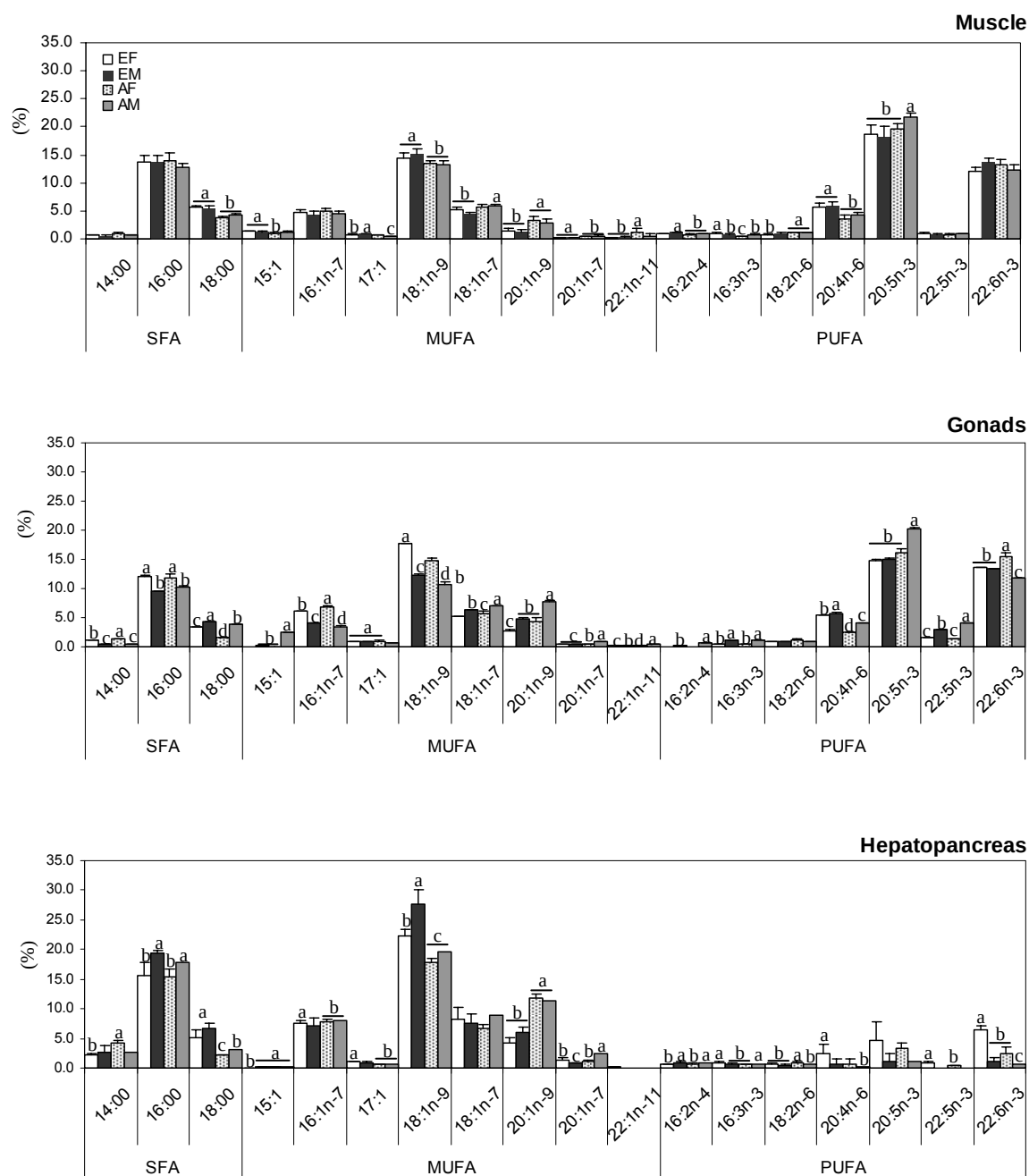


Figure 4.1.1 Fatty acid profile (%) of edible tissues of *H. gammarus* and *H. americanus*. Different letters represent statistical differences ($p < 0.05$), whereas bars without letters indicate no significant differences. Abbreviations: (EF) female *H. gammarus*; (EM) male *H. gammarus*; (AF) female *H. americanus*; (AM) male *H. americanus*; (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids.

Amino acid profile

The amino acid profile of edible tissues of homarids is shown in figure 4.1.2. Proteins of both lobsters contained high amounts of nonessential amino acids (NEAA), such as glutamic acid, aspartic acid, and glycine. Particularly, male gonads (*H. americanus*) had the highest values of NEAA (Table 4.1.2).

Muscle and hepatopancreas had similar concentration patterns of essential amino acids (EAA), dominated by arginine, leucine, and lysine, although generally higher contents were detected in muscle. Also, the amino acid content in muscle was very homogeneous between sexes and species.

The amino acid pattern was more heterogeneous in hepatopancreas and gonads according to sex and species. In hepatopancreas, *H. gammarus* had more tyrosine and taurine than *H. americanus*, but less histidine. Methionine was more concentrated in hepatopancreas of female *H. gammarus* compared to male, whereas the opposite results were found in *H. americanus* hepatopancreas.

In general, gonads of European lobster had higher concentration of most amino acids than the American counterpart. The most striking differences were found between sexes of both species, in which females had a higher concentration of most amino acids than males (except taurine). In this tissue the major essential amino acids were leucine, arginine, valine, and lysine.

The ratio of EAA to NEAA did not vary in muscle (Table 4.1.2), whereas in hepatopancreas significant differences were found between species (higher in *H. gammarus*), and in gonads both sex and species affected this ratio (higher in *H. gammarus* and in females). With regard to the amino acid score presented in figure 4.1.3, the highest scores were obtained for histidine, phenylalanine + tyrosine and threonine, in all tissues. Particularly, female gonads of European lobsters had the highest scores. The limiting amino acids were leucine (hepatopancreas of males; ovaries of *H. gammarus* and both gonads of *H. americanus* and methionine (hepatopancreas of both homarids; gonads of *H. americanus*); whereas tryptophan and cysteine were not quantified.

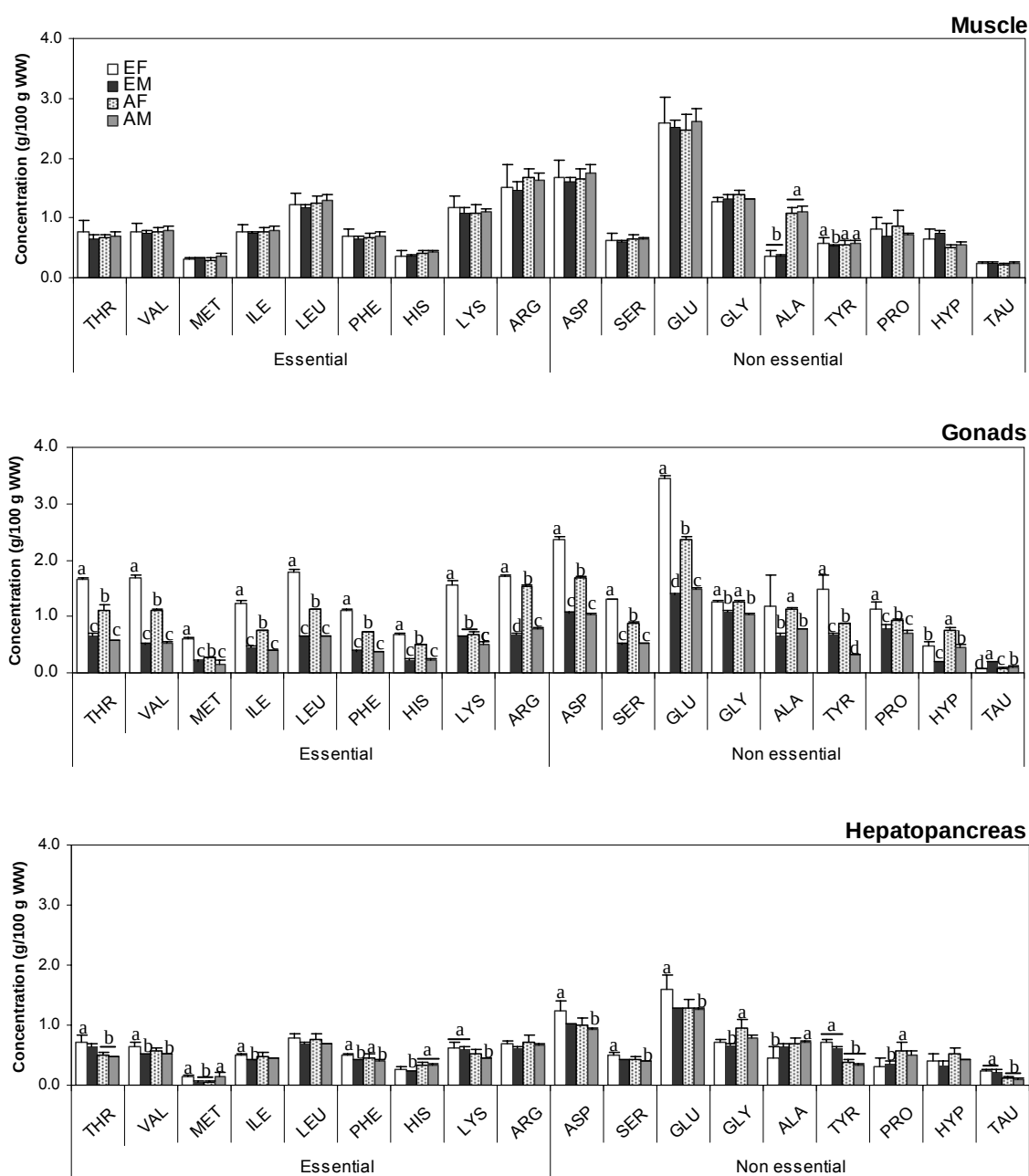


Figure 4.1.2 Amino acid profile ($\text{g } 100 \text{ g}^{-1}$ of wet weight) of edible tissues of *H. gammarus* and *H. americanus*. Different letters represent statistical differences ($p < 0.05$), whereas bars without letters indicate no significant differences. Abbreviations: (EF) female *H. gammarus*; (EM) male *H. gammarus*; (AF) female *H. americanus*; (AM) male *H. americanus*; (THR) threonine; (VAL) valine; (MET) methionine; (ILE) isoleucine; (LEU) leucine; (PHE) phenylalanine; (HIS) histidine; (LYS) lysine; (ARG) arginine; (ASP) aspartic acid; (SER) serine; (GLU) glutamic acid; (GLY) glycine; (ALA) alanine; (TYR) tyrosine; (PRO) proline; (HYP) hydroxyproline; (TAU) taurine.

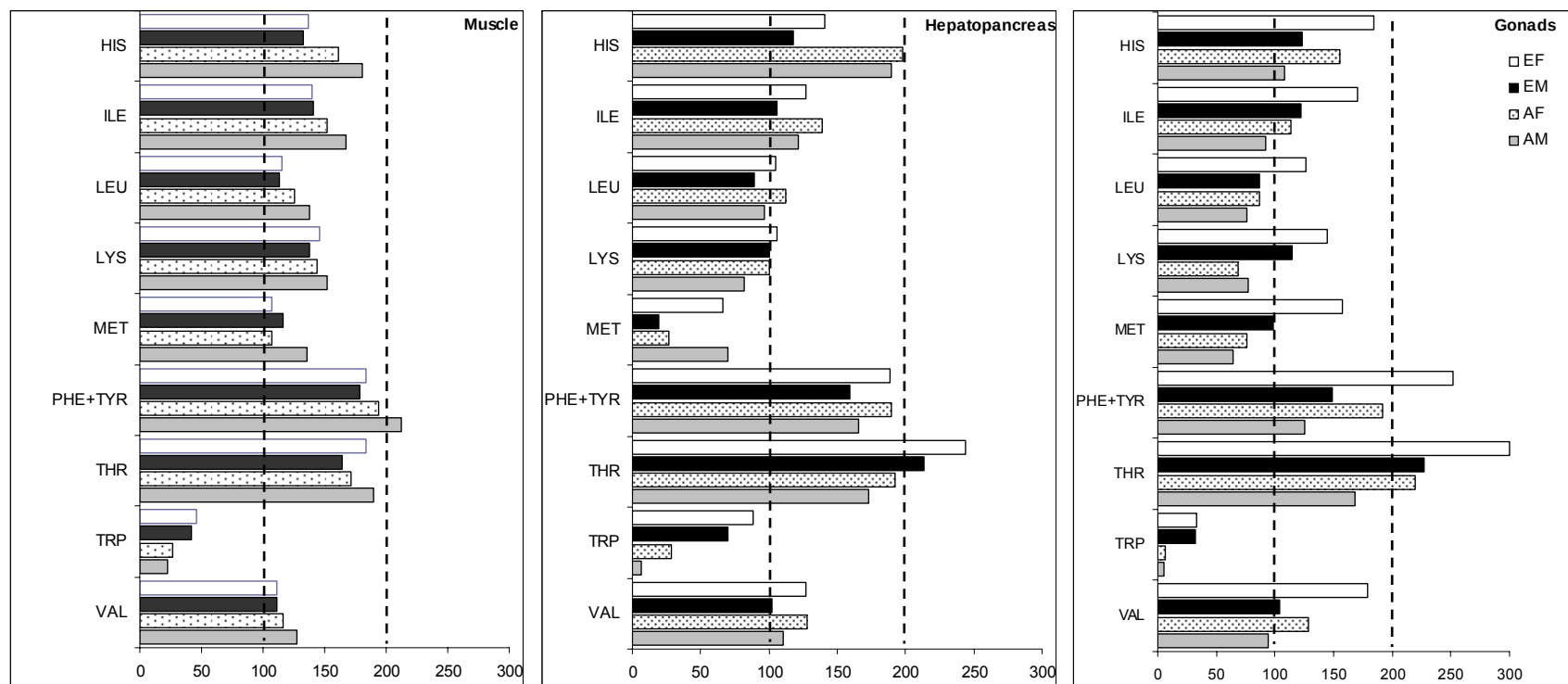


Figure 4.1.3 Representation of the essential amino acid score (%) of edible tissues of *H. gammarus* and *H. americanus*. Dotted lines represent 100 and 200 % scores. Abbreviations are as in figure 4.1.2.

Discussion

Chemical composition

This study showed significant differences in the chemical composition of the edible tissues of both homarid species. The most important variations were found in tissues involved in regulatory processes (hepatopancreas and gonads), in opposition to structural tissues (muscle). In crustaceans, hepatopancreas is the major lipid storage organ, having the highest fat content compared to the other tissues (Ying *et al.*, 2006), whereas muscle is a protein-rich tissue low in fat. These characteristics are essential considering that muscle is involved in locomotion and prey hunting. The high protein and cholesterol levels found in female gonads indicate that these nutrients, together with proteins and fatty acids, are important for maturation of crustacean ovaries (Çelik *et al.*, 2004).

The fatty acid patterns obtained for *H. americanus* in muscle and hepatopancreas were consistent with previous results (Floreto *et al.*, 2000). Variations in the fatty acid content between species, such as European lobster having proportionally more stearic acid (all tissues), oleic acid (hepatopancreas), and n-6 PUFA (all tissues), are most probably related to different diets. Other factors can also influence the distribution of lipids and fatty acids in crustaceans, namely, metabolism, moulting cycle, captivity duration, seawater temperature, and salinity (Çelik *et al.*, 2004). PUFA from the n-3 series, especially DHA and EPA, have been identified in the past few decades as essential nutrients for marine animals in general (Rainuzzo *et al.*, 1997; Sargent *et al.*, 1999). Previous studies showed that n-3 fatty acids, such as DHA and EPA, are essential for the maturation and reproduction of crab and lobsters (Castell and Covey, 1976; Ying *et al.*, 2006; Takeuchi and Murakami, 2007) and might even be implicated in the moulting process (Floreto *et al.*, 2000). This probably explains the fatty acid pattern in the gonads, which was dominated by PUFA, and the differences between females and males, especially in the European lobsters. Therefore, differences between sexes and tissues are probably related to the cumulative effects of diets, moulting cycle, and reproductive metabolism.

In this study gonads and muscle had more PUFA than hepatopancreas, but less MUFA and SFA. It has been suggested that gonads use essential fatty acids from hepatopancreas to synthesize MUFA contained in the yolk, where these fatty acids may come from triacylglycerols in the hepatopancreas (Ying *et al.*, 2006). Our results suggest that the depletion of essential fatty acids in hepatopancreas to be used by gonads can also occur in males, as DPA was absent in male hepatopancreas but accumulated in male gonads.

As far as amino acids are concerned, their profiles in muscle of both lobster species are similar to those of other decapod crustaceans, such as *Carcinus maenas* and *Parapenaeus longirostris* (Naczek *et al.*, 2004; Rosa and Nunes, 2005). However, few studies have dealt with the amino acid composition in hepatopancreas and gonads of crustaceans. Most studies concerned the dietary requirements during larval stages in order to formulate efficient diets to use in aquaculture.

Amino acids are the building blocks of proteins and are also fuel sources and precursors for other biological molecules. Arginine, leucine, and methionine are necessary for moulting of *H.*

americanus (Floreto *et al.*, 2000). Most amino acids, such as glutamic acid, aspartic acid, leucine, and arginine, were more concentrated in female gonads, particularly in European lobsters, which is probably related to their physiological role, but also the life cycle, as female *H. gammarus* had more developed gonads and one individual had already spawned. For example, arginine is highly important as the main reserve of ATP in crustaceans, whereas aspartic acid is part of the urea cycle and contributes to the formation of purines and pyrimidines (Hird, 1986). Both functions seem to be essential in a regulating organ that must invest energy and nutrients in the development of eggs. On the other hand, taurine was less concentrated in gonads compared to the remaining tissues. Taurine plays an essential role in anaerobic energy metabolism via the formation of tauropeine, which is an end product of glycolysis (Gäde and Grieshaber, 1986).

Nutritional quality

The human health aspects of eating seafood have primarily been linked to marine lipids because epidemiological studies have evidenced that seafood consumption has a potential protective role against coronary heart diseases, mainly attributed to the effects of long-chain polyunsaturated fatty acids and their cardioprotective action (Kris-Etherton *et al.*, 2002). The majority of studies concerning the benefits of seafood consumption consider only the muscle as edible tissue. Yet, several tissues can be consumed in different parts of the world. Crabs and lobsters are especially appreciated for their viscera, namely, hepatopancreas and gonads. To address the nutritional quality of each edible tissue of clawed lobsters several parameters, including indices, were considered, and the results clearly indicated differences between tissues, species, and sexes.

Total fat contents of muscle and gonads were below 5 %, a value generally considered to characterize a low-fat food (Sikorski, 2000) in contrast to hepatopancreas, which contained 11.8-25.5 %. All edible tissues had an n-6/n-3 fatty acid ratio in the range of the recommended values: the U.K. Department of Health stipulated an ideal n-6/n-3 ratio < 4.0 for human consumption (UKDH, 1994). On the other hand, the PUFA to SFA ratio in male hepatopancreas (PUFA/SFA = 0.2) was below the recommended minimum value of 0.45 set by that department. The ratio of n-3 to n-6 has been cited as an excellent index to compare the relative nutritional value of lipids, where high values correspond to better quality foods. The ratios obtained in muscle (4.1-6.2), gonads (4.1-5.4), and hepatopancreas (1.7-3.1) indicate that gonads and muscle had higher quality than hepatopancreas. Compared to other crustacean species, such as the crabs *Callinectes sapidus* (muscle, 2.32; hepatopancreas, 1.57; Çelik *et al.*, 2004) and *Carcinus mediterraneus* (muscle, 1.4; Cherif *et al.*, 2008), all edible tissues of clawed lobsters had higher nutritional quality. Hepatopancreas had the highest IA and IT values compared to muscle and gonads. Nevertheless, hepatopancreas IA (0.40-0.53) was lower than those of other food items, such as lamb (1.00), beef (0.72), pork (0.69), and rabbit (0.82), but similar to values obtained with chicken (0.50; Rosa and Nunes, 2005).

According to the amino acid score, proteins available in the edible tissues of both lobster species were well balanced in the essential amino acid composition, and the EAA to NEAA ratios were particularly good in female gonads of European lobsters. Tryptophan and the sulfur-containing

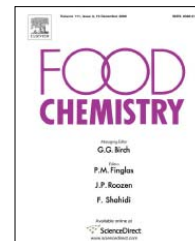
amino acid cysteine are lost during the acid hydrolysis of meat products (Spindler *et al.*, 1984) and, therefore, were not quantified. Both lobster species had good levels of taurine (2-aminoethanesulfonic acid), which is a biologically active compound in foods. Despite not being regarded as an essential nutrient, taurine can be beneficial under certain circumstances. Taurine plays an important role in several essential processes such as membrane stabilization, osmoregulation, antioxidation, development of the central nervous system and retina, and reduction of cholesterol absorption (Murakami *et al.*, 2002; Militante and Lombardini, 2004).

Nowadays, one of the major concerns about food quality and nutrition in developed countries is the cholesterol content. In this regard, muscle (31-43 mg 100 g⁻¹) and hepatopancreas (42.5-103.5 mg 100 g⁻¹) of both clawed lobsters had less cholesterol content than other commercially important decapod species, such as the crab *C. maenas* (muscle, 57.4 mg 100 g⁻¹), crayfish *Nephrops norvegicus* (muscle, 97 mg 100 g⁻¹), and shrimp *Pandalus borealis* (muscle, 107 mg 100 g⁻¹) (Skonberg and Perkins, 2002; Oehlenschläger, 2006). Whereas, gonads (105-114 mg 100 g⁻¹) had the highest cholesterol concentration in both clawed lobsters. Yet these concentrations are similar to the muscle of squid, *Todarodes sagittatus* (140 mg 100 g⁻¹) and are considered to be moderate values for human consumption (Oehlenschläger, 2006).

Sub-chapter 4.2

Essential elements and contaminants of clawed lobsters

Barrento, S., Marques, A., Teixeira, B., Vaz-Pires, P., Carvalho, M.L., and Nunes, M. L. 2008. Essential elements and contaminants in edible tissues of European and American lobsters. *Food Chemistry*, 111: 862-867.



In several European countries, clawed lobsters such as the European (*Homarus gammarus*) and American (*H. americanus*) ones are widely consumed. Yet, information about essential elements and contaminants in both species is still scarce. Therefore, the aim of this work was to characterize the elemental content in the edible part (muscle, hepatopancreas, gonads and roe) of both homarids and to compare them with the daily intake recommendations and maximum allowed levels. Two techniques were employed: energy dispersive X-ray fluorescence (EDXRF) to quantify Cl, S, K, Ca, Fe, Cu, Zn, As, Se, Br and Sr; and flame atomic-absorption spectrometry (FAAS) to analyse Na, Mg, Mn, Cd, Hg and Pb. Significant differences were found in the elemental composition of edible tissues of both species, likely reflecting the distinct physiological role of those tissues: muscle (higher: Na, Mg, Ca and Sr; lower: Fe, Se, Cd); hepatopancreas (higher: Fe, Cu, Br and Cd); gonads (lower: Cl, Ca, Zn and Hg); and roe (higher: Na and Br; lower: K and As). Statistical differences in the elemental composition of each tissue were found between both homarids: muscle (Na, Se, As and Hg); hepatopancreas (Na, Mg, Fe, Cu, Se, Br and Hg); and gonads (S and Zn). Since the geographical distribution of both species is different, the differences likely reflect distinct elemental composition in the aquatic environment and, consequently in the feed chain. Both lobster species were rich sources of Na, Cl, Cu, Zn and Se for human consumption. Regarding contaminants, only Cd was detected at high concentrations in the hepatopancreas of both homarids. Despite Cd values were well above the maximum level set by the European Commission for crustaceans' muscle, so far any limit value was set for crustaceans' hepatopancreas. Nonetheless, the present study recommends avoiding or moderately consuming this tissue in homarids.

Keywords: *Homarus gammarus*, *H. americanus*, EDXRF, FAAS, macroelements, trace elements, contaminants.

Introduction

In the last years, the health benefits related with seafood consumption have been extensively publicized. Seafood is rich in protein, contains low cholesterol and high percentage of (n-3) polyunsaturated fatty acids, liposoluble vitamins and essential minerals (Tahvonen *et al.*, 2000; Adeyeye, 2002; Chen *et al.*, 2006; Zalloua *et al.*, 2007). Epidemiological studies have indicated that populations with a rich seafood diet have low risk of coronary heart disease, hypertension and cancer (Simopoulos, 1997). In recent years, attention has been focused on determination of elements in seafood due to nutritional benefits of essential elements and toxicological concerns related to anthropogenic influx of contaminants. Lack of essential elements (e.g. Na, Mg, Cl, P, S, K, Ca, Mn, Fe, Cu, Zn, Se) leads to improper enzyme mediated metabolic functions and results in organ malfunctions, chronic diseases and ultimately death (Simopoulos, 1997; FAO/WHO, 2002). Other trace elements, such as Br and Sr, are still being categorised as essentials (Oehlenschläger, 1997). Therefore, regular intake of these elements via food ingestion is vital. On the other hand, environmental contaminants (e.g. As, Cd, Hg and Pb) are a major concern in aquatic environments, since they cause severe human health disorders (Carvalho *et al.*, 2005; Francesconi, 2007).

Clawed lobsters (*Homarus gammarus* and *H. americanus*) are worldwide highly prized delicacies (Holmyard and Franz, 2006). In Southern Europe, especially in coastal areas of Mediterranean countries (e.g. Italy, France, Spain, Greece and Portugal), these crustaceans are widely consumed. The European lobster *H. gammarus* production in 2005 was 2,326 t in all EU countries (EUROSTAT, 2007). This species typically inhabits the North Eastern Atlantic coast, Mediterranean Sea and Black Sea down to 100 m depth (Ingle, 1997; Holmyard and Franz, 2006). In order to satisfy the high consuming demands in Europe, American lobster *H. americanus* has been extensively imported from USA and Canada (16,000 t, corresponding to 140 million euros economic value; data from 2006; INFOFISH, 2007). *H. americanus* is mostly found along the North Western Atlantic coast down to 50 m depth (Holmyard and Franz, 2006).

Several studies have documented crustaceans' mineral value (Skonberg and Perkins, 2002; Gökoğlu and Yerlikaya, 2003; Küçükgülmez *et al.*, 2006) and contaminants content (Páez-Osuna and Ruiz-Fernández, 1995ab; Páez-Osuna and Bdjorquez-Leyva, 1999; Kádár *et al.*, 2006; Mishra *et al.*, 2007; Mohapatra *et al.*, 2007). Yet, as far as clawed lobsters are concerned there is still limited information on their essential elements and contaminants composition. This knowledge is fundamental, especially considering the high consumption of both homarids, but also because the edible tissues of *H. americanus* are available in a wide variety of forms for human consumption (Holmyard and Franz, 2006). These forms include processed hepatopancreas, presented as a green coloured meat (named tomalley), roe and muscle. Since each tissue has distinct functions, it is assumed that their mineral content also varies. In this context, the aim of the present work was to quantify the essential elements and contaminants contents in the muscle, hepatopancreas, gonads and roe of *H. americanus* and *H. gammarus*, and to compare such values with the daily intake recommendations and maximum limit levels.

Material and methods

Biological material

Twenty-one live American lobsters *H. americanus* (origin: USA and Canada; 580.1 ± 58 g average weight) and 20 European lobsters *H. gammarus* (origin: Scotland; 587 ± 81 g average weight) were purchased from a local company soon after importation and immediately transported to the laboratory. Animals were kept under refrigerated conditions during one hour to decrease their metabolism before being euthanized. The muscle tissue (from claws, legs and abdomen), hepatopancreas, gonads and roe (whenever present) were individually separated and weighed. Each tissue was subsequently homogenised with a grinder, vacuum packed and stored at -20 °C, whereas the remaining was frozen at -80 °C and subsequently freeze-dried for 48 h at -50 °C and low pressure (approximately 10^{-1} atm). Samples were powdered and stored at -20 °C under controlled moisture conditions (vacuum packed) until further analyses.

Element analyses

Energy dispersive X-ray fluorescence (EDXRF) spectrometer was used to quantify Cl, S, K, Ca, Fe, Cu, Zn, As, Se, Br and Sr. The EDXRF technique consists of an X-ray tube equipped with a changeable secondary target, normally of molybdenum (EXTRA II A, Atomika Instruments, Temple). The characteristic radiations emitted by the elements in the sample were detected by a Lithium drifted Silicon [Si (Li)] detector, with a 30 mm^2 active area and an $8\text{ }\mu\text{m}$ beryllium window. The energy resolution was 135 eV at 5.9 keV and the acquisition system was a Nucleus PCA card. Quantitative calculations were made by the fundamental parameters method (Mantler and Kawahara, 2004). Experimental parameters were obtained by calibration, using standard reference materials. The X-ray generator was operated at 50 kV and 20 mA and a typical acquisition time of 1000 s was used. Each sample powder (~ 1 g) was pressed into pellets of 2 cm diameter without any chemical treatment. A minimum of three pellets (replicates) per sample were glued onto Mylar films, on sample holders, and placed directly in the X-ray beam. There are several advantages of using EDXRF to determine elemental content: no sample preparation, less time consuming compared to conventional techniques, low amount of sample required, no need to use liquid reagents, thus eliminating reagent waste and reducing potential contamination. The elements Na, Mg, Mn, Cd and Pb were measured by flame atomic-absorption spectrometry (FAAS), with a Variant Spectr AA 20 spectrometer, according to official analytical methods (AOAC, 2005). The procedure is based on sample incineration (5 g for Na, Mg and Mn; 10 g for Cd and Pb) and dissolution in HNO_3 . A minimum of three replicate analyses was performed per sample. Concentrations were calculated from linear calibration plots obtained by measurement of the absorbance of standard solutions of NaNO_3 , $\text{Mg}(\text{NO}_3)_2$, $\text{Mn}(\text{NO}_3)_2$, $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$ diluted in HNO_3 (0.5 M), all supplied by Merck. The element Hg was also measured in triplicate by flame atomic- absorption spectrometry (FAAS), but with AMA 254 Mercury Analyser spectrometer that uses the Hg vapour generation technique spectrometer, according to official methods of analysis (AOAC, 2005). The procedure is based on combustion of dry sample (hepatopancreas, 3 mg; muscle, 6 mg; and gonads, 10 mg), pre-concentration of Hg by amalgamation with gold and

atomic-absorption spectrometry. A minimum of three replicate analyses was performed per sample. Concentrations were calculated from linear calibration plots obtained by measurement of the absorbance of an Hg standard solution (Hg diluted in HNO₃, 0.5 M) supplied by Merck. Two groups of elements were distinguished in the analyses: elements considered essential for life maintenance (macroelements: Na, Mg, Cl, S, K and Ca; trace elements: Mn, Fe, Cu, Zn, Se, Br and Sr), and elements considered harmful for animals (contaminants: As, Cd, Hg and Pb), which are related with an exogenous influence (Belitz and Grosch, 1999; Francesconi, 2007).

Accuracy levels

Accuracy was checked with certified biological material (Table 4.2.1). The elemental concentrations obtained for canned matrix meat (SMRD-2000; Swedish Meats R&D and Scan Foods/National Food Administration, Sweden; FAAS), non defatted lobster hepatopancreas (LUTS-1; National Research Council of Canada; FAAS), oyster tissue (SRM 1566; United States National Bureau of Standards; EDXRF), freeze-dried animal blood (IAEA-A-13; International Atomic Energy Agency; EDXRF) and lobster hepatopancreas (TORT-2; National Research Council of Canada; FAAS) were compared with certified values. The detection limits (DL) of each element (Table 4.2.1) were determined by two means: (a) EDXRF - with the signal-to-noise approach, where the equipment compares the signal of each element with blank samples and established the minimum concentration at which the element is reliably detected; (b) FAAS - with the residual standard deviation (RSD) of the response and the slope (S) of the calibration curve of each standard solution used ($DL = 3.3 \times RSD/S$). The FAAS technique requires large amount of sample, which was not possible in gonads and roe.

Nutritional quality and potential risks for consumers

To evaluate the elemental nutritional quality and potential risks of clawed lobsters' consumption, intake of elements per 100 g serving portion was estimated for the edible tissues (wet weight) (similar to the procedures used by the Food and Agriculture Organization of the United Nations, by the World Health Organization of the United Nations and by the United States Food and Drug Administration/USFDA for food risk-benefit analysis). For each homarid species, the average intake (AI) of each element per 100 g edible serving portion was calculated using the edible contributions of each tissue: *H. americanus* (muscle: 84.1 %; hepatopancreas: 14.2 %; gonads: 1.7 %) and *H. gammarus* (muscle: 85.7 %; hepatopancreas: 12.3 %; gonads: 2.0 %). For each homarid species the following equation was used to calculate the average intake (AI) of each element per 100 g portion: $AI/portion = (AIM \times ECM) + (AIH \times ECH) + (AIG \times ECG)$, where M = muscle, H = hepatopancreas, G = gonads, EC = edible contribution. The edible contribution of roe was not considered as this tissue was only available in few animals. The values of average intake of the essential element Na, Mg, Cl, K, Ca, Mn, Fe, Cu, Zn and Se were compared with the daily dietary reference intake (DRI) of adult females and males aged between 19 and 50 years set by the USA Food and Nutrition Board of the Institute of Medicine (IOM, 1997; 2001 and 2004). The contaminants Cd, Hg and Pb were compared with the maximum allowed level (ML) set by the

European Commission (EC), whilst As was evaluated in terms of the action level (AL; similar meaning to the ML) set by the United States Food and Drug Administration (USFDA). For each element, the ratio between the average intakes per portion and the DRI, ML or AL was calculated.

Table 4.2.1 Elemental concentration ($\mu\text{g g}^{-1}$ DW; $n = 4$) and detection limits of certified reference material ($\pm$ sd) analysed by FAAS and EDXR.

Elements	Present work	Certified value	Detection limit
Na ^(a)	8346 $\pm$ 280	8533 $\pm$ 281	0.37
Mg ^(b)	91 $\pm$ 2	90 $\pm$ 4	0.05
Cl ^(c)	10200 $\pm$ 500	10000 ^(d)	100
S ^(c)	8200 $\pm$ 500	7600 ^(d)	100
K ^(c)	10000 $\pm$ 80	9690 $\pm$ 50	10
Ca ^(c)	1350 $\pm$ 50	1500 $\pm$ 200	20
Mn ^(b)	1.28 $\pm$ 0.03	1.20 $\pm$ 0.13	0.04
Fe ^(c)	210 $\pm$ 15	195 $\pm$ 34	3
Cu ^(c)	64 $\pm$ 4	63 $\pm$ 4	1
Zn ^(c)	830 $\pm$ 40	852 $\pm$ 14	1
As ^(c)	13 $\pm$ 1	13 $\pm$ 2	1
Se ^(c)	2.3 $\pm$ 0.5	2.1 $\pm$ 0.5	0.8
Br ^(e)	22 $\pm$ 2	22 $\pm$ 3	0.8
Sr ^(c)	9.9 $\pm$ 0.8	10 $\pm$ 1	0.8
Cd ^(f)	27 $\pm$ 0	26.7 $\pm$ 0.6	0.01
Hg ^(f)	0.28 $\pm$ 0.00	0.27 $\pm$ 0.06	0.02
Pb ^(f)	0.35 $\pm$ 0.06	0.35 $\pm$ 0.13	0.02

Superscripts: (a) canned matrix meat (SMRD-2000; FAAS); (b) non defatted lobster hepatopancreas (LUTS-1; FAAS); (c) oyster tissues (SRM 1566; EDXRF); (d) non-certified values provided by United States National Bureau of Standards; (e) freeze-dried animal blood (IAEA-A-13; EDXRF); (f) Lobster hepatopancreas (TORT-2; FAAS).

Statistical analysis

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (sd). Differences in the elemental concentration of edible tissues of *H. americanus* and *H. gammarus* were tested with analysis of variance (ANOVA) and Student's t-test. Whenever necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements, followed by non-parametric analysis of variance (Kruskal-Wallis or Mann-Whitney tests), if transformed data could not met these assumptions. All statistical analyses were tested at 0.05 level of probability with the software STATISTICA™ 6.1.

Results and discussion

Element composition in the edible tissues of each species

The mean elemental concentrations ($\mu\text{g g}^{-1}$ wet weight) in the edible tissues of *H. americanus* and *H. gammarus* are listed in table 4.2.2.

Table 4.2.2 Mean elemental composition ($\pm$ sd) ($\mu\text{g g}^{-1}$ wet weight) in the tissues of *H. americanus* and *H. gammarus*.

Elements	<i>H. americanus</i>			<i>H. gammarus</i>				<i>H. americanus</i> vs. <i>H. gammarus</i> (p-value)		
	Muscle	Hepat.	Gonads	Muscle	Hepat	Gonads	Roe	Muscle	Hepat	Gonads
Macroelements										
Na	3228 $\pm$ 329a	2842 $\pm$ 473b	1498 $\pm$ 7b	1962 $\pm$ 1090b	1608 $\pm$ 499b	1506 $\pm$ 32b	4006 $\pm$ 76a	0.004	0.008	0.326
Mg	252 $\pm$ 31a	158 $\pm$ 29b	112 $\pm$ 18b	222 $\pm$ 183ab	82 $\pm$ 48b	92 $\pm$ 26b	396 $\pm$ 209a	0.821	0.005	0.420
Cl	4419 $\pm$ 456a	4681 $\pm$ 305a	2316 $\pm$ 286b	4043 $\pm$ 695a	4756 $\pm$ 998a	2455 $\pm$ 478b	4803 $\pm$ 1496a	0.050	0.804	0.436
S	1716 $\pm$ 272a	1955 $\pm$ 300a	1320 $\pm$ 617a	1695 $\pm$ 251a	2029 $\pm$ 190a	2168 $\pm$ 301a	1696 $\pm$ 549a	0.808	0.476	0.003
K	2629 $\pm$ 185a	2694 $\pm$ 279a	2135 $\pm$ 589a	2613 $\pm$ 259a	2534 $\pm$ 494a	2314 $\pm$ 532a	644 $\pm$ 253b	0.821	0.339	0.474
Ca	473 $\pm$ 128a	293 $\pm$ 50b	173 $\pm$ 47c	456 $\pm$ 118a	335 $\pm$ 97b	173 $\pm$ 53c	446 $\pm$ 208ab	0.663	0.199	0.944
Trace elements										
Mn	0.61 $\pm$ 0.24b	2.7 $\pm$ 0.4a	2.1 $\pm$ 0.2a	0.40 $\pm$ 0.34b	1.9 $\pm$ 1.2a	2.2 $\pm$ 0.3a	0.80 $\pm$ 0.04ab	0.131	0.317	0.976
Fe	BDL	29 $\pm$ 4a	12 $\pm$ 8b	BDL	16 $\pm$ 3a	16 $\pm$ 5a	7.0 $\pm$ 3.4b	-	0.000	0.324
Cu	10 $\pm$ 2b	127 $\pm$ 87a	15 $\pm$ 10b	9.6 $\pm$ 5.2c	270 $\pm$ 141a	14 $\pm$ 7c	34 $\pm$ 4b	0.525	0.007	0.880
Zn	29 $\pm$ 4a	31 $\pm$ 6a	17 $\pm$ 5b	26 $\pm$ 3b	41 $\pm$ 11a	30 $\pm$ 8ab	35 $\pm$ 4a	0.004	0.009	0.000
Se	BDL	1.8 $\pm$ 0.6a	3.3 $\pm$ 2.0a	BDL	1.1 $\pm$ 0.3a	2.6 $\pm$ 1.8a	1.0 $\pm$ 0.2a	-	0.001	0.622
Br	23 $\pm$ 2b	48 $\pm$ 14a	25 $\pm$ 3b	22 $\pm$ 3c	74 $\pm$ 21b	26 $\pm$ 7c	114 $\pm$ 9a	0.411	0.002	0.888
Sr	34 $\pm$ 11a	14 $\pm$ 6b	12 $\pm$ 5b	35 $\pm$ 14a	20 $\pm$ 15ab	12 $\pm$ 8b	20 $\pm$ 4ab	0.784	0.151	0.833
Contaminants										
As	11 $\pm$ 3b	20 $\pm$ 5a	12 $\pm$ 4b	17 $\pm$ 3a	24 $\pm$ 5a	17 $\pm$ 3a	11 $\pm$ 1b	0.000	0.086	0.006
Cd	0.02 $\pm$ 0.00b	11 $\pm$ 5a	-	0.02 $\pm$ 0.01b	11 $\pm$ 9a	-	-	0.086	0.922	-
Hg	0.10 $\pm$ 0.02a	0.13 $\pm$ 0.03a	BDL	0.15 $\pm$ 0.04a	0.09 $\pm$ 0.02a	BDL	BDL	0.000	0.006	-
Pb	0.10 $\pm$ 0.00a	0.11 $\pm$ 0.03a	-	0.10 $\pm$ 0.00a	0.10 $\pm$ 0.06a	-	-	-	0.431	-

In each line, different letters indicate significant differences ($p < 0.05$) between the edible tissues of each homarid species. Additionally, the elements' composition in each tissue were compared between both homarids; results are presented as p-values. Abbreviations: (BDL) below detection limit.

Quantification of Pb and Cd was not possible in the gonads due to the small tissue sample obtained. Sodium, Cl, S and K were the most abundant elements in all tissues. Generally, significant differences were found in most elements' content amongst tissues of each homarid species, except S and Pb, suggesting distinct physiological role of each tissue. Sulfur is present in amino acids (e.g. cysteine, methionine), polypeptides (e.g. glutathione), proteins and enzymes, which might explain its ubiquity at similar concentrations in all tissues (Walker *et al.*, 2001). Similarly, the identical content of Pb in all homarids' tissues is likely explained by the fact that Pb is a toxic contaminant related with an environmental input and without any physiological function (Al-Mohanna and Subrahmanyam, 2001).

The muscle had significantly higher concentrations of Na, Mg, Ca and Sr. Since this tissue is mostly involved in the mechanical movements of appendages and claws, it is likely these elements are vital for neuromuscular functions. In contrast, Fe and Se were below the detection limit (BDL) in this tissue and Cd was close to BDL.

The hepatopancreas had statistically higher concentration of Fe, Cu, Br and Cd. The hepatopancreas is a multifunctional vital organ responsible for the regulation of essential mono and divalent ions and for detoxification of contaminants (Chavez-Crooker *et al.*, 2003). Earlier studies reported high contents of Cu in the hepatopancreas of other crustaceans, such as blue crab, swim crab, Chinese mitten crab and shrimps (Gökoğlu and Yerlikaya, 2003; Chen *et al.*, 2006; Kádár *et al.*, 2006). Copper levels can reflect the active accumulation of this element by homarids for incorporation into the respiratory pigment haemocyanin found in crustacean's haemolymph (Lee and Shiao, 2002). Previous studies with *H. americanus* reported elevated levels of contaminants, namely Cd, in the hepatopancreas (Chou *et al.*, 2000; Engel *et al.*, 2001). The high concentration of Cd found in the hepatopancreas likely reflects the detoxifying role of this organ, particularly in the isolation of dietary contaminants and in complexing them with organic solutes, such as metallothionein, glutathione or inorganic anions (Chavez-Crooker *et al.*, 2003). In this way, the hepatopancreas reduces the load of contaminants that reach the haemolymph, tissues and organs. This role is also evidenced by the lower levels of Cd obtained in the remaining tissues.

Gonads are the reproductive organs where high nutritional investments are made to produce the reproductive cells. Selenium was detected at high concentrations in this organ, likely due to the important role of this element in the modulation of growth and development, and in oxidative stress defence (FAO/WHO, 2002). In contrast, low contents of Cl, Ca, Zn and Hg were found in gonads. The roe had significantly higher Na and Br contents and lower contents of K and Hg.

Differences in elements composition between species

No statistical differences between *H. americanus* and *H. gammarus* were found in the content of Cl, K, Ca, Mn, and Sr in all edible tissues (Table 4.2.2). Such result likely reflects similar vital biochemical mechanisms in both homarids regulating these elements (Skinner *et al.*, 2004). *H. americanus* had significantly higher Na concentrations in the muscle and higher Na, Mg, Fe and Se

content in the hepatopancreas. In contrast, *H. gammarus* had statistically higher Cu and Br values in the hepatopancreas and higher S and Zn levels in gonads.

These differences might reflect distinct elemental requirements for the metabolism of each species or to different elemental availability in the aquatic environment, as the species are captured in different areas of the Atlantic Ocean. Worldwide, Se availability in sediments and live organisms show great variability due to distinct environmental conditions and agricultural practices (FAO/WHO, 2002). USA and Canada are seleniferous areas, whereas low Se concentrations are found in European countries (Popilac and Prpic-Majic, 2002). According to FAO/WHO, (2002), Se content in marine fish captured in USA is usually higher than the average international values. This fact may clarify the higher contents of Se found in the hepatopancreas of *H. americanus*.

Among contaminants, variations between both homarids were only found in the concentration of As and Hg: *H. gammarus* had statistically higher As and Hg content in muscle and lower Hg level in hepatopancreas than *H. americanus*. Such differences may indicate the degree of pollution in sediments and seawater of both regions of the Atlantic Ocean (Sadiq, 1992). Arsenic and Hg enter in coastal waters by sewage and other wastewater effluents, power plant cooling water discharges, vehicle emissions, petroleum and petrochemical industrial wastes, storm drain outfalls and solid waste landfills (Al-Mohanna and Subrahmanyam, 2001). Contaminants cannot be regulated by crustaceans and, therefore, bioaccumulation of these elements occurs in specimens' edible tissues (Al-Mohanna and Subrahmanyam, 2001).

Nutritional quality and potential risks for consumers

Clawed lobsters are important diet sources in coastal areas of Mediterranean European countries. Therefore, intake of trace elements from *H. americanus* and *H. gammarus*, especially toxic elements, is of great concern for human health. The results obtained in table 4.2.3 indicate that both species are good sources of Na, Cl, Cu, Zn and Se, being *H. americanus* a better source of Na and Se than *H. gammarus*. Sodium plays an important role in many physiological processes: signal transduction in the human central nervous system, osmoregulation, arterial pressure maintenance and optimal salt and water balance in the body (Walker *et al.*, 2001). Zinc is an essential component of several enzymes, participating in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids (FAO/WHO, 2002). Additionally, this element has also an essential role in the process of gene expression, particularly in polynucleotide transcription (FAO/WHO, 2002). Fish, fruit and green leaf vegetables are considered poor sources of Zn ($<10 \text{ mg kg}^{-1}$), whilst lean meat, cereals and legumes are rich Zn sources ($25\text{-}50 \text{ mg kg}^{-1}$, compared to 30 mg kg^{-1} in both clawed lobsters; FAO/WHO, 2002).

In both lobsters' species Cu and Se concentrations were above the DRI. Nevertheless, the tolerable upper limit (UL) set by the USDA for Cu (10 mg day^{-1}) is well above the results found in the present study ($2.7\text{-}4.0 \text{ mg } 100 \text{ g}^{-1}$ portion). Copper is found in several enzymes, including the cytochrome c oxidase and the superoxide dismutase, and is used for biological electron transport (Walker *et al.*, 2001).

Table 4.2.3 Average content of each element in 100 g edible serving portion (considering the contribution of all tissues) for each homarid species, and ratio between the average intake of each element per portion and the recommended daily dietary reference intakes.

Elements	DRI/ML/AL	<i>H. americanus</i>		<i>H. gamarus</i>	
		AI/portion	%	AI/portion	%
Macroelements					
Na	1500 mg	314	21	213	14
Mg*	(420-320) mg	24	(5.7-7.5)	22	(5.4-7.0)
Cl	2300 mg	442	19	417	18
S	NS	174	-	173	-
K	4700 mg	263	5.6	240	5.1
Ca	1000 mg	44	4.4	44	4.4
Trace elements					
Mn	(2.3-1.8) mg	0.09	(4.1-5.2)	0.06	(2.7-3.8)
Fe	(8-18) mg	0.63	(7.8-3.5)	0.42	(5.2-2.3)
Cu	900 µg	2706	301	4048	450
Zn	(11-8) mg	2.9	(27-37)	2.9	(26-36)
Se	55 µg	103	187	70	128
Br	NS	2.6	-	3.7	-
Sr	NS	3.1	-	3.2	-
Contaminants					
As	7.6 mg	1.2	16	1.7	23
Cd	50 µg	156	321	119	237
Hg	50 µg	11	22	2.8	5.5
Pb	50 µg	10	20	8.8	18

Daily recommended intake values (DRI) correspond to Na, Mg, Cl, K, Ca, Mn, Fe, Cu, Zn and Se; maximum level (ML) refers to Cd, Hg and Pb and, the action level refers to As. Values in brackets for Mg, Mn, Fe and Zn refer to differences in gender: the first and second values correspond to adult male and female aged between 19 and 50 years, respectively; Abbreviations: (AI) average intake; (NS) value not set; (*) data refer to female and male adults aged between 31 and 50 years.

Despite the UL value for Se has not been set, it is important to stress that a normal adult can ingest up to 216 µg Se per day without any toxicological effect (FAO/WHO, 2002), which is higher than values found in the edible tissues of both clawed lobsters (55-70 µg 100 g⁻¹ portion). Several authors reported the Se protective action, especially selenite, (Se₂O₂⁻³), on many toxicological effects of Cd and Hg (Gasiewicz and Smith, 1978; Early *et al.*, 1992; Skowerski *et al.*, 1997a, b). Selenium is also implicated in the protection of body tissues against oxidative stress, maintenance of defense against infection and modulation of growth and development (FAO/WHO, 2002).

Considering contaminants (As, Cd, Hg and Pb), only Cd content in both homarids was well above the ML/AL per 100 g portion (Table 4.2.3). The concentration obtained for As, Hg and Pb was in the range of the typical concentrations found in crustaceans muscle: 5-98 mg 100 g⁻¹ (As), 2-20 µg 100 g⁻¹ (Hg) and 2-10 µg 100 g⁻¹ (Pb; Francesconi, 2007).

In the present study, the main contribution to the high Cd concentration in both species was the hepatopancreas, whilst the Cd concentration in the muscle and gonads was below the ML (Table 4.2.2). Cadmium is toxic to many organisms since the ion form combines with sulphhydryl (thiol) groups of many enzymes preventing their normal function (Walker *et al.*, 2001). Yet, Cd absorption by humans is influenced by nutritional factors (*e.g.* Fe levels; Francesconi, 2007), and many toxicological effects of Cd are attenuated in the presence of Se (Gasiewicz and Smith, 1978; Early *et al.*, 1992; Skowerski *et al.*, 1997a, b). Since both homarids have high Fe and Se concentrations in the hepatopancreas (Table 4.2.2), the toxicological effects of Cd may be reduced. Moreover, despite *H. gammarus* and *H. americanus* are widely consumed in coastal areas of Mediterranean countries, the annual per capita consumption of these food items is relatively low (109 g in Spain; 64 g in France; 64 g in Italy; 23 g in Greece; 10 g in Portugal; data from 2005; EUROSTAT, 2007). Therefore, the risk of Cd contamination to homarids consumers is low.

Conclusions

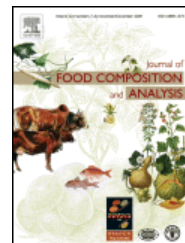
In general, Na, Cl, S and K were the most abundant elements in all tissues, evidencing their essential character to *H. americanus* and *H. gammarus*. Differences found in the elemental composition amongst tissues of the same species likely reflect distinct physiological roles of the tissues. Variations detected between both homarid species likely reflect distinct availability of elements in the aquatic environment, as the animals are distributed in different regions of the Atlantic Ocean. Both species can also require distinct elements to their metabolism.

Considering the daily intake recommendations, both homarids are good sources of Na, Cl, Cu, Zn, and Se. As to contaminants, only Cd was above the action level in the hepatopancreas of both homarids. Despite the low risk of Cd intake, consumers should avoid or moderately consume this tissue in both species. Future studies should evaluate the mineral composition of other crustacean species with relevance for human consumption.

Sub-chapter 4.3

C. pagurus biochemical composition: population differences

Barrento, S., Marques, A., Teixeira, B., Mendes, R., Bandarra, N., Vaz-Pires, P., and Nunes, M.L. (*in press*). Chemical composition, cholesterol, fatty acid and amino acid in two populations of brown crab *Cancer pagurus*: ecological and human health implications. *Journal of Food Composition and Analysis*.



The brown crab *Cancer pagurus*, is a valued decapod species captured mainly in UK and France. In Portugal, Scottish crabs and females are less expensive than crabs caught in the English Channel and males. In this work the proximate chemical composition, cholesterol, fatty acid and amino acid content of female and male *C. pagurus* edible tissues from the Scottish coast and English Channel were compared. There was no evidence that the fishing ground influenced the chemical composition of tissues, but there were significant differences between tissues and sexes. Muscle was richer in protein, but poorer in fat and cholesterol than gonads and hepatopancreas. Ovaries had more protein, fat, cholesterol and amino acids than testis. The fatty acid profiles in muscle and gonads were dominated by PUFA, while hepatopancreas was richer in MUFA and SFA. Lower n-3 fatty acid content and n-3/n-6 fatty acid ratio in hepatopancreas contributed to higher index of atherogenicity and thrombogenicity. Considering the biochemical composition there is no reason for price differentiation between crabs from different locations. As far as sex is concerned the principal difference that might increase male crabs' value is the meat yield content of claws, which was higher than females' claws.

Keywords: crustaceans, *Cancer pagurus*, hepatopancreas, gonads, muscle, cholesterol, fatty acids, amino acids, nutritional quality.

Introduction

Worldwide, crustaceans are highly appreciated and luxury seafood items. Though its frequent consumption is not in general advised either because of allergenic reactions or the supposedly high cholesterol content, there is a growing number of studies promoting its consumption (Gökoolu and Yerlikaya, 2003; Rosa and Nunes, 2003a; Küçükgülmez *et al.*, 2006; Chen *et al.*, 2007; Teixeira *et al.*, 2008; Sub-chapters 2.1, 4.2, 4.5). Regardless of this controversy, crustaceans are widely appreciated and consumed in Mediterranean countries. In particular, the crab *Cancer pagurus* is a valued decapod species in France, Spain, Portugal and Italy, where the muscle from claws and abdomen and the brown meat (gonads and hepatopancreas) are appreciated. In these countries the market trend is still the commercialization of live crabs harvested throughout Britain (31,079 t), Ireland (11,525 t) and France (5,724 t; Tully *et al.*, 2006; data from 2007, EUROSTAT, 2009). The major export countries to Portugal are UK and France. This species has different market prices according to the fishing ground and sex. In fact, crabs caught off the English Channel are usually more expensive than those caught off Welsh, Scottish and Irish coasts, presumably due to the intrinsic quality of these populations (e.g. bigger animals facing less hours of transport and therefore having more vigor at arrival) (Sub-chapter 2.1). In addition, males are more expensive than females (Brown and Bennet, 1980).

It is well known that the biochemical composition of edible tissues of marine invertebrates is influenced by its nutritional habits, age, sex, season, seawater temperature and salinity (Chapelle, 1977; Oliveira *et al.*, 2007; Souchet and Laplante, 2007). Since crab populations from different fishing grounds might have distinct biochemical patterns in the edible tissues, the purpose of this study was to determine the nutritional quality of *C. pagurus* harvested in the English Channel and Scottish coast by determining the proximate chemical composition, amino acid and fatty acid profiles, and cholesterol content. Data was also analyzed to evaluate the nutritional quality of *C. pagurus* edible tissues to human consumption by calculating the indices of atherogenicity and thrombogenicity, the essential amino acid scores and two ratios related with fatty acid content: (n-3/n-6) and, PUFA/SFA (polyunsaturated fatty acids/saturated fatty acids).

Material and methods

Biological material

Twenty *Cancer pagurus* caught off the Scottish coast in summer (SC; 10 females and 10 males) and twenty specimens from the English Channel (EC; 10 females and 10 males) were live purchased and transported to the laboratory. The biological material was the same used in previous published studies (sub-chapters 4.4 and 4.5). Animals were kept under refrigerating conditions (~4 °C) during one hour to decrease their metabolism and stunned before being euthanized by piercing the two nerve centres by means of a stainless steel rod. The rod was inserted through one of the eyes and through the vent as recommended by the Codex Alimentarius Commission (FAO/WHO, 1983). Several parameters were recorded for each crab: sex, total weight (Omega PP 50\1; d=1 g), claw muscle, gonad and hepatopancreas weight (Mettler PM 400;

d=0.001 g), carapace width and length (digital caliper Comecta; 0.01 mm), and ovarian maturity stage. The ovarian maturity scale was adapted from Edwards, (1979). Females were classified as immature/no ovary development (Stage 1: S1); initial ovary development (Stage 2: S2); developing orange ovaries extending into carapace (Stage 3: S3); and ripe, carapace full of bright red ovary material (Stage 4: S4). Gonadosomatic (GSI) and Hepatosomatic (HI) indices were calculated as well as claws muscle meat yield (MY) and the total meat yield (TMY). The GSI, HI and MY, were calculated as follows: (tissue wet weight / body wet weight) $\times$ 100; the TMY was calculated according to the formula: (gonad wet weight + hepatopancreas wet weight + claw muscle wet weight) / (body wet weight) $\times$ 100. For each tissue the edible content was also calculated as follows: (tissue wet weight / sum of all edible tissues wet weight) $\times$ 100. For the biochemical analyses, the edible tissues of at least two crabs were pooled taking into account sex, ovarian maturity stage and location, and were homogenized with a grinder (Retsch Grindomix GM200, Düsseldorf, Germany; 5000 rpm; material: PP cup and stainless steel knives), vacuum packed and stored at -20 °C. whereas the remaining was frozen at -80 °C and subsequently freeze-dried for 48 h at -50 °C and low pressure (approximately 10^{-1} atm; Heto power dry LL 3000). Samples were powdered and stored at -20 °C under controlled moisture conditions (vacuum packed) until further analyses.

Proximate chemical composition and energy content

Moisture, ash, protein and lipid contents were determined in duplicate for each sample, according to the AOAC (2005) methods that were validated with reference material (Table 4.3.1). Briefly, the moisture content was obtained by drying the sample overnight at 105 °C (laboratory heater, P-Selecta 207); ash was quantified after combustion for 16 h at 550 °C (muffle furnace, Heraeus Hanau, TYPMR170); crude protein content was determined by the Kjeldahl method using an automatic distillation and titration unit (VELP Scientifica, UDK152) with a conversion factor of 6.25; and total lipid content was determined with the Soxhlet extraction method using ethyl ether (40-60 °C; 7 h; heater plate SBS Instruments PC6L). The energy content was estimated as: proteins, 4.27 kcal g⁻¹ wet weight; lipids, 9.02 kcal g⁻¹ wet weight; 1 kcal = 4.184 kJ (FAO, 1987).

Cholesterol

Cholesterol content was determined in duplicate for each sample, and was based on the modified procedure of Naeemi *et al.* (1995). Briefly, each sample (300 mg of dry weight) was combined with 260 μ L of the internal standard solution (5 α -cholestane Sigma; 5 mg mL⁻¹ cyclohexane, Merck), 3mL of saturated methanolic potassium hydroxide solution (2 M, Merck), and 3 mL of methanol pro analysis (Merck). Following heating (80 °C; 30 min water bath), samples were cooled and supplied with 250 μ L of magnesium chloride solution (1 M, Merck) and 1.5 mL of cyclohexane pro analysis (Merck). Samples were shaken and centrifuged (1500g; 4 min, centrifuge Sigma 2k15) until phase separation. The moisture content of the upper phase was removed with anhydrous sodium sulfate (Panreac). The cholesterol in the upper phase (2 μ L) was separated by gas chromatography (Varian Star 3400 Cx) using helium as carrier gas at a flow rate of 1 mL min⁻¹ in a flame ionization

detector and a fused silica capillary CP-Sil 8 CB column (30 m length $\times$ 0.25 mm internal diameter, 0.25 μ m film thickness; J&W Scientific). The temperatures of the oven, injector, and detector were 280, 285, and 300 $^{\circ}$ C, respectively. Cholesterol was identified and quantified by comparison with the calibration curve of a pure cholesterol standard with 5 α -cholestane (Sigma). Cholesterol/cholestane peak area ratios obtained with the Varian software were plotted with cholesterol concentrations, and a straight line was fitted to data points by linear regression.

Fatty acid analysis

Fatty acid methyl esters (FAME) profile from non polar and polar lipids was determined in duplicate for each sample, and was based on the experimental procedure of Cohen *et al.* (1988). Each sample (300 mg of dry weight) was dissolved in 5 mL of acetyl chloride/methanol (1:19 v/v; Merck), shaken, and heated (80 $^{\circ}$ C; 1 h). After cooling, 1 mL of Milli-Q distilled water and 2 mL of n-heptane pro analysis (Merck) were added, and samples were shaken and centrifuged (2000g; 5min, Sigma 2k15) until phase separation. The moisture content of the upper phase was removed with anhydrous sodium sulfate (Panreac). An aliquot (2 μ L) of the upper phase was then injected onto a gas chromatograph (Varian Star 3800 Cp) equipped with an autosampler and fitted with a flame ionization detector at 250 $^{\circ}$ C for FAME analysis. The separation was carried out with helium as carrier gas at a flow rate of 1 mL min $^{-1}$, in a capillary column DB-Wax (30m length $\times$ 0.32 mm internal diameter; 0.25 μ m film thickness; Hewlett-Packard) programmed at 180 $^{\circ}$ C for 5 min, raised to 220 at 4 $^{\circ}$ C min $^{-1}$, and maintained at 220 $^{\circ}$ C for 25 min, with the injector at 250 $^{\circ}$ C. FAME were identified by comparing retention times with those of Sigma standards. Quantitative data was calculated using the peak area ratio (percent of total fatty acids) and the Varian software. The fatty acid determination method was validated with reference material (Table 4.3.1).

Amino acids

Amino acid concentration was determined in duplicate for each sample. To extract total amino acids (protein bound + free), 40-90 mg of sample was placed in 10 mL ampoules with 3 mL of 6 M HCl (Merck) with 0.1 % phenol (Merck), according to the method described by AOAC (2005). Ampoules were vacuum-sealed, and samples were hydrolyzed at 110 $^{\circ}$ C for 24 h; hydrolysates were filtered (0.45 μ m pore size, Life sciences) and dissolved with Milli-Q distilled water to 20 mL. Samples were frozen at -80 $^{\circ}$ C and freeze-dried during 48 h at -50 $^{\circ}$ C under low pressure (approximately 10 $^{-1}$ atm). Samples were dissolved in 5 mL of 0.1 M HCl (Merck) and stored at -80 $^{\circ}$ C until amino acid separation. After thawing samples were filtered (0.2 μ m pore size Life sciences) and separation was performed with high-performance liquid chromatography (Agilent 1100 HPLC) using pre-column o-phthalaldehyde and 3-mercaptopropionic acid in borate buffer (OPA, Agilent Technologies) and 9-fluorenylmethyl chloroformate in acetonitrile (FMOC; Agilent Technologies) derivatization, a Phenomenex Gemini ODS C18 guard column (4 $\times$ 3mm), and a Phenomenex Gemini ODS C18 110A column (4.6 $\times$ 150 mm, 5 μ m). The solvents and gradient conditions were those described by Henderson *et al.* (2000). Detection wavelengths were set at UV 338 and 262 nm and fluorescence 340/450 and 266/305 nm. The identity and quantity of the amino acids were

assessed by comparison with the retention times and peak areas of standard amino acids (Sigma-Aldrich) using norvaline as internal standard.

Nutritional quality

The propensity of crab's tissue to promote the incidence of coronary heart disease, indices of atherogenicity (IA) and thrombogenicity (IT) were calculated by using the Ulbricht and Southgate (1991) equations:

$$IA = [12:0 + (4 \times 14:0) + 16:0] / [(n-6 + n-3) \text{ PUFA} + \text{MUFA}];$$

$$IT = (14:0 + 16:0 + 18:0) / [(0.5 \times \text{MUFA}) + (0.5 \times (n-6) + 3 \times (n-3) + (n-3)/n-6)]$$

Where MUFA means monounsaturated fatty acids and PUFA means polyunsaturated fatty acids. Two ratios related with fatty acid content were also calculated: (n-3/n-6) and PUFA/SFA (saturated fatty acids), in order to allow comparisons with the United Kingdom Department of Health recommendations (UKDH, 1994). Essential amino acid scores (AS) were also calculated with respect to reference amino acid requirements for adults: isoleucine (30 mg g⁻¹ protein), leucine (59 mg g⁻¹ protein), lysine (45 mg g⁻¹ protein), methionine (16 mg g⁻¹ protein), phenylalanine+tyrosine (38 mg g⁻¹ protein), threonine (23 mg g⁻¹ protein), valine (39 mg g⁻¹ protein) and histidine (15 mg g⁻¹ protein) (FAO/WHO/UNU, 2007). This was achieved by comparing the content of each essential amino acid in the protein/diet with its requirements, according to the equation: AS (%) = (mg of amino acid per g of protein/mg of amino acid requirement) × 100. The ratio of essential amino acids to non essential amino acids was also calculated.

Table 4.3.1 Summary of reference material elemental concentration ($\mu\text{g g}^{-1}$ DW; n=4) and detection limits (DL) of certified reference material ($\pm$ standard deviation) analyzed by the different techniques.

Elements	Technique	DL	Certified biological material	Certified	Present work	
Moisture	Drying	nd	canned matrix meat - SMRD 2000	68.8 ± 0.1	68.7 ± 0.0	
Protein	Kjeldahl	nd	canned matrix meat - SMRD 2000	1.63 ± 0.05	1.62 ± 0.06	
Total lipids	Soxhlet	0.01	canned matrix meat - SMRD 2000	14.3 ± 0.4	14.4 ± 0.1	
Ash	Combustion	0.1	canned matrix meat - SMRD 2000	2.65 ± 0.07	2.69 ± 0.03	
Fatty acids	14:0	GC	0.4-1	beef pork fat blend - BCR-163	2.29 ± 0.04	2.23 ± 0.04
	16:0	GC	0.4-1	beef pork fat blend - BCR-163	25.96 ± 0.30	25.39 ± 0.017
	16:1	GC	0.4-1	beef pork fat blend - BCR-163	2.58 ± 0.16	2.22 ± 0.03 ^a
	18:0	GC	0.4-1	beef pork fat blend - BCR-163	18.29 ± 0.16	17.65 ± 0.06
	18:1	GC	0.4-1	beef pork fat blend - BCR-163	38.34 ± 0.36	38.65 ± 0.16 ^b
	18:2	GC	0.4-1	beef pork fat blend - BCR-163	7.05 ± 0.17	7.19 ± 0.03 ^c
	18:3	GC	0.4-1	beef pork fat blend - BCR-163	0.86 ± 0.14	0.81 ± 0.01 ^d
Amino acids	HPLC	nd	no reference material used	nd	nd	
Cholesterol	GC	nd	no reference material used	nd	nd	

Reference materials suppliers and location: SMRD-2000 (Swedish Meats R&D and Scan Foods/National Food Administration, Sweden), BCR-163 (Institute for Reference Materials and Measurement, Belgium). Abbreviations: (GC) Gas Chromatography; (HPLC) High Performance Liquid Chromatography; (nd) not determined (a) 16:1n-7 + 16:1n-5; (b) 18:1n-9 + 18:1n-7; (c) 18:2n-6; (d) 18:3n-3.

Statistics

Main differences among tissues, sexes and locations were tested with analysis of variance (ANOVA). Whenever necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements, followed by nonparametric analysis of variance (Kruskal-Wallis), if transformed data could not meet these assumptions. The logarithmic transformation was used whenever the standard deviation was proportional to the means (*i.e.* there is a constant coefficient of variation), whereas the square root transformation was used when the group variances were proportional to the means, that is when the variances increase as the means increase (Zar, 1999). All statistical analyses were tested at 0.05 level of probability with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Results

Proximate chemical composition

The biometric data and proximate chemical composition of brown crab is presented in tables 4.3.2 and 4.3.3. Muscle and hepatopancreas were responsible by the major edible contribution. Muscle represented the highest content in male crabs (50.7 - 52.5 %) independently of location compared to females (38.3 - 42.4 %), whereas the contribution of gonads from both locations was higher in females (14.2 - 15.4 %) than in males (4.6 - 6.0 %). The content of hepatopancreas (42.9 - 46.3 %) was constant between sexes and locations.

Table 4.3.2 Biometric data of *C. pagurus* from both locations and sexes (average $\pm$ standard deviation).

	SF	EF	SM	EM
Weight (g)	748 $\pm$ 25	829 $\pm$ 155	751 $\pm$ 27	977 $\pm$ 104
CL (mm)	107.38 $\pm$ 1.77	109.93 $\pm$ 5.88	98.44 $\pm$ 3.71	110.16 $\pm$ 3.71
CW (mm)	156.30 $\pm$ 5.93	163.32 $\pm$ 2.26	168.75 $\pm$ 9.41	177.20 $\pm$ 5.96
TMY (%)	22.5 $\pm$ 3.8	23.5 $\pm$ 2.2	22.0 $\pm$ 9.9	23.8 $\pm$ 3.6

Abbreviations: (SF) females from the Scottish coast; (EF) females from the English Channel; (SM) males from the Scottish coast; (EM) males from the English Channel; (CL) carapace length; (CW) carapace width; (TMY) total meat yield. For each location and sex n=10.

Regarding the proximate chemical composition (Table 4.3.3), significant differences were detected among tissues (Table 4.3.3). Few sex and location differences were observed in the proximate chemical composition of muscle and hepatopancreas. On the contrary, gonads chemical composition showed marked differences between sexes independently of location, as females had more proteins, fat, cholesterol and energy than males but less ash and moisture. Muscle and ovaries had high protein content compared to hepatopancreas. Muscle also had the lowest fat content and cholesterol concentration compared to gonads and hepatopancreas. Concerning the female maturation stage, crabs from both locations were mainly in the S3 stage (SC: 40 %; EC: 50 %), while the remaining specimens were S2 (SC: 30 %; EC: 25 %) and S4 (SC: 30 %; EC: 25 %).

Table 4.3.3 Proximate chemical composition (%), cholesterol content (mg 100 g⁻¹ wet weight) and energy content (kcal 100 g⁻¹ tissue wet weight) in edible tissues of *Cancer pagurus* (average $\pm$ standard deviation).

Muscle	SF	EF	SM	EM	p
MY	8.5 $\pm$ 1.0a	9.1 $\pm$ 3.6a	11.9 $\pm$ 1.1a	12.4 $\pm$ 2.1a	<0.001
EC	38.3 $\pm$ 5.2b	42.4 $\pm$ 5.4b	50.7 $\pm$ 5.0a	52.5 $\pm$ 5.2a	<0.001
Moisture	77.8 $\pm$ 2.1a	77.4 $\pm$ 1.9a	74.6 $\pm$ 2.2b	77.3 $\pm$ 1.3a	0.001
Ash	2.1 $\pm$ 0.2a	2.2 $\pm$ 0.2a	1.9 $\pm$ 0.1b	2.2 $\pm$ 0.2a	0.003
Proteins	16.4 $\pm$ 4.2b	18.8 $\pm$ 1.3ab	20.5 $\pm$ 1.7a	17.9 $\pm$ 0.8b	0.001
Fat	0.3 $\pm$ 0.1a	0.4 $\pm$ 0.1a	0.2 $\pm$ 0.1a	0.4 $\pm$ 0.1a	0.050
Cholesterol	37.3 $\pm$ 4.7a	40.6 $\pm$ 4.7a	37.0 $\pm$ 9.1a	40.7 $\pm$ 3.6a	0.233
Energy	85.9 $\pm$ 10.1a	86.7 $\pm$ 7.6a	95.4 $\pm$ 10.3a	87.9 $\pm$ 4.8a	0.324
Hepatopancreas	SF	EF	SM	EM	p
HI	10.4 $\pm$ 1.8a	9.3 $\pm$ 3.4a	10.2 $\pm$ 2.2a	10.2 $\pm$ 2.3a	0.806
EC	46.3 $\pm$ 4.5a	43.3 $\pm$ 4.7a	43.3 $\pm$ 6.2a	42.9 $\pm$ 6.3a	0.503
Moisture	67.6 $\pm$ 6.7a	69.8 $\pm$ 5.6a	61.8 $\pm$ 7.3ab	61.1 $\pm$ 2.3b	0.006
Ash	3.0 $\pm$ 0.4b	3.1 $\pm$ 0.9b	5.0 $\pm$ 1.2a	6.6 $\pm$ 0.6a	0.006
Proteins	12.2 $\pm$ 1.6ab	9.4 $\pm$ 4.6b	13.3 $\pm$ 1.9ab	14.3 $\pm$ 1.8a	<0.001
Fat	10.2 $\pm$ 3.6a	10.0 $\pm$ 1.6a	12.2 $\pm$ 3.4a	10.8 $\pm$ 2.3a	0.056
Cholesterol	116.6 $\pm$ 27.3a	115.6 $\pm$ 57.1a	112.4 $\pm$ 10.1a	126.7 $\pm$ 13.0a	0.518
Energy	167.5 $\pm$ 52.7a	154.9 $\pm$ 24.9a	195.7 $\pm$ 46.9a	184.5 $\pm$ 15.3a	0.103
Gonads	SF	EF	SM	EM	p
GSI	3.6 $\pm$ 2.0a	3.6 $\pm$ 3.1a	1.4 $\pm$ 0.7b	1.1 $\pm$ 0.5b	<0.001
EC	15.4 $\pm$ 6.4a	14.2 $\pm$ 6.0a	6.0 $\pm$ 3.2b	4.6 $\pm$ 1.6b	<0.001
Moisture	56.8 $\pm$ 1.6b	58.2 $\pm$ 1.8b	80.0 $\pm$ 0.2a	78.2 $\pm$ 0.3a	0.005
Ash	1.6 $\pm$ 0.0b	1.6 $\pm$ 0.0b	2.7 $\pm$ 0.0a	2.6 $\pm$ 0.0a	0.002
Proteins	25.5 $\pm$ 0.0a	25.1 $\pm$ 0.1b	13.1 $\pm$ 0.0d	13.8 $\pm$ 0.1c	<0.001
Fat	3.1 $\pm$ 0.5a	3.4 $\pm$ 0.7a	0.9 $\pm$ 0.0b	1.1 $\pm$ 0.2b	0.034
Cholesterol	170.5 $\pm$ 5.2a	169.7 $\pm$ 13.7a	84.6 $\pm$ 4.3b	85.9 $\pm$ 4.9b	<0.001
Energy	192.3 $\pm$ 1.4a	186.1 $\pm$ 3.2a	82.1 $\pm$ 3.2b	90.1 $\pm$ 1.5b	<0.001

Abbreviations: (MY) claw muscle yield (%); (EC) edible content (%), (HI) hepatosomatic index; (GSI) gonadosomatic index; (SF) females from the Scottish coast; (EF) females from the English Channel; (SM) males from the Scottish coast; (EM) males from the English Channel. In each row different letters indicate significant differences between sexes and locations ($p < 0.05$). The number of replicates for each location and sex were: muscle ($n=10$); hepatopancreas ($n=10$), ovaries ($n=6$) and testis ($n=4$).

Fatty acids

In all tissues the main SFA, MUFA and PUFA were respectively palmitic acid (16:0), oleic acid (18:1n-9) and, eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acids (DHA; 22:6n-3) (Table 4.3.4). The fatty acids profile of muscle showed differences mainly according to location, as crabs from the SC had higher proportion of 14:0, 18:1n-9 and 18:2n-6, and lower 18:1n-7 and 16:4n-3 ($p < 0.01$) than those of the EC, independently of sex. The proportion of n-3, the ratios n-3/n-6, PUFA/SFA and IT were constant in respect to sex and location (Table 4.3.4).

Table 4.3.4 Fatty acid profile (% of total fatty acids) of edible tissues of crabs from each location and sex (average $\pm$ standard deviation).

Muscle	SF	EF	SM	EM	p
14:0	1.2 $\pm$ 0.2a	0.53 $\pm$ 0.10b	1.1 $\pm$ 0.1a	0.54 $\pm$ 0.13b	0.000
15:0	0.53 $\pm$ 0.10a	0.56 $\pm$ 0.07a	0.57 $\pm$ 0.09a	0.56 $\pm$ 0.09a	0.836
16:0	9.5 $\pm$ 0.7a	9.0 $\pm$ 0.3a	9.6 $\pm$ 0.6a	10.9 $\pm$ 0.3a	0.052
18:0	5.1 $\pm$ 0.5a	4.9 $\pm$ 0.2a	4.4 $\pm$ 0.5a	4.6 $\pm$ 0.5a	0.050
SFA	17.4$\pm$0.9a	15.7$\pm$0.5b	16.7$\pm$1.0ab	17.6$\pm$0.4a	0.001
16:1n-7	4.2 $\pm$ 0.4b	4.9 $\pm$ 0.2b	4.6 $\pm$ 0.9ab	5.7 $\pm$ 0.6a	0.001
16:1n-5	0.55 $\pm$ 0.07ab	0.64 $\pm$ 0.08a	0.59 $\pm$ 0.08ab	0.49 $\pm$ 0.07b	0.002
18:1n-9	17.0 $\pm$ 0.8a	14.7 $\pm$ 0.6b	16.8 $\pm$ 1.0a	15.0 $\pm$ 1.09b	0.000
18:1n-7	5.1 $\pm$ 0.3b	6.2 $\pm$ 0.3a	5.3 $\pm$ 0.5b	6.0 $\pm$ 0.6a	0.000
20:1n-9	2.3 $\pm$ 0.5a	1.7 $\pm$ 0.2b	2.0 $\pm$ 0.4ab	1.1 $\pm$ 0.3c	0.000
MUFA	30.8$\pm$1.7a	29.8$\pm$2.1a	30.9$\pm$1.5a	29.0$\pm$1.0a	0.056
16:3n-4	1.1 $\pm$ 0.4a	1.2 $\pm$ 0.3a	1.5 $\pm$ 0.2a	1.3 $\pm$ 0.7a	0.154
16:3n-3	2.6 $\pm$ 0.5b	2.9 $\pm$ 0.5b	2.8 $\pm$ 0.4b	4.2 $\pm$ 0.8a	0.000
16:4n-3	0.22 $\pm$ 0.04c	0.42 $\pm$ 0.19b	0.25 $\pm$ 0.09c	0.71 $\pm$ 0.06a	0.000
18:2n-6	0.99 $\pm$ 0.07a	0.73 $\pm$ 0.08b	1.1 $\pm$ 0.1a	0.60 $\pm$ 0.22b	0.000
20:2n-6	1.3 $\pm$ 0.1a	1.1 $\pm$ 0.1a	1.1 $\pm$ 0.3a	0.63 $\pm$ 0.16b	0.000
20:4n-6	7.0 $\pm$ 0.5ab	7.4 $\pm$ 0.6a	7.5 $\pm$ 1.0a	6.3 $\pm$ 0.4b	0.000
20:5n-3	21.3 $\pm$ 2.5ab	24.1 $\pm$ 1.5a	19.5 $\pm$ 2.3b	21.4 $\pm$ 1.5ab	0.001
22:5n-3	1.1 $\pm$ 0.2a	1.1 $\pm$ 0.2a	1.2 $\pm$ 0.4a	1.2 $\pm$ 0.2a	0.459
22:6n-3	10.9 $\pm$ 1.5b	10.7 $\pm$ 0.7b	11.5 $\pm$ 1.3ab	12.2 $\pm$ 0.6a	0.039
PUFA	48.4$\pm$2.7a	50.7$\pm$2.4a	48.9$\pm$1.8a	50.5$\pm$1.8a	0.095
n-3	36.6 $\pm$ 2.8a	36.5 $\pm$ 1.8a	35.8 $\pm$ 2.4a	35.4 $\pm$ 1.9a	0.648
n-6	9.6 $\pm$ 0.5ab	10.1 $\pm$ 1.0a	10.3 $\pm$ 1.3a	8.8 $\pm$ 0.6b	0.006
(n-3/n-6)	3.8 $\pm$ 0.3a	3.6 $\pm$ 0.5a	3.5 $\pm$ 0.5a	4.0 $\pm$ 0.4a	0.072
PUFA/SFA	2.8 $\pm$ 0.2a	3.2 $\pm$ 0.2a	2.9 $\pm$ 0.3a	2.9 $\pm$ 0.1a	0.053
IA	0.19 $\pm$ 0.01a	0.15 $\pm$ 0.01b	0.18 $\pm$ 0.01a	0.18 $\pm$ 0.01a	0.000
IT	0.12 $\pm$ 0.01a	0.11 $\pm$ 0.00a	0.12 $\pm$ 0.01a	0.12 $\pm$ 0.01a	0.058
Hepat.	SF	EF	SM	EM	p
14:0	4.3 $\pm$ 0.7a	2.7 $\pm$ 0.8b	3.3 $\pm$ 0.7ab	2.4 $\pm$ 0.5b	0.000
15:0	0.72 $\pm$ 0.11b	1.2 $\pm$ 0.2a	0.79 $\pm$ 0.20b	0.84 $\pm$ 0.41ab	0.001
16:0	12.6 $\pm$ 1.22b	12.57 $\pm$ 1.1b	12.8 $\pm$ 1.2ab	14.7 $\pm$ 1.4a	0.013
18:0	3.2 $\pm$ 0.7a	3.0 $\pm$ 0.1a	2.7 $\pm$ 0.6a	3.2 $\pm$ 0.1a	0.105
SFA	21.8$\pm$2.6a	20.5$\pm$1.8a	20.5$\pm$1.8a	22.0$\pm$2.0a	0.390
16:1n-7	9.0 $\pm$ 0.8ab	9.4 $\pm$ 2.3ab	8.8 $\pm$ 1.6b	11.6 $\pm$ 1.1a	0.021
16:1n-5	0.27 $\pm$ 0.03c	0.93 $\pm$ 0.18a	0.25 $\pm$ 0.07c	0.56 $\pm$ 0.15b	0.000
18:1n-9	17.8 $\pm$ 2.4a	17.7 $\pm$ 3.1a	15.7 $\pm$ 2.0ab	11.6 $\pm$ 1.5b	0.000
18:1n-7	5.7 $\pm$ 0.5ab	7.0 $\pm$ 1.5a	5.3 $\pm$ 0.7b	6.5 $\pm$ 1.1ab	0.009
20:1n-9	6.7 $\pm$ 1.3a	5.0 $\pm$ 0.8a	4.6 $\pm$ 0.8a	2.6 $\pm$ 0.1b	0.005
MUFA	46.3$\pm$5.3a	44.7$\pm$6.9ab	38.8$\pm$4.5b	35.8$\pm$3.2b	0.003
16:3n-4	1.0 $\pm$ 0.1a	1.0 $\pm$ 0.1a	1.1 $\pm$ 0.2a	1.1 $\pm$ 0.2a	0.513
16:3n-3	0.52 $\pm$ 0.10a	0.6 $\pm$ 0.07a	0.56 $\pm$ 0.12a	0.50 $\pm$ 0.02a	0.533

Abbreviations: (SF) female crabs from the Scottish coast; (EF) female crabs from the English Channel; (SM) male crabs from the Scottish coast; (EM) male crabs from the English Channel; (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity; (IT) index of thrombogenicity; (hepat.) hepatopancreas. In each row different letters indicate significant differences between sexes and locations ($p < 0.05$). The number of replicates for each location and sex were: muscle ($n=10$); hepatopancreas ($n=10$), ovaries ($n=6$) and testis (4).

Table 4.3.4 (cont.)

Hepat.	SF	EF	SM	EM	p
16:4n-3	1.2±0.4b	1.6±0.7b	1.7±0.5b	4.2±1.2a	0.001
18:2n-6	0.91±0.07a	0.79±0.13ab	0.90±0.13a	0.62±0.10b	0.000
20:2n-6	1.6±0.2a	1.3±0.3ab	1.6±0.7a	0.67±0.15b	0.001
20:4n-6	2.6±0.5a	3.2±0.7a	3.7±1.6a	3.1±0.5a	0.193
20:5n-3	5.5±1.5b	7.0±2.6ab	7.4±1.7ab	10.3±2.0a	0.002
22:5n-3	0.8±0.2b	0.95±0.31b	1.3±0.3a	1.1±0.2ab	0.002
22:6n-3	6.5±2.1b	7.9±2.4ab	10.4±1.3a	9.3±0.7ab	0.005
PUFA	24.2±4.2b	28.0±8.2ab	32.8±2.4a	35.0±3.6a	0.002
n-3	15.4±4.2b	17.8±6.6ab	21.5±2.7ab	23.3±2.8a	0.010
n-6	5.8±0.7a	6.2±0.8a	6.9±2.3a	5.9±0.4a	0.512
(n-3/n-6)	2.6±0.7b	2.8±0.6b	3.5±1.5ab	3.9±0.3a	0.011
PUFA/SFA	1.1±0.3b	1.4±0.5ab	1.6±0.2ab	1.7±0.3a	0.001
IA	0.44±0.09a	0.34±0.07b	0.39±0.05ab	0.38±0.05ab	0.034
IT	0.27±0.06a	0.23±0.05a	0.21±0.04a	0.22±0.03a	0.061
Gonads	SF	EF	SM	EM	p
14:0	1.9±0.4a	1.6±0.0a	0.61±0.03b	0.86±0.42ab	0.026
15:0	0.89±0.15a	0.80±0.03ab	0.43±0.02c	0.49±0.16bc	0.002
16:0	11.7±1.1a	11.3±0.4a	8.7±0.4a	10.7±3.0a	0.051
18:0	2.5±0.1b	2.5±0.0b	5.5±0.4a	6.1±0.6a	0.036
SFA	17.6±1.8a	16.8±0.5a	16.6±0.9a	19.4±4.3a	0.054
16:1n-7	0.16±0.02a	0.14±0.01a	0.02±0.00b	0.05±0.00b	<0.001
16:1n-5	0.59±0.02b	0.69±0.11a	0.63±0.02ab	0.58±0.12ab	<0.001
18:1n-9	17.3±0.5a	17.0±0.7a	13.0±0.5b	11.5±1.7b	0.033
18:1n-7	4.8±0.4a	5.5±0.4a	5.2±0.2a	5.4±0.6a	0.149
20:1n-9	2.4±0.2a	2.0±0.1b	2.4±0.2a	1.7±0.0b	0.005
MUFA	34.3±0.7a	35.0±0.7a	32.2±2.1ab	30.0±0.2b	0.015
16:3n-4	0.98±0.30a	1.1±0.1a	0.44±0.01b	1.1±0.2a	0.008
16:3n-3	0.51±0.03b	0.55±0.01b	1.2±0.1a	1.0±0.2a	<0.001
16:4n-3	2.4±0.3ab	1.9±0.1bc	1.5±0.1c	2.9±0.1a	<0.001
18:2n-6	0.87±0.02a	0.73±0.04b	0.64±0.03bc	0.50±0.10c	<0.001
20:2n-6	0.69±0.09b	0.60±0.03b	1.3±0.1a	0.54±0.01b	<0.001
20:4n-6	4.9±0.2b	4.9±0.3b	9.2±0.4a	5.7±0.0b	<0.001
20:5n-3	13.9±0.4b	14.8±1.0b	19.7±0.9a	18.3±0.9a	<0.001
22:5n-3	1.5±0.2a	1.6±0.1a	1.12±0.0b	0.85±0.10b	<0.001
22:6n-3	14.2±1.6ab	13.8±0.1ab	15.5±0.9a	11.2±1.5b	<0.001
PUFA	43.2±2.4a	42.6±1.3a	48.6±3.6a	43.1±0.7a	0.054
n-3	31.6±2.0b	31.7±1.2b	38.2±1.8a	32.2±2.2b	0.003
n-6	7.6±0.3b	7.6±0.3b	11.9±0.5a	8.8±1.4b	<0.001
(n-3/n-6)	4.2±0.1a	4.2±0.0a	3.2±0.0b	3.7±0.3b	0.004
PUFA/SFA	2.5±0.4ab	2.5±0.2b	2.9±0.1a	2.3±0.5b	<0.001
IA	0.26±0.04a	0.24±0.01ab	0.14±0.01b	0.20±0.08ab	0.048
IT	0.14±0.02a	0.13±0.01a	0.11±0.00a	0.15±0.04a	0.100

Abbreviations: (SF) female crabs from the Scottish coast; (EF) female crabs from the English Channel; (SM) male crabs from the Scottish coast; (EM) male crabs from the English Channel; (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity; (IT) index of thrombogenicity; (hepat.) hepatopancreas. In each row different letters indicate significant differences between sexes and locations ($p < 0.05$). The number of replicates for each location and sex were: muscle ($n=10$); hepatopancreas ($n=10$), ovaries ($n=6$) and testis (4).

In contrast, hepatopancreas revealed few differences related either with sex or location as only 22:5n-3 had a lower proportion in females than in males and 16:1n-5 had a higher proportion in crabs from EC and among these, females had more 16:1n-5 than males.

Most differences found in gonads were related to sex, as 16:1n-7, 18:1n-9, 22:5n-3 and, the ratio n-3 to n-6 were all proportionally higher in females than in males. On the other hand 18:0, 16:3n-3 and 20:5n-3 were proportionally higher in males. The only population differences were observed in SC crabs which had proportionally more 20:1n-9. The fatty acid profiles were statistically different between tissues (Table 4.3.5). Muscle and gonads had a similar pattern dominated by PUFA, followed by MUFA and SFA, while hepatopancreas had proportionally more MUFA and SFA. Hepatopancreas had proportionally more 14:0, 15:0, 16:1n-7, 20:1n-9, and 20:2n-6 than muscle and gonads, but less 16:1n-5, 20:4n-6, and 20:5n-3 ($p<0.01$). The proportion of n-3, n-6 and the ratio n-3/n-6 was lower in hepatopancreas than in muscle and gonads, whereas, IA and IT were higher.

Table 4.3.5 Fatty acid profile (% of total fatty acids) of each edible tissue of crabs from both location and sexes (average $\pm$ standard deviation).

Fatty acids	Muscle	Hepatopancreas	Gonads	p
14:0	0.86 $\pm$ 0.34b	3.33 $\pm$ 0.97a	1.24 $\pm$ 0.65b	<0.001
15:0	0.56 $\pm$ 0.09b	0.88 $\pm$ 0.28a	0.65 $\pm$ 0.24b	<0.001
16:0	9.82 $\pm$ 0.88b	12.97 $\pm$ 1.39a	10.45 $\pm$ 1.72ab	0.001
18:0	4.74 $\pm$ 0.52a	3.00 $\pm$ 0.52b	4.11 $\pm$ 1.70ab	<0.001
16:1n-7	4.85 $\pm$ 0.82b	9.46 $\pm$ 1.78a	0.09 $\pm$ 0.07c	<0.001
16:1n-5	0.56 $\pm$ 0.09a	0.48 $\pm$ 0.31b	0.62 $\pm$ 0.06a	<0.001
18:1n-9	15.96 $\pm$ 1.36a	16.24 $\pm$ 3.20a	14.84 $\pm$ 2.60a	0.264
18:1n-7	5.61 $\pm$ 0.64ab	6.03 $\pm$ 1.17a	5.14 $\pm$ 0.44b	<0.001
20:1n-9	1.78 $\pm$ 0.58b	5.06 $\pm$ 1.66a	2.21 $\pm$ 0.29b	<0.001
16:3n-4	1.26 $\pm$ 0.43a	1.04 $\pm$ 0.15ab	0.84 $\pm$ 0.34b	0.005
16:3n-3	3.13 $\pm$ 0.85a	0.54 $\pm$ 0.09b	0.83 $\pm$ 0.34b	<0.001
16:4n-3	0.40 $\pm$ 0.23b	1.92 $\pm$ 1.21a	2.07 $\pm$ 0.55a	<0.001
18:2n-6	0.85 $\pm$ 0.24a	0.83 $\pm$ 0.15ab	0.71 $\pm$ 0.14b	0.030
20:2n-6	1.00 $\pm$ 0.29b	1.36 $\pm$ 0.51a	0.87 $\pm$ 0.36b	<0.001
20:4n-6	7.03 $\pm$ 0.81a	3.12 $\pm$ 1.01b	6.46 $\pm$ 2.09a	<0.001
20:5n-3	21.42 $\pm$ 2.49a	7.17 $\pm$ 2.45c	16.71 $\pm$ 2.77b	<0.001
22:5n-3	1.14 $\pm$ 0.26ab	1.01 $\pm$ 0.32b	1.28 $\pm$ 0.29a	0.021
22:6n-3	11.37 $\pm$ 1.20b	8.31 $\pm$ 2.66c	14.06 $\pm$ 1.82a	<0.001
SFA	16.90 $\pm$ 1.04b	21.18 $\pm$ 2.10a	17.46 $\pm$ 1.96b	<0.001
MUFA	30.15 $\pm$ 1.72c	42.24 $\pm$ 6.53a	32.96 $\pm$ 2.18b	<0.001
PUFA	49.52 $\pm$ 2.32a	29.15 $\pm$ 6.45b	44.88 $\pm$ 3.61a	<0.001
n-3	36.08 $\pm$ 2.24a	18.85 $\pm$ 5.25b	33.94 $\pm$ 3.56a	<0.001
n-6	9.70 $\pm$ 1.04a	6.21 $\pm$ 1.33b	9.24 $\pm$ 2.10a	<0.001
(n-3/n-6)	3.76 $\pm$ 0.44a	3.11 $\pm$ 1.02b	3.77 $\pm$ 0.46a	<0.001
PUFA/SFA	0.20 $\pm$ 0.05ab	0.18 $\pm$ 0.07b	0.25 $\pm$ 0.07a	0.002
IA	0.17 $\pm$ 0.02b	0.39 $\pm$ 0.08a	0.21 $\pm$ 0.07b	<0.001
IT	0.12 $\pm$ 0.01b	0.24 $\pm$ 0.05a	0.13 $\pm$ 0.02b	<0.001

Abbreviations: as in table 4.3.4. In each row different letters indicate significant differences between tissues ($p<0.05$). The number of replicates for each tissue were: muscle (n=40); hepatopancreas (n=40), gonads (n=20).

Amino acids

Non essential amino acids (NEAA) dominated the protein content in all tissues of crabs from both locations and sexes, namely glutamic acid, aspartic acid and glycine (Table 4.3.6). As to essential amino acids (EAA), all tissues had mostly arginine, leucine and threonine. Generally, amino acid concentration in the edible tissues showed few differences in respect to population and more in relation to sex. In muscle, males had more methionine and taurine than females. On the other hand, SM crabs had higher concentration of aminoacids like valine, leucine, asparagine and serine. In hepatopancreas most differences were related to sex, in which male crabs had usually higher concentration of several aminoacids than females like histidine, lysine and arginine. The only difference observed between populations was detected for proline, with EC crabs showing higher concentration than SC crabs. All identified amino acids in female gonads from both locations had higher concentration than males on a wet weight basis.

Table 4.3.6 Amino acid concentration ($\text{g } 100 \text{ g}^{-1}$ of wet weight) of edible tissues of *C. pagurus* from each location and sex (average $\pm$ standard deviation).

Muscle	SF	EF	SM	EM	p
Thr	0.99 $\pm$ 0.03a	0.98 $\pm$ 0.00a	1.06 $\pm$ 0.09a	0.94 $\pm$ 0.02a	0.099
Val	0.69 $\pm$ 0.03b	0.70 $\pm$ 0.01b	0.77 $\pm$ 0.04a	0.70 $\pm$ 0.00b	0.037
Met	0.18 $\pm$ 0.01b	0.19 $\pm$ 0.03b	0.26 $\pm$ 0.02a	0.24 $\pm$ 0.03a	0.003
Ile	0.67 $\pm$ 0.02b	0.68 $\pm$ 0.03ab	0.75 $\pm$ 0.04a	0.70 $\pm$ 0.00a	0.012
Leu	1.11 $\pm$ 0.03b	1.13 $\pm$ 0.04b	1.25 $\pm$ 0.06a	1.14 $\pm$ 0.01b	0.002
Phe	0.63 $\pm$ 0.04ab	0.63 $\pm$ 0.01b	0.70 $\pm$ 0.04a	0.63 $\pm$ 0.00b	0.037
His	0.36 $\pm$ 0.03ab	0.35 $\pm$ 0.02b	0.40 $\pm$ 0.03a	0.35 $\pm$ 0.00b	0.024
Lys	0.89 $\pm$ 0.07a	0.96 $\pm$ 0.04a	1.00 $\pm$ 0.07a	0.99 $\pm$ 0.05a	0.144
Arg	1.64 $\pm$ 0.02b	1.67 $\pm$ 0.07ab	1.79 $\pm$ 0.07a	1.63 $\pm$ 0.01b	0.004
Asp	1.49 $\pm$ 0.02b	1.54 $\pm$ 0.02b	1.73 $\pm$ 0.09a	1.52 $\pm$ 0.02b	<0.001
Ser	0.67 $\pm$ 0.02b	0.68 $\pm$ 0.01b	0.74 $\pm$ 0.04a	0.64 $\pm$ 0.00c	<0.001
Glu	2.50 $\pm$ 0.01b	2.49 $\pm$ 0.07b	2.79 $\pm$ 0.11a	2.59 $\pm$ 0.06ab	0.008
Gly	1.34 $\pm$ 0.04ab	1.39 $\pm$ 0.02a	1.24 $\pm$ 0.08b	1.09 $\pm$ 0.03c	<0.001
Ala	0.91 $\pm$ 0.02a	0.93 $\pm$ 0.02a	0.98 $\pm$ 0.06a	0.92 $\pm$ 0.01a	0.222
Tyr	0.62 $\pm$ 0.03b	0.63 $\pm$ 0.01b	0.71 $\pm$ 0.04a	0.66 $\pm$ 0.01ab	0.003
Pro	0.73 $\pm$ 0.10a	0.73 $\pm$ 0.04a	0.75 $\pm$ 0.04a	0.82 $\pm$ 0.06a	0.212
Hyp	0.39 $\pm$ 0.06a	0.45 $\pm$ 0.04a	0.45 $\pm$ 0.06a	0.44 $\pm$ 0.02a	0.333
Tau	0.33 $\pm$ 0.03c	0.34 $\pm$ 0.03c	0.43 $\pm$ 0.03b	0.50 $\pm$ 0.01a	<0.001
EAA/NEAA	0.80 $\pm$ 0.01a	0.80 $\pm$ 0.01a	0.81 $\pm$ 0.01a	0.80 $\pm$ 0.01a	0.054
TAA	16.2 $\pm$ 0.6ab	16.5 $\pm$ 0.5ab	17.8 $\pm$ 0.9a	16.5 $\pm$ 0.0b	0.032
Hepatopancreas	SF	EF	SM	EM	p
Thr	0.75 $\pm$ 0.12ab	0.67 $\pm$ 0.03b	0.75 $\pm$ 0.02a	0.79 $\pm$ 0.02a	0.030
Val	0.55 $\pm$ 0.09ab	0.52 $\pm$ 0.01b	0.60 $\pm$ 0.05a	0.65 $\pm$ 0.02a	0.012
Met	0.03 $\pm$ 0.03b	0.06 $\pm$ 0.04ab	0.12 $\pm$ 0.04a	0.16 $\pm$ 0.04a	0.001
Ile	0.47 $\pm$ 0.09ab	0.44 $\pm$ 0.01b	0.52 $\pm$ 0.03a	0.58 $\pm$ 0.01a	0.012

Abbreviations: (SF) female crabs from the Scottish coast; (EF) female crabs from the English Channel; (SM) male crabs from the Scottish coast; (EM) male crabs from the English Channel; (Thr) threonine; (Val) valine; (Met) methionine; (Ile) isoleucine; (Leu) leucine; (Phe) phenylalanine; (His) histidine; (Lys) lysine; (Arg), arginine; (Asp) aspartic acid; (Ser) serine; (Glu) glutamic acid; (Gly) glycine; (Ala) alanine; (Tyr) tyrosine; (Pro) proline; (Hyp) hydroxyproline; (Tau) taurine; (EAA/NEAA) essential amino acids/nonessential amino acids; (TAA) total amino acids. In each row different letters indicate significant differences per tissue ($p < 0.05$). The number of replicates for each location and sex were: muscle ($n=10$); hepatopancreas ($n=10$), ovaries ($n=6$) and testis (4).

Table 4.3.6 (cont.).

Hepatopancreas	SF	EF	SM	EM	p
Leu	0.75±0.15ab	0.70±0.01b	0.81±0.05a	0.89±0.00a	0.020
Phe	0.46±0.10ab	0.42±0.01b	0.50±0.03a	0.54±0.01a	0.046
His	0.29±0.06b	0.27±0.00b	0.35±0.02a	0.40±0.02a	0.010
Lys	0.54±0.12c	0.54±0.01c	0.60±0.02b	0.72±0.00a	0.020
Arg	0.92±0.18c	0.88±0.03c	1.08±0.11b	1.26±0.06a	0.001
Asp	0.98±0.19abc	0.90±0.01c	1.08±0.06b	1.17±0.04a	0.046
Ser	0.47±0.09ab	0.43±0.00b	0.48±0.03a	0.50±0.01a	0.045
Glu	1.30±0.25ab	1.15±0.04b	1.39±0.09a	1.51±0.02a	0.033
Gly	0.58±0.09ab	0.54±0.02b	0.61±0.04a	0.67±0.02a	0.025
Ala	0.57±0.10ab	0.53±0.01b	0.60±0.02a	0.63±0.02a	0.029
Tyr	0.52±0.10ab	0.52±0.00b	0.58±0.02a	0.63±0.02a	0.018
Pro	0.35±0.09b	0.47±0.02a	0.39±0.02b	0.46±0.04a	0.025
Hyp	0.33±0.03a	0.32±0.01a	0.28±0.05a	0.26±0.04a	0.064
Tau	0.54±0.02a	0.31±0.02b	0.58±0.06a	0.59±0.01a	0.019
EAA/NEAA	0.84±0.02b	0.87±0.03ab	0.89±0.01b	0.93±0.01a	0.001
TAA	10.4±1.9ab	9.7±0.1b	11.3±0.7ab	12.4±0.2a	0.020
Gonads	SF	EF	SM	EM	p
Thr	2.03±0.03a	1.99±0.03a	0.81±0.01b	0.87±0.00b	<0.001
Val	1.55±0.00a	1.56±0.03a	0.68±0.02b	0.73±0.01b	<0.001
Met	0.43±0.01a	0.39±0.02a	0.09±0.00b	0.12±0.01b	<0.001
Ile	1.32±0.01a	1.30±0.02a	0.49±0.01b	0.53±0.01b	<0.001
Leu	2.04±0.01a	1.98±0.04a	0.75±0.01b	0.79±0.01b	<0.001
Phe	1.23±0.00a	1.19±0.02a	0.51±0.01b	0.52±0.01b	<0.001
His	0.72±0.01a	0.72±0.02a	0.29±0.00b	0.30±0.00b	<0.001
Lys	1.13±0.04a	1.18±0.03a	0.81±0.02b	0.88±0.09b	<0.001
Arg	2.13±0.02a	2.06±0.06a	0.96±0.02b	1.03±0.04b	<0.001
Asp	2.37±0.02a	2.26±0.04a	1.13±0.02c	1.22±0.01b	<0.001
Ser	1.64±0.01a	1.59±0.03a	0.66±0.01b	0.69±0.00b	<0.001
Glu	3.60±0.07a	3.45±0.01a	1.64±0.03c	1.82±0.01b	<0.001
Gly	1.30±0.01a	1.34±0.04a	0.87±0.02b	0.83±0.06b	<0.001
Ala	1.28±0.01a	1.28±0.04a	0.67±0.01b	0.68±0.00b	<0.001
Tyr	1.41±0.01a	1.36±0.02a	0.55±0.00b	0.57±0.00b	<0.001
Pro	1.30±0.06a	1.28±0.06a	0.70±0.02b	0.74±0.03b	<0.001
Hyp	0.69±0.04a	0.52±0.01a	0.34±0.01b	0.37±0.02b	<0.001
Tau	0.73±0.02a	0.76±0.01a	0.49±0.01b	0.52±0.02b	<0.001
EAA/NEAA	0.88±0.01a	0.77±0.00b	0.90±0.00a	0.78±0.02b	0.009
TAA	26.9±0.1a	26.2±0.5a	12.4±0.2b	13.2±0.2b	<0.001

Abbreviations: (SF) female crabs from the Scottish coast; (EF) female crabs from the English Channel; (SM) male crabs from the Scottish coast; (EM) male crabs from the English Channel; (Thr) threonine; (Val) valine; (Met) methionine; (Ile) isoleucine; (Leu) leucine; (Phe) phenylalanine; (His) histidine; (Lys) lysine; (Arg), arginine; (Asp) aspartic acid; (Ser) serine; (Glu) glutamic acid; (Gly) glycine; (Ala) alanine; (Tyr) tyrosine; (Pro) proline; (Hyp) hydroxyproline; (Tau) taurine; (EAA/NEAA) essential amino acids/nonessential amino acids; (TAA) total amino acids. In each row different letters indicate significant differences per tissue ($p < 0.05$). The number of replicates for each location and sex were: muscle ($n=10$); hepatopancreas ($n=10$), ovaries ($n=6$) and testis (4).

The amino acid concentration on a wet weight basis showed marked differences mainly between hepatopancreas and remaining tissues, independently of sex and location (Table 4.3.7), *i.e.* the concentration of total amino acids was lower in hepatopancreas than in muscle and gonads, except for taurine and histidine. The EAA/NEAA ratio was lower in muscle. All tissues had an excellent S-score for all amino acids except methionine (Table 4.3.8). Hepatopancreas had the lowest methionine concentration, especially females, followed by testis and muscle. Ovaries were a good source of this amino acid.

Table 4.3.7 Amino acid concentration (g 100 g⁻¹ of wet weight) comparison between tissues of *C. pagurus* from both location and sexes and with the respective p values (average ± standard deviation).

Amino acid	Muscle	Hepatopancreas	Gonads	p
Thr	0.99±0.06a	0.74±0.07b	1.30±0.59a	<0.001
Val	0.71±0.04a	0.58±0.07b	1.04±0.43a	<0.001
Met	0.22±0.04a	0.09±0.06b	0.23±0.16a	<0.001
Ile	0.70±0.04a	0.50±0.07b	0.83±0.41a	<0.001
Leu	1.16±0.06a	0.79±0.10b	1.26±0.63ab	<0.001
Phe	0.65±0.04a	0.48±0.06b	0.79±0.35a	<0.001
His	0.36±0.03a	0.32±0.06a	0.46±0.22a	0.142
Lys	0.96±0.07a	0.60±0.10b	0.97±0.18a	<0.001
Arg	1.68±0.08a	1.03±0.18b	1.43±0.56ab	<0.001
Asp	1.57±0.11a	1.03±0.14b	1.62±0.58a	<0.001
Ser	0.68±0.04a	0.47±0.05b	1.05±0.48a	<0.001
Glu	2.59±0.14a	1.34±0.18b	2.43±0.92a	<0.001
Gly	1.26±0.13a	0.60±0.07b	1.04±0.24a	<0.001
Ala	0.94±0.04a	0.58±0.06b	0.92±0.31a	<0.001
Tyr	0.65±0.04a	0.55±0.08b	0.89±0.41ab	<0.001
Pro	0.76±0.07a	0.38±0.07b	0.94±0.29a	<0.001
Hyp	0.43±0.05a	0.30±0.04b	0.45±0.14a	<0.001
Tau	0.40±0.08b	0.56±0.04a	0.60±0.13a	<0.001
EAA/NEAA	0.80±0.01b	0.88±0.04a	0.82±0.06a	0.045
TAA	16.73±0.83a	10.96±1.40b	18.24±6.98a	<0.001

Abbreviations as in table 4.3.6. In each row different letters indicate significant differences per tissue (p<0.05). The number of replicates for each tissue were: muscle (n=40); hepatopancreas (n=40), gonads (n=20).

Table 4.3.8 Essential amino acid scores (%) of edible tissues of *C. pagurus* from both locations and sexes.

Muscle	SF	EF	SM	EM
Val	117	95	96	100
Thr	287	227	226	228
Phe+Tyr	220	176	181	188
Met	75	63	81	83
Lys	134	113	109	123
Leu	125	102	104	108
Ile	149	122	123	129
His	160	123	129	129
Hepatopancreas	SF	EF	SM	EM
Val	116	123	113	119
Thr	269	270	239	243
Phe+Tyr	212	218	210	218
Met	13	32	54	68
Lys	99	111	98	115
Leu	105	110	102	107
Ile	129	136	128	138
His	158	164	170	186
Gonads	SF	EF	SM	EM
Val	156	159	134	136
Thr	345	345	270	275
Phe+Tyr	272	268	214	207
Met	106	98	44	53
Lys	99	106	137	142
Leu	136	134	97	97
Ile	172	173	126	128
His	189	191	147	145

Abbreviations as in table 4.3.6. The number of replicates for each location and sex were: muscle (n=10); hepatopancreas (n=10), ovaries (n=6) and testis (4).

Discussion

The nutritive and commercial value of seafood depends on the chemical composition of the muscle, gonad, liver, etc., their proportion in the specimen weight, and factors related to fishing (Sikorski *et al.*, 2000). However, most studies concerning the benefits of seafood in general, and crustaceans in particular, only consider the muscle as edible tissue (Sikorski *et al.*, 2000). Since, the brown meat of *C. pagurus* (hepatopancreas and gonads) is highly appreciated in Southern Europe, it is fundamental to address the nutritional quality of crabs considering all edible parts in order to make wise decisions about consumption. Moreover, the deliberate price difference according to fishing ground location also needs to be further clarified.

As far as the meat yield is concerned, no differences were observed between both fishing ground location, whereas this study confirmed that males, independently of location, contributed with a higher proportion of white meat from claws than females of the same size. This difference is due to a sexual dimorphism, in which males have bigger claws than females (Edwards, 1979) and justifies the higher price of males. On the other hand, females were responsible by a larger proportion of

gonads though with greater variation in the gonadosomatic index due to the different maturation stages found.

Most differences in the chemical composition were observed between tissues and for each tissue between sexes, whereas few differences were observed due to fishing ground location. On the other hand, previous results obtained in sub-chapters 4.4, for *C. pagurus* elemental composition revealed that crabs from the SC have more macro and trace elements than EC crabs. However, as far as the meat yield and biochemical composition are concerned there is no reason for price differentiation between crabs from different locations. The main reason for the price distinction is most probably due to a condition factor as suggested previously in sub-chapter 2.1.

Other studies have also reported lack of differences in some chemical compounds in respect to location, e.g. cholesterol in hepatopancreas of snow crabs, *Chionoecetes opilio* (Souchet and Laplante, 2007).

The results obtained in this study are in agreement with the common knowledge that seafood is a source of high-quality protein with well-balanced essential amino acids' composition, high in essential PUFA and with low cholesterol levels but in this case, with different magnitudes according to tissue and sex. The proximate chemical composition of edible tissues generally reflects their physiological functions, metabolic needs and available diet (Rosa and Nunes, 2003b; Vinagre *et al.*, 2007). Several studies have reported similar results with decapods, like the crabs *Callinectes sapidus* (Çelik *et al.*, 2004), *Maja brachydactyla* (Bodin *et al.*, 2007), *Carcinus mediterraneus* (Cherif *et al.*, 2008) and the penaeid shrimp *Fenneropenaeus indicus* (Vasquez-Boucard *et al.*, 2004).

Muscle is a structural tissue with a high protein content and poor fat. Hepatopancreas is a multifunctional organ responsible for the absorption and storage of ingested materials, such as lipids and cholesterol in decapod crustaceans (Gibson and Barker, 1979). Gonads are responsible for the development of reproductive cells and, therefore, there is a high energy investment in this organ confirmed by the high protein, fat and cholesterol levels (Wen *et al.*, 2001; Çelik *et al.*, 2004).

The higher amount of MUFA detected in hepatopancreas lipids compared to muscle is similar to other decapods (Chapelle, 1977; Harrison, 1990; Floreto *et al.*, 2000; Celik *et al.*, 2004; Latyshev *et al.*, 2009). The major fatty acids differences found among tissues were registered for 16:1n-7 and 20:1n-9, which might be associated with different metabolic needs of each tissue and with biosynthesis occurring in hepatopancreas, similar to results reported by Latyshev *et al.* (2009) for 5 crab species of the north-western Pacific Ocean, namely *Paralithodes camtschaticus*, *P. platypus*, *Chionoecetes opilio*, *C. angulatus* and *C. japonicus*. In general, the fatty acid profile of gonads was similar to muscle. The major differences were detected in the higher proportion of 16:4n-3 and DHA in gonads but lower values of EPA. This indicates that 16:4n-3 and DHA might be important in gonads maturation. The higher EPA values in muscle might be related to its physiological function as an important structural component of cell membranes (Lilly and Bottino, 1981).

One of the major concerns about food quality and nutrition in developed countries is the cholesterol, fat and PUFA contents. Cholesterol content of *C. pagurus* muscle (37 - 47 mg 100 g⁻¹) was lower than other commercially important decapod species, such as the crayfish *Nephrops norvegicus* (97 mg 100 g⁻¹) and shrimp *Pandalus borealis* (107 mg 100 g⁻¹; Oehlenschläger, 2006). Among all analyzed tissues, ovaries had the highest cholesterol concentration (169.7-170.5 mg 100 g⁻¹). Nonetheless, since the current dietary recommendations suggest a cholesterol intake below 200-300 mg per day and *C. pagurus* is an occasional food item in the diet, all tissues pose minor risks.

Seafood is considered as a low fat food as many fish species and almost all shellfish contains less than 2.5 % of total fat in the muscle, and only a short number of species has more than 15 % (Nunes *et al.*, 2003). The present study confirms that this conclusion only applies to muscle. The fat content of *C. pagurus* showed marked differences between tissues and sexes (in gonads only). The total fat in muscle (0.2 - 0.4 %) and gonads of both sexes (0.9 - 1.1 %, males; 3.1 - 3.4 %, females) were below 5 %, a value generally considered to characterize a low fat food, in contrast to hepatopancreas (10.0 - 12.2 %).

The ratio of *n*-3/*n*-6 is an excellent index to compare the relative nutritional value of lipids, where high values generally correspond to better quality foods. Compared to other crustacean species, like the crabs *Callinectes sapidus* (muscle, 2.32; hepatopancreas, 1.57) and *Carcinus mediterraneus* (muscle, 1.4), all edible tissues of *C. pagurus* had higher nutritional quality in respect to this ratio (Çelik *et al.*, 2004; Cherif *et al.*, 2008). The United Kingdom Department of Health stipulated a minimum value of PUFA/SFA ratio of 0.45 (UKDH, 1994). In this regard all edible tissues had ratios in the range of the recommended values. Despite hepatopancreas had the highest IA and IT values compared to muscle and gonads, such values were lower than those of other food items, such as lamb (1.00), beef (0.72), pork (0.69), rabbit (0.82) and chicken (0.50) (Rosa and Nunes, 2003a).

Both muscle and ovaries were better sources of proteins and total amino acids than hepatopancreas. The amino acid pattern found in all tissues of *C. pagurus* was characterized by higher amounts of non essential amino acids. The lower amino acid content of hepatopancreas is in agreement with results found in literature for other crustaceans, like shrimps, lobsters and crabs (Rosa and Nunes, 2003a; Naczek *et al.*, 2004; Chen *et al.*, 2007, Sub-chapter 4.1). In general, crustacean's muscle contains more non-essential amino acids, which are synthesized or modified in the body, whereas this difference is not so evident in hepatopancreas. Ovaries were a better source of amino acids than testis considering the wet weight. Nevertheless on dry bases differences are less significant.

All tissues had S-scores above 100 %, except for methionine. Similar results were obtained in the muscle of Chinese mitten crab *Eriocheir sinensis* (Chen *et al.*, 2007). High levels of taurine were found in *C. pagurus* hepatopancreas. Despite so far, there is not a recommended dietary intake value for taurine, its ingestion is beneficial to human and animal consumption as it promotes reduced cardiovascular disease risk (Park *et al.*, 1998; Militante and Lombardini, 2004; Elvevoll *et*

al., 2008). Humans only have limited ability for biosynthesis of taurine by the liver and reabsorption by kidneys, and therefore taurine needs to be acquired with the diet (Militante and Lombardini, 2004).

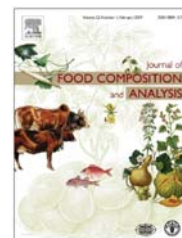
Conclusions

Most differences in the proximate chemical composition, fatty acid and amino acid content in the edible tissues of female and male *Cancer pagurus* from the Scottish coast and English Channel were related to tissues and sexes. There was not clear evidence that the fishing ground influenced the biochemical composition in a significant way. *C. pagurus* muscle is rich in proteins and poor in fat, while hepatopancreas had high fat, cholesterol and energy contents. Though gonads were rich in protein, this tissue also had high levels of cholesterol and energy. All edible tissues were valuable sources of essential amino acids. The fatty acid profile in muscle and gonads showed a pattern dominated by PUFA, while hepatopancreas was richer in MUFA and SFA. This pattern associated with lower n-3 content in hepatopancreas, a lower n-3/n-6 fatty acid ratio and higher n-6/n-3 fatty acid ratio contributed to higher indices of atherogenicity and thrombogenicity. Nonetheless, comparatively to other food items, like poultry, all edible tissues can be considered as balanced and nutritious food. Yet, previous studies highlighted that Cd content in hepatopancreas can be above the maximum permissible concentration and action levels, which can be a concern for heavy consumers (Sub-chapters 4.1 and 4.5). Therefore, it is recommended that future studies should take into consideration separate tissues to evaluate crustaceans' nutritional quality, particularly those with crabs, and to perform consumers' risk-benefit assessment analyses.

Sub-chapter 4.4

C. pagurus elemental composition: population differences

Barrento, S., Marques, A., Teixeira, B., Anacleto, P., Carvalho, M.L., Vaz-Pires, P., and Nunes, M. L. 2009. Macro and trace elements in two populations of brown crab *Cancer pagurus*: ecological and human health implications. *Journal of Food Composition and Analysis*, 22: 65-71.



Cancer pagurus is a widely appreciated crustacean in Southern European countries, where they have different market prices depending on the catching area and animal sex. Yet, the mineral composition of this species is still poorly understood. Therefore, the aim of this study was to quantify the content of essential elements in edible tissues of specimens from the Scottish Coast and English Channel and to compare such values with the dietary reference intakes. Crabs caught off the Scottish Coast had higher Na, Cl, K, Cu, Zn and Se content, while specimens harvested in the English Channel showed higher Mn concentrations. Females were better sources of Cu, Zn and Se, whereas males had higher concentrations of Na, Cl, K, Ca and Mn. Hepatopancreas showed higher contents of most elements, except Mn, Zn and Se than muscle and gonads. These results are certainly related to the crabs' physiological needs and the bioavailability of the elements in water and diet. In general, *C. pagurus* is an excellent source of macro (Na, Cl and Ca) and trace elements (Fe, Cu, Zn and Se) for human consumption. The present study highlights the importance to take into consideration all edible tissues of crustaceans to evaluate their nutritional quality given consumers' habits.

Keywords: crustaceans, *Cancer pagurus*, muscle, hepatopancreas, gonads, food composition, minerals, FAAS, EDXRF, English Channel crabs, Scottish Coast crabs, environment, food safety.

Introduction

Worldwide, crustaceans are high-valued and appreciated food items, representing an important economic source. In the last decade, the chemical composition and nutritional value of crustaceans have been investigated worldwide (e.g., Adeyeye, 2002; Skonberg and Perkins, 2002; Gökölu and Yerlikaya, 2003; Çelik *et al.*, 2004; Naczki *et al.*, 2004; Küçükgülmez *et al.*, 2006; Chen *et al.*, 2007; Vilasoa-Martínez *et al.*, 2007), and consumption benefits to human health have been much promoted (Oehlenschläger, 1997; Bügel *et al.*, 2001). Yet, only in recent years, attention has been paid to crustaceans' composition of essential elements. (e.g., Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Se), and this information is still rather disperse.

The lack of essential elements in humans leads to improper enzyme-mediated metabolic functions and results in organ malfunctions, chronic diseases and ultimately death (Simopoulos, 1997; FAO/WHO, 2002). The brown crab *Cancer pagurus* is one of the most consumed crustaceans in Southern European countries, such as France, Spain, Italy and Portugal (EUROSTAT, 2007). In these countries the white meat (muscle), brown meat (hepatopancreas) and gonads are consumed separately or as a mixture. This species is mostly harvested in the English Channel, Welsh, Scottish and Irish coasts (worldwide representing 84 % of total captures; 37,480 t; data from 2006; EUROSTAT, 2007), and transported mostly live to South Europe. This species has different market prices according to the fishing ground and sex. Crabs caught off the English Channel are usually more expensive than those caught off Welsh, Scottish and Irish coasts, presumably due to the intrinsic quality of these populations (e.g., bigger animals, more resistant to transport). In addition, larger males are usually more expensive than smaller males and females (Brown and Bennett, 1980). Considering that the chemical composition of a particular species varies according to sex, diet, habitat and tissue (Davies *et al.*, 1981; Bodin *et al.*, 2007; Mohapatra *et al.*, 2007), it is imperative to understand these variations on the essential element composition of *C. pagurus*. Therefore, the aim of this study was to quantify the essential elements Na, Mg, Cl, K, Ca, Mn, Fe, Cu, Zn and Se in the muscle, hepatopancreas and gonads of female and male *C. pagurus* from two fishing grounds (English Channel and Scottish Coast) and to compare such values with the recommended dietary allowance.

Material and methods

Biological material

Twenty inter-moult *C. pagurus* from the Scottish Coast (SC: 10 females and 10 males) and twenty inter-moult specimens from the English Channel (EC: 10 females and 10 males), were purchased live from a local importer and transported to the laboratory. Animals were kept under refrigerated conditions (~4 °C) for 1 h to decrease their metabolism before being euthanized. Muscle (from claws), hepatopancreas and gonads from each animal were individually separated and weighted. For each tissue the edible content was calculated as follows: (tissue wet weight / sum of all edible tissues wet weight) × 100. Each tissue was subsequently homogenized with a grinder (Retasch Grindomix GM200; 5000 rpm; material: PP cup and stainless steel knives) vacuum packed and stored at -20 °C, whereas the remaining was frozen at -80 °C and subsequently freeze-dried for 48

h at -50 °C and low pressure (approximately 10^{-1} atm). Samples were powdered and stored at -20 °C under controlled moisture conditions (vacuum packed) until further analyses. Plastic tools were generally used to process the crabs in order to avoid any contamination from processing equipment with trace elements measured.

Elemental analysis

Energy dispersive X-ray fluorescence (EDXRF) was used to quantify the essential elements Cl, K, Ca, Fe, Cu, Zn and Se. The EDXRF technique consists of an X-ray tube equipped with a molybdenum secondary exciter (EXTRA II A, Atomika Instruments, Temple). The characteristic radiations emitted by the elements in the sample were detected by a Lithium drifted Silicon [Si (Li)] detector, with a 30 mm² active area and an 8 µm beryllium window. The energy resolution was 135 eV at 5.9 keV and the acquisition system was a Nucleus PCA card. Quantitative calculations were made with the fundamental parameters method (Custódio *et al.*, 2003). The X-ray generator was operated at 50 kV, 20 mA and an acquisition time of 1000 s. Each sample powder (~1 g) was pressed into pellets of 2 cm diameter without any chemical treatment. A minimum of three pellets (replicates) per sample were glued onto Mylar films, on sample holders, and placed directly in the X-ray beam. There are several advantages of using EDXRF to determine elemental content: multi-elemental determination, simple sample preparation, less time-consuming compared to conventional techniques, low amount of sample required, no need to use liquid reagents, thus eliminating reagent waste and reducing potential contamination.

Flame atomic-absorption spectrometry (FAAS), through the spectrometer Variant Spectr AA 20, was employed to quantify Na, Mg and Mn according to official analytical methods (AOAC, 2005). The procedure is based on sample incineration (5 g) and further solubilization in HNO₃. Concentrations were calculated from linear calibration plots obtained by measurement of standard solutions absorbance: NaNO₃ (Merck) dissolved in HNO₃ (0.5 M); Mg (NO₃)₂ (Merck) dissolved in HNO₃ (0.5 M); and Mn (NO₃)₂ (Merck) dissolved in HNO₃ (0.5 M). Male gonads were not analyzed with the FAAS technique due to lack of sample material. A minimum of three replicate analyses was performed per sample.

Accuracy tests

Accuracy was checked by analyses of certified biological material. The elemental concentrations obtained for canned matrix meat (SMRD-2000; Swedish Meats R&D and Scan Foods/ National Food Administration, Sweden; FAAS), non-defatted lobster hepatopancreas (LUTS-1; National Research Council of Canada; FAAS), oyster tissue (SRM1566; National Bureau of Standards; EDXRF), freeze-dried animal blood (IAEA-A-13; International Atomic Energy Agency; EDXRF) and lobster hepatopancreas (TORT-2; National Research Council of Canada; FAAS) FAAS were compared with certified values. The detection limits (DL) of each element (Table 4.4.1) were determined by two means: (a) EDXRF, with the signal-to-noise approach, where the equipment compares the signal of each element with blank samples and established the minimum concentration at which the element is reliably detected; (b) FAAS, with the residual standard

deviation (RSD) of the response and the slope (S) of the calibration curve of each standard solution used ($DL=3.3 \times RSD/S$).

Table 4.4.1 Elemental concentration ($\mu\text{g g}^{-1}$ DW; $n=4$) and detection limits of certified reference material ($\pm$ sd) analyzed by FAAS and EDXRF.

Element	Present work	Certified value	Detection limit
Na ^(a)	8346 $\pm$ 280	8533 $\pm$ 281	0.37
Mg ^(b)	91 $\pm$ 2	90 $\pm$ 4	0.05
Cl ^(c)	10200 $\pm$ 500	10000 ^(d)	100
K ^(c)	10000 $\pm$ 80	9690 $\pm$ 50	10
Ca ^(c)	1350 $\pm$ 50	1500 $\pm$ 200	20
Mn ^(b)	1.28 $\pm$ 0.03	1.20 $\pm$ 0.13	0.04
Fe ^(c)	210 $\pm$ 15	195 $\pm$ 34	3
Cu ^(c)	64 $\pm$ 4	63 $\pm$ 4	1
Zn ^(c)	830 $\pm$ 40	852 $\pm$ 14	1
Se ^(c)	2.3 $\pm$ 0.5	2.1 $\pm$ 0.5	0.8

(a) canned matrix meat (SMRD-2000; FAAS); (b) non defatted lobster hepatopancreas (LUTS-1; FAAS); (c) oyster tissues (SRM 1566; EDXRF); (d) non-certified values provided by United States National Bureau of Standards.

Nutritional quality and potential risks to consumers

To evaluate the elemental nutritional quality and potential consumption risks of *C. pagurus* from both locations, intake of elements per 100 g serving portion was estimated for the edible tissues (wet weight). For each location and sex, the average intake (AI) of each element per 100 g edible portion was calculated. The average intake values of all elements were compared with the dietary reference intake (DRI) of adult females and males aged between 19 and 50 years set per day by USA Food and Nutrition Board of the Institute of Medicine (IOM, 1997; 2001; 2004 and 2008). For each element, the ratio between the average intakes per portion and the DRI was calculated.

Statistical analysis

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (sd). Differences in elements' concentration between sexes, fishing grounds and tissues were tested with analysis of variance (ANOVA). Whenever necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements, followed by non-parametric analysis of variance (Kruskal-Wallis), if transformed data could not meet these assumptions. Principal Component Analysis (PCA) was also employed to reduce the multidimensional data sets of the several elements to lower dimensions, thus simplifying the presentation and interpretation of data. All statistical analyses were tested at 0.05 level of probability with the software STATISTICATM 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Results and discussion

Elemental composition in crabs from the English Channel and Scottish Coast

The biometric data of all specimens analyzed are displayed in table 4.4.2.

Table 4.4.2 Biometric data of all specimens of *C. pagurus* analyzed.

Biometrics	SF	SM	EF	EM
Weight (g)	748±25	751±27	829±155	977±104
CL (mm)	107.38±1.77	98.44±3.71	109.93±5.88	110.16±3.71
CW (mm)	156.30±5.93	168.75±9.41	163.32±2.26	177.20±5.96
TMY (%)	22.5±3.8	23.5±2.2	22.0±9.9	23.8±3.6
ECM (%)	38.3±5.2	50.7±5.0	42.4±5.4	52.5±5.2
ECH (%)	46.3±4.5	43.3±6.2	43.3±4.7	42.9±6.3
ECG (%)	15.4±6.4	6.0±3.2	14.2±6.0	4.6±1.6

Abbreviations: (SF) female crabs from the Scottish coast; (SM) male crabs from the Scottish coast; (EF) female crabs from the English channel; (EM) male crabs from the English channel; (CL) cephalothorax length; (CW) cephalothorax width; (ECM) edible contributions of muscle; (ECH) edible contributions of hepatopancreas; (ECG) edible contributions of gonads.

In general, major differences in the elemental composition of *C. pagurus* were detected in hepatopancreas and gonads between fishing grounds and sexes, while few variations occurred in the muscle (Table 4.4.3).

Usually, crabs caught off the SC had statistically higher concentrations in the hepatopancreas (Cl, K, Zn and Se) and gonads (Na and Cu), while specimens harvested in the EC showed higher Mn content in hepatopancreas and gonads (Table 4.4.3). These results could indicate that Scottish coastal waters have less Mn and more Na, Cl, K, Cu, Zn and Se in a bio available form.

Sex differences in the elemental composition were also observed: females usually had higher contents in the muscle (Fe and Se), hepatopancreas (Cu) and gonads (Fe, Zn and Se), while in males the most relevant amounts were found in the hepatopancreas (Ca) and gonads (Cl, K and Ca) (Table 4.4.3). Since these elements are essential to crabs, it seems that females have a higher physiological need of trace elements (e.g., Fe, Cu, Zn and Se), while males require more macroelements (e.g., Cl, K and Ca). Additionally, *C. pagurus* has distinct migration patterns between female and male crabs, which might explain differences in elemental composition. Large females tend to migrate long distances, thus diversifying their feeding grounds, while males are more stationary, staying within the same habitats (Woll, 2006). Earlier studies by MacFarlane *et al.* (2000) with semaphore crab also reported higher Cu and Zn accumulation in females compared to males. The higher Ca content in male crabs is likely because this species has a sexual dimorphism, in which males have bigger claws and harder exoskeletons (composed by calcium phosphate; Woll, 2006). Particularly during the pre moult period of *C. pagurus*, hepatopancreas accumulates Ca that is likely used in the exoskeleton calcification (Luquet and Marin, 2004).

Table 4.4.3 Mean elemental composition ($\mu\text{g g}^{-1}$ wet weight) in female and male *C. pagurus* tissues from the Scottish Coast and English Channel.

Elements		Muscle				Hepatopancreas				Gonads			
		SF	SM	EF	EM	SF	SM	EF	EM	SF	SM	EF	EM
Macroelements													
Na	A	3025a	1741a	2400a	2240a	2721b	3790a	2605abc	1845c	2184a	-	1784b	-
	sd	774	889	485	1351	288	642	1253	333	220	-	150	-
Mg	A	290a	190a	305a	175a	343b	450ab	362ab	601a	168a	-	140a	-
	sd	67	99	78	116	80	110	201	177	17	-	33	-
Cl	A	2830ab	2436b	2998ab	3234a	18798a	15243a	4688b	4643b	3804b	11136a	2900c	4513b
	sd	401	329	402	252	6268	3870	1211	1050	113	386	399	709
K	A	1652a	1792a	1750a	1801a	5991a	5410a	1762c	2342b	1865c	4938a	1617c	2399b
	sd	186	210	167	200	1331	548	318	225	73	122	205	372
Ca	A	220a	244a	278a	225a	7894b	23136a	4193c	12495a	195c	565a	153c	324b
	sd	26	43	106	21	486	10578	800	1197	14	30	28	55
Trace elements													
Mn	A	0.26a	0.24a	0.28a	0.32a	1.4c	2.4abc	2.1b	3.4a	2.4b	-	3.2a	-
	sd	0.03	0.04	0.03	0.15	0.3	1.3	0.3	0.7	0.1		0.1	-
Fe	A	3.4ab	3.1b	4.3a	BDL	58a	61a	11b	38a	19a	4.5c	13b	BDL
	sd	0.4	0.4	0.8	0.2	18	20	3	11	1	0.6	1	0.5
Cu	A	9.3a	7.5a	10a	6.7a	166a	53b	58ab	13c	12a	14a	8.1b	4.8c
	sd	2.1	0.9	3	1.1	112	3	11	7	1	0	0.5	0.5
Zn	A	55ab	59a	56ab	52b	80a	57a	21b	18b	90a	33c	72b	16d
	sd	4	3	5	4	13	15	4	2	4	1	3	1
Se	A	0.98a	0.92ab	1.1a	BDL	5.3a	3.5a	1.1b	BDL	2.6a	1.4c	1.8b	0.84d
	sd	0.14	0.19	0.3	0.12	2.1	0.9	0.5	0.13	0.2	0.1	0.1	0.07

Abbreviations: (SF) female crabs from the Scottish coast; (SM) male crabs from the Scottish coast; (EF) female crabs from the English channel; (EM) male crabs from the English channel; (A) average; (sd) standard deviation; (BDL) below detection limit. In each row different letters indicate significant differences in element concentration per tissue ($p < 0.05$).

The elemental composition of crabs' edible tissues from the same fishing ground and sex also varied considerably (Table 4.4.3). Generally, elements were more concentrated in hepatopancreas, except Mn (higher in female gonads of crabs from both locations), Zn (higher in male muscle and female gonads from crabs of the EC) and Se (higher in gonads from crabs of the EC). Earlier studies with blue crab, swim crab, Chinese mitten crab, king crab and shrimps reported comparatively higher Na, Ca and Cu, but lower Mg, K, Fe and Zn contents in hepatopancreas than in muscle (Jop *et al.*, 1997; Turoczy *et al.*, 2001; Gököolu and Yerlikaya, 2003; Kádár *et al.*, 2006; Küçükgülmez *et al.*, 2006; Chen *et al.*, 2007). Hepatopancreas is a midgut gland implicated in the digestion, storage and detoxification of several inorganic and organic contaminants, and in the regulation of several cations like Ca, Zn and Cu (Al-Mohanna and Subrahmanyam, 2001; Chavez-Crooker *et al.*, 2003; Zanotto and Wheathy, 2003; Luquet and Marin, 2004). Higher levels of Cu in hepatopancreas can reflect an accumulation of this element for incorporation into the respiratory pigment haemocyanin found in crustacean's haemolymph (Lee and Shiau, 2002).

The high levels of Mn, Se and Zn found in ovaries of *C. pagurus* are likely related to particular physiological needs of this tissue. Manganese has been shown to be essential in several animal species, including crustaceans, since at least two enzymes are known to contain Mn-pyruvate carboxylase (indirectly implicated in the production of ATP) and superoxide dismutase (anti-oxidative stress enzyme) (Goldhaber, 2003). Zinc is essential for the synthesis of nucleic acids that are required in the production of sexual cells and has an essential role in the synthesis and degradation of carbohydrates, lipids and proteins (FAO/WHO, 2002). Selenium is known to participate in the modulation of animal growth and development, and participates in the oxidative defence (FAO/WHO, 2002).

When the data set of seven elements' concentration was subjected to PCA (in order to include data from male gonads), using concentrations of elements as dependent variables, two factors with initial eigenvalues greater than one were found. Factors one and two yielded 82.5 % of explainable results, with Cl, K, Fe, Cu and Se loading heavily on the first factor, and Ca and Zn loading heavily in factor two (Figure 4.4.1). A factor scores plot for this data is presented in figure 4.4.1a, illustrating clear separations between fishing ground, tissues and sexes. PCA using all elements, excluding male gonads, was also analyzed (Fig. 4.4.2) and revealed a similar pattern (clear separation between fishing ground, tissues and sexes), in which Mg and Mn were correlated with elements loading heavily on factor two, while Na was correlated with elements loading heavily on factor one.

In both PCA, crabs' muscle from both fishing grounds and sexes are clustered together, while hepatopancreas are separated in four clusters (by sexes and origins) and gonads are separated by sexes. Female gonads are closer to the muscle cluster, while male gonads are closer to the female hepatopancreas from crabs caught off English Channel (Figure 4.4.1a). These results are certainly related to the elements intake route, which in aquatic invertebrates is either from seawater or food (Rainbow, 2002). The proportion from each intake route varies with the invertebrate physiological

needs/endogenous factors (e.g., sex, age, condition and tissue) and the bioavailability of the elements in water and diet, which is influenced by external environmental factors (e.g., season, location, substrate, depth, water salinity, temperature and anthropogenic influence) (Jop *et al.*, 1997; Wang and Fisher, 1999; Rainbow and Black, 2001; Turoczy *et al.*, 2001; Roast *et al.*, 2002; Ni *et al.*, 2005; Fabris *et al.*, 2006; Kádár *et al.*, 2006; Küçükgülmez *et al.*, 2006). The present study concerns two endogenous factors (sex and tissue) and one external factor (location).

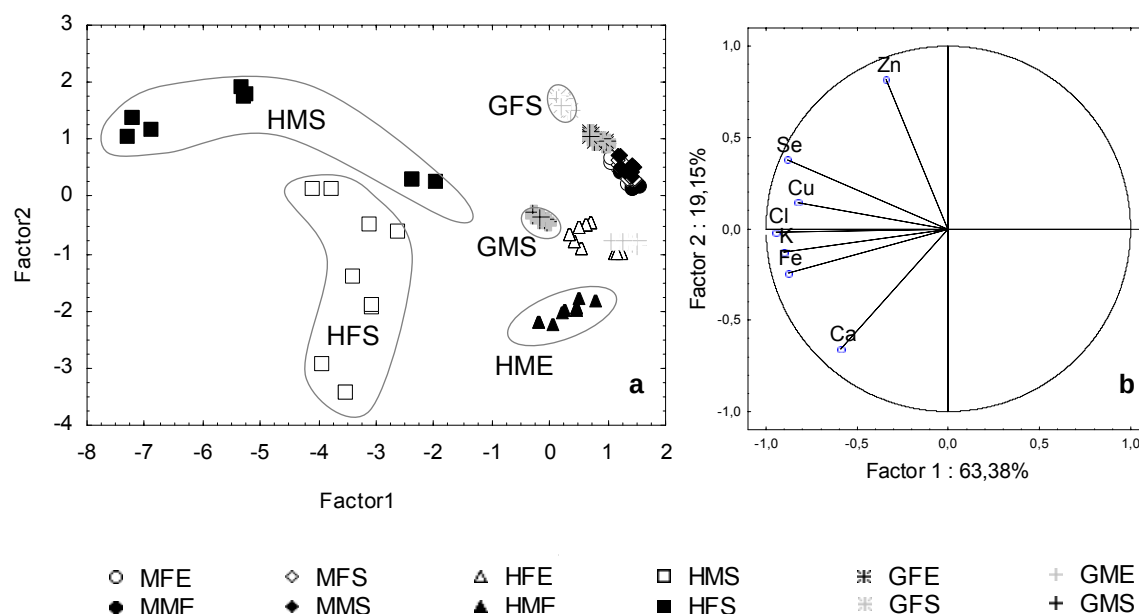


Figure 4.4.1 (a) Result of PCA analysis using concentrations of Cl, K, Ca, Fe, Cu, Zn and Se as the dependent variable; (b) The standardized component loadings for factor one and two of analysis 4.4.1a. Abbreviations: (MFE) muscle from female crabs of the English Channel; (MME) muscle from male crabs of the English Channel; (MFS) muscle from female crabs of the Scottish Coast; (MMS) muscle from male crabs of the Scottish Coast; (HFE) hepatopancreas from female crabs of the English Channel; (HME) hepatopancreas from male crabs of the English Channel; (HFS) hepatopancreas from female crabs of the Scottish Coast; (HMS) hepatopancreas from male crabs of the Scottish Coast; (GFE) gonads from female crabs of the English Channel; (GME) gonads from male crabs of the English Channel; (GFS) gonads from female crabs of the Scottish Coast; (GMS) gonads from male crabs of the Scottish Coast.

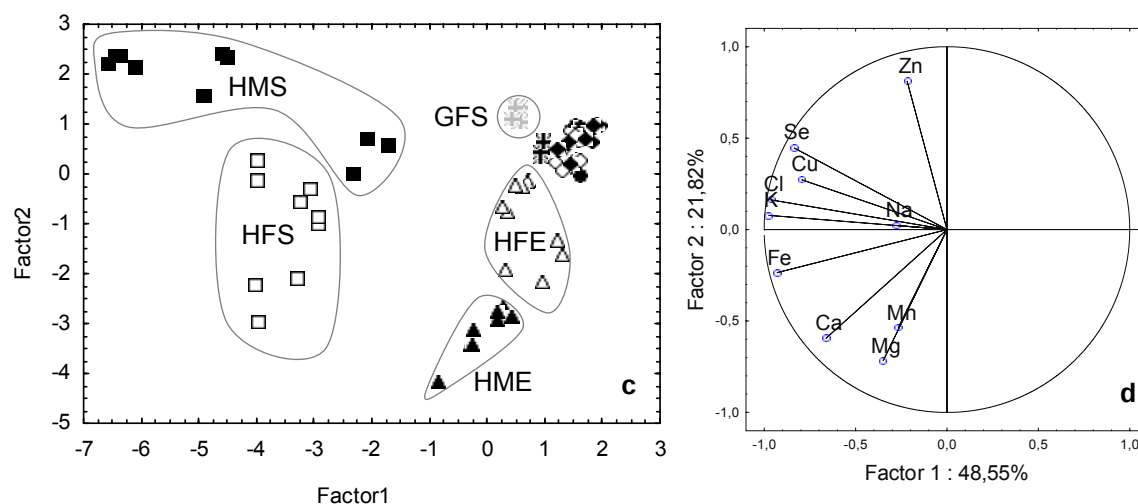


Figure 4.4.2 (a) Result of PCA analysis using concentrations of all ten elements as the dependent variable. (b) the standardized component loadings for factor one and two of analysis 4.2.2a. Male gonads of both locations are not presented in the PCA due to the lack of Na, Mn and Mg data. Symbols as in figure 4.4.1.

Comparison with daily intake recommendations

So far, most studies and nutrition tables only refer the muscle composition (excluding hepatopancreas and gonads), which is a considerable lack of information, especially considering the consumption habits and marketing profile of crustaceans. In order to fill this gap, the elements' concentration of each edible tissue was compared with the dietary reference intakes (DRI) set per day by the USA Food and Nutrition Board of the Institute of Medicine (IOM, 1997; 2001, 2004 and 2008). for adult females and males aged between 19 and 50 years (Table 4.4.4). All elements quantified in this study are essential to human health, thus a regular intake normally via food is vital (Oehlenschläger, 1997; Goldhaber, 2003). However, toxicity may occur when intakes are too high (Goldhaber, 2003). The average yield contributions of each edible tissue from *C. pagurus* of both fishing grounds are shown in table 4.4.4.

Crabs from both fishing grounds are excellent sources of macro (Na, Cl and Ca) and trace elements (Fe, Cu, Zn and Se). Generally, crabs harvested in the SC were better sources of most elements studied than animals from the EC, except Mg and Mn. Considering both sexes, females are better sources of Mg (muscle), Fe (muscle and gonads), Cu (muscle and hepatopancreas), Zn (hepatopancreas and gonads) and Se (all tissues), whereas males are better suppliers of Mg (hepatopancreas), Cl (gonads), K (gonads), Ca (hepatopancreas and gonads), Mn (hepatopancreas) and Fe (hepatopancreas). Hepatopancreas has greater contribution of most elements than muscle and gonads, except Na, Mn and Zn. Considering the elemental composition of common food items (dairy products, meat, fish, cereals and fruits), *C. pagurus* hepatopancreas is a good source of Ca, Fe, Cu, Zn and Se (FAO/WHO, 2002; Porto and Oliveira, 2006).

Particularly, male hepatopancreas is an excellent Ca supply, accounting for more than 100% of the daily intake recommendation. Calcium is nutritionally very important (up to 1.9 % Ca is available in human body), as it provides rigidity to the skeleton and plays a role in many metabolic processes (FAO/WHO, 2002). Iron has several vital functions in humans, such as carrier of oxygen to tissues by red blood cell haemoglobin, transport medium for electrons within cells, and part of important enzyme systems in various tissues (FAO/WHO, 2002).

Copper is found in several enzymes, including the cytochrome c oxidase and the superoxide dismutase, and is used for biological electron transport (Walker *et al.*, 2001). Zinc is an essential trace element for all living species, since is an important component of several enzymes (Goldhaber, 2003), and plays an essential role in a number of biological processes involved in growth and development (FAO/WHO, 2002). Selenium has an important role in the protection of body tissues against oxidative stress, against many toxicological effects of Cd and Hg, maintenance of defences against infection, modulation of growth and development (Skowerski *et al.*, 1997; FAO/WHO, 2002).

Table 4.4.4 Average percentage of macro and trace elements per 100 g portion of crabs caught off Scottish Coast (SC) and English Channel (EC).

Elements	DRI	Location	Muscle		Hepatopancreas		Gonads	
			F	M	F	M	F	M
Macro elements								
Na	1500 mg	SC	20	18	18	25	15	-
		EC	16	17	17	12	12	-
Mg*	(420-320) mg	SC	(7.0-9.1)	(4.6-6.0)	(8.2-11)	(11-14)	(4.1-5.4)	-
		EC	(7.3-9.5)	(4.4-5.7)	(7.0-9.1)	(15-19)	(3.4-4.4)	-
Cl	2300 mg	SC	12	11	82	66	17	48
		EC	13	14	20	20	13	20
K	4700 mg	SC	3.5	3.8	13	12	4.0	11
		EC	3.7	3.8	3.7	5.0	3.4	5.1
Ca	1000 mg	SC	2.2	2.4	79	231	2.0	5.6
		EC	2.8	2.2	42	125	1.5	3.2
Trace elements								
Mn	(2.3-1.8) mg	SC	(1.3-1.7)	(0.87-1.1)	(6.1-7.8)	(10-13)	(10-13)	-
		EC	(1.3-1.7)	(1.3-1.7)	(8.7-11)	(15-19)	(13-17)	-
Fe	(8-18) mg	SC	(4.3-1.9)	(3.9-1.7)	(73-32)	(76-34)	(24-11)	(5.6-2.5)
		EC	(5.4-2.4)	-	(14-6.1)	(48-21)	(16-7.2)	(3.0-1.3)
Cu	0.9 mg	SC	103	83	1843	594	130	151
		EC	116	75	644	149	90	53
Zn	(11-8) mg	SC	(50-69)	(54-74)	(73-100)	(52-71)	(82-113)	(30-41)
		EC	(51-70)	(47-65)	(19-26)	(16-23)	(65-90)	(15-20)
Se	0.055 mg	SC	178	168	966	633	467	262
		EC	197	-	207	-	323	154

Data considering the dietary reference intakes (DRI) set per day by the IOM (1997; 2001; 2004 and 2008). Values in brackets for Mg, Mn, and Zn refer to genders DRI: the first and second values correspond to adult males and females aged between 19 and 50 years, respectively; (*) data refer to female and male adults aged between 31 and 50 years. Abbreviations: (F) females; (M) males.

Despite the importance of these elements to human health, they can become toxic at high concentrations. In the present study, Ca, Cu, Zn and Se were above the DRI in some samples. Still, Ca and Zn were below the maximum daily intake that pose no risk to human health (UL; Ca: 2500 mg/day; Zn: 40 mg/day) set by the IOM (2008), Cu was below the permissible maximum tolerable daily intake for humans (PMTDI: 35 mg/day) set by the World Health Organization of the United Nations, and Se was below the highest dose level tested at which a critical adverse effect does not occur (NOAEL: 0.85 mg/day), set by the Environmental Protection Agency, USA (Goldhaber, 2003). Therefore, the consumption of edible crab does not constitute a health problem due to the high intake of Ca, Cu, Zn and Se.

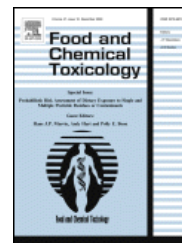
Conclusions

C. pagurus is an excellent source of macro (Na, Cl and Ca) and trace elements (Fe, Cu, Zn and Se). Despite crabs caught off the Scottish Coast have usually lower market price than animals from the English Channel, most of their elemental content was higher. Differences in the elemental composition of all tissues were also detected between sexes, as generally females had higher content of trace elements and male crabs had more macro elements. Generally, hepatopancreas showed higher content and variations of most elements than muscle and gonads. The differences found in the present study strongly suggest the need to take into consideration all edible tissues of crustaceans to evaluate their nutritional quality given consumers' habits.

Sub-chapter 4.5

Accumulation of non essential elements in *C. pagurus*: population differences

Barrento, S., Marques, A., Teixeira, B., Carvalho, M.L., Vaz-Pires, P., and Nunes, M. L. 2009. Accumulation of elements (S, As, Br, Sr, Cd, Hg, Pb) in two populations of *Cancer pagurus*: ecological implications to human consumption. Food and Chemical Toxicology 47: 150-156.



The brown crab *Cancer pagurus* is highly appreciated in Southern European countries and edible tissues are consumed separately or as mixtures. This species is mostly harvested along the Scottish Coast and English Channel and has different market prices depending on the catching area and sex. The aim of this study was to quantify and characterize the contents of S, As, Br, Sr, Cd, Hg and Pb in muscle, hepatopancreas, gonads and gills of female and male crabs from both catching areas. Additionally, the accumulation patterns were evaluated according to hazards for human consumption and from an ecological point of view. Crabs caught off the Scottish Coast had more S and As (gonads), while specimens from the English Channel showed more Cd (gonads) and Br (gonads and muscle). The elemental bioavailability and physiological needs likely explain these differences. Independently of catching area and sex, brown crabs' muscle and gonads are safe food items as far as contaminants are concerned. Yet, Cd in hepatopancreas was always above the level set by international regulating organizations. Consequently, future risk assessment studies should evaluate Cd concentration in all edible tissues of crustaceans prior to the extensive use of processed tissues in food products.

Keywords: *Cancer pagurus*, hepatopancreas, gonads, muscle, elements, hazards.

Introduction

It is globally accepted that seafood is important in a healthy and balanced diet (WHO, 2003). Seafood is an important source of valuable nutrients (e.g. long chain n-3 poly-unsaturated fatty acids, high quality proteins, vitamins and minerals) and is low in saturated fat (Kris-Etherton *et al.*, 2002; Nesheim and Yaktine, 2007). However, seafood can contribute to human exposure to chemical contaminants like As, Hg, Cd and Pb resulting from man-made processes or from natural deposits (Sioen *et al.*, 2008). As a consequence, severe health impacts can arise from consuming contaminated aquatic organisms (Hashmi *et al.*, 2002).

In Southern European countries (e.g. France, Spain, Italy and Portugal) the brown crab *Cancer pagurus* is widely consumed, particularly the white meat (muscle), brown meat (hepatopancreas and gonads). This species is mostly captured in the English Channel, Welsh, Scottish and Irish coasts, representing worldwide 84 % of its total captures (total: 37,480 t; data from 2006; EUROSTAT, 2007). Brown crabs have different market prices depending on the capture origin and animal sex: crabs caught off the English Channel and bigger males are usually more expensive (Brown and Bennett, 1980). These differences are related to importers' convictions that Scottish crabs are more susceptible to stress and, consequently, to higher mortalities, than crabs from the English Channel (Uglow *et al.*, 1986). On the other hand, male crabs have bigger claws with more meat yield than females, which is a crucial issue for retailers and restaurant managers.

Despite several published studies report the presence of contaminants in the tissues of crustaceans, only few evaluated their risks for human consumption (e.g. Turoczy *et al.*, 2001; Sivaperumal *et al.*, 2007), and none concerning the brown crab. Previous studies with marine crabs, like *Pseudocarcinus gigas*, reported that the accumulation of contaminants was strongly affected by the geographical location, sex and tissue (Turoczy *et al.*, 2001). Therefore, the aim of the present work was to study the presence of the elements S, Br, Sr, total As, Cd, total Hg and Pb in the muscle, hepatopancreas, gonads and gills of *C. pagurus* captured in the English Channel and in the Scottish Coast. Data were examined according to sex, tissue and capture location. The accumulation pattern was discussed considering the risks for human consumption and from an environmental point of view.

Material and methods

Biological Material

Twenty *C. pagurus* caught off the Scottish coast (SC; 10 females: 748 ± 25 g; 10 males: 751 ± 27 g) and twenty specimens from the English Channel (EC; 10 females: 829 ± 155 g; 10 males: 977 ± 104 g) were live purchased from a local importer and transported to the laboratory. Animals were kept under refrigerated conditions (~ 4 °C) during one hour to decrease their metabolism before being euthanized. The muscle (from claws), hepatopancreas, gonads and gills from each animal were individually separated and weighted. Each tissue was subsequently homogenized with a grinder (Retasch Grindomix GM200; 5000 rpm; material: PP cup and stainless steel knives) vacuum packed and stored at -20 °C, whereas the remaining was frozen at -80 °C and

subsequently freeze-dried for 48 h at -50 °C and low pressure (approximately 10^{-1} atm). Samples were powdered and stored at -20 °C under controlled moisture conditions (vacuum packed) until further analyses. Plastic tools were generally used to process the crabs in order to avoid any contamination from processing equipment with trace elements measured.

Elemental analyses

Two techniques were used: (a) energy dispersive X-ray fluorescence (EDXRF), to quantify S, total As, Br and Sr; and (b) flame atomic-absorption spectrometry (FAAS), to quantify Cd, total Hg and Pb. The EDXRF technique consists of an X-ray tube equipped with a molybdenum secondary exciter (EXTRA II A, Atomika Instruments, Temple). The characteristic radiations emitted by the elements in the sample were detected by a Lithium drifted Silicon [Si (Li)] detector, with 30 mm² active area and 8 µm beryllium window. The energy resolution was 135 eV at 5.9 keV and the acquisition system was a Nucleus PCA card. Quantitative calculations were made by the fundamental parameters method (Custódio *et al.*, 2003). The X-ray generator was operated at 50 kV, 20 mA and acquisition time of 1000 s. Each sample powder (~1 g) was pressed into pellets of 2 cm diameter without any chemical treatment. A minimum of three pellets (replicates) per sample were glued onto Mylar films, on sample holders, and placed directly in the X-ray beam. The spectrometer Varian Spectr AA 20 was used to quantify the contaminants Cd and Pb with the FAAS technique, according to the official analytical methods (AOAC, 2005). The procedure is based on sample incineration (10 g) and dissolution of tissues in HNO₃. A minimum of three replicate analyses was performed per sample. Concentrations were calculated from linear calibration plots obtained by measurement of standard solutions absorbance: Cd(NO₃)₂ and Pb(NO₃)₂ (Merck; 1 g L⁻¹ dissolved in 0.5 M HNO₃). Total Hg was measured in triplicate with an AMA 254 Mercury Analyser spectrometer that uses the mercury vapour generation technique. The procedure is based on dry sample decomposition (hepatopancreas, 3 mg; muscle, 6 mg; and gonads, 10 mg), by combustion, preconcentration of mercury by amalgamation with gold and atomic absorption spectrometry. Concentrations were calculated from linear calibration plots obtained by measurement of the absorbance of an Hg standard solution (Merck; 1 g⁻¹ dissolved in 0.5 M HNO₃).

Accuracy tests

Accuracy was checked through analysis of certified biological material: oyster tissues (SRM 1566; United States National Bureau of Standards; EDXRF); freeze dried animal blood (IAEA-A-13, International Atomic Energy Agency; EDXRF); and non defatted lobster hepatopancreas (TORT-2; National Research Council of Canada; FAAS). The detection limits (DL) of each element (Table 4.5.1) were determined by two means: (a) EDXRF, with the signal-to-noise approach, where the equipment compares the signal of each element with blank samples and established the minimum concentration at which the element is reliably detected; (b) FAAS, with the residual standard deviation (RSD) of the response and the slope (S) of the calibration curve of each standard solution

used ($DL=3.3 \times RSD/S$). The FAAS technique requires large amount of sample, which was not possible in gonads and roe, therefore data of Cd, Hg and Pb are not shown for these tissues.

Table 4.5.1 Elemental concentration ($\mu\text{g g}^{-1}$ DW; $n=4$) and detection limits of certified reference material ($\pm$ sd) analyzed by FAAS and EDXRF.

Element	Technique	Certified Biological material	Present work	Certified value	Detection limit
S ^(c)	EDXRF	oyster tissues (SRM 1566)	8200 $\pm$ 500	7600 ^(d)	100
As ^(c)	EDXRF		13 $\pm$ 1	13 $\pm$ 2	1
Br ^(e)	EDXRF	Freeze-dried animal blood (IAEA-A-13)	22 $\pm$ 2	22 $\pm$ 3	0.8
Sr ^(c)	EDXRF	oyster tissues (SRM 1566)	9.9 $\pm$ 0.8	10 $\pm$ 1	0.8
Cd ^(f)	FAAS	lobster hepatopancreas (TORT-2)	27 $\pm$ 0	26.7 $\pm$ 0.6	0.01
Hg ^(f)	FAAS		0.28 $\pm$ 0.00	0.27 $\pm$ 0.06	0.02
Pb ^(f)	FAAS		0.35 $\pm$ 0.06	0.35 $\pm$ 0.13	0.02

(c) oyster tissues (SRM 1566; EDXRF); (d) non-certified values provided by United States National Bureau of Standards; (e) freeze-dried animal blood (IAEA-A-13; EDXRF); (f) lobster hepatopancreas (TORT-2; FAAS).

Potential hazards to consumers

To evaluate the potential hazards of brown crab consumption, the concentration of contaminants (As, Cd, Hg and Pb) in each tissue and in whole crab were compared to the action level (AL; set for crustaceans by the United States Food and Drug Administration, USFDA) and to the maximum level (ML; set for crustaceans' muscle by the European Commission, EC). The action level represents the value at which the USFDA take legal action to remove contaminated products from the market. The results are presented as percentage using the following equations:

a) ratio of a contaminant in a particular tissue

$$(\%) = (\text{contaminant concentration in the tissue/ML or AL}) \times 100;$$

b) ratio of a contaminant in whole crab

$$(\%) = [(\text{muscle yield} \times \text{contaminant concentration in muscle}) + (\text{hepatopancreas yield} \times \text{contaminant concentration in hepatopancreas}) + (\text{gonads yield} \times \text{contaminant concentration in gonads})] / (\text{ML or AL}) \times 100.$$

c) tissue yield = (tissue wet weight)/(body wet weight)

Statistical analysis

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (sd). Differences in elements' concentration between sex, capture origin and tissues were tested with analysis of variance (ANOVA) followed by multiple comparisons test (Tukey HSD). Whenever necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, differences were analysed with non-parametric analysis of variance (Kruskal-Wallis) followed by non-parametric multiple comparisons test (Mann-Whitney). Principal component analysis (PCA) was also employed to reduce the multidimensional data sets of the several elements to lower dimensions, thus simplifying

the presentation and interpretation of data. All statistical analyses were tested at 0.05 level of probability with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Results

Elements in female and male crabs from both locations

Brown crabs from both locations had different meat yields according to tissue and sex. Male crabs' muscle yield (50.7 % in SC, 52.5 % in EC) was greater than females (38.3 % in SC, 42.4 % in EC). On the other hand, male crabs had less gonads' yield (6.0 % in SC, 4.6 % in EC) than females (15.4 % in SC, 14.2 % in EC), and hepatopancreas' yield was similar in males (43.3 % in SC, 42.9 % in EC) and females (46.3 % SC, 43.3 % in EC).

Among the seven elements studied, S was the most abundant element on a wet weight basis in all crabs' tissues ranging between 1073 and 3061 $\mu\text{g g}^{-1}$ (Figure 4.5.1), whereas Pb was the lowest element (below the detection limit, except in gills; results not shown in Figure 4.5.1). Male crabs of both populations had more Pb in gills than females ($0.21 \pm 0.02 \mu\text{g g}^{-1}$ in SC males and $0.34 \pm 0.03 \mu\text{g g}^{-1}$ in EC males; $0.14 \pm 0.01 \mu\text{g g}^{-1}$ in SC females and $0.11 \pm 0.01 \mu\text{g g}^{-1}$ in EC females).

Statistical differences in crabs' elemental concentrations were found between tissues, capture origins and sex. In general, *C. pagurus* had more Br and Pb in gills ($p < 0.05$); higher Sr and Cd contents in hepatopancreas ($p < 0.05$); and more Hg in hepatopancreas and muscle ($p < 0.05$) (Figure 4.5.1).

Within tissues, a PCA analysis was applied to differentiate capture origins and sex (Figure 4.5.2). Clear cluster separation is evidenced in gonads (Figure 4.5.2A) and gills (Figure 4.5.2B), where factors one and two yielded a total of 89 and 77 % of explainable results, respectively. Gonads PCA shows four distinct clusters with all elements loading heavily in factor one, except Sr. These results are corroborated by the significant differences ($p < 0.05$) in elements' concentration found in gonads, as crabs caught off the SC had more S and As but less Cd than crabs caught off the EC (Figure 4.5.1). Also, in gonads, male crabs from EC had low As and Hg, while female crabs from SC had high Br and Sr.

Gills PCA shows four distinct clusters corresponding to females and males of both populations (Figure 4.5.2B) with all elements loading heavily on factor one, except As and Hg. Significant differences ($p < 0.05$) found in gills elemental concentrations confirm this pattern (Figure 4.5.1), as females from both locations had more Cd but less Br and Pb than males. Additionally, in gills, EC females had more Hg, but less Pb and As than SC females, whereas EC males had more Pb and less Hg than SC males.

In muscle and hepatopancreas the only evident cluster corresponds to males from the EC (Figure 4.5.2C and D), where factors one and two yielded a total of 69 % and 65 % of explainable results, respectively. In both tissues all elements loaded on factor one, except S and Sr (muscle) and S and Cd (hepatopancreas). ANOVA results confirm PCA analyses in both tissues. In fact, in muscle, EC male crabs had less As and Hg, but more S and less Cd than females; EC crabs had also more Br

than SC specimens ($p < 0.05$; Figure 4.5.1). As to hepatopancreas, EC male crabs had low As and Hg, and male crabs had more Sr than female specimens (both populations; Figure 4.5.1).

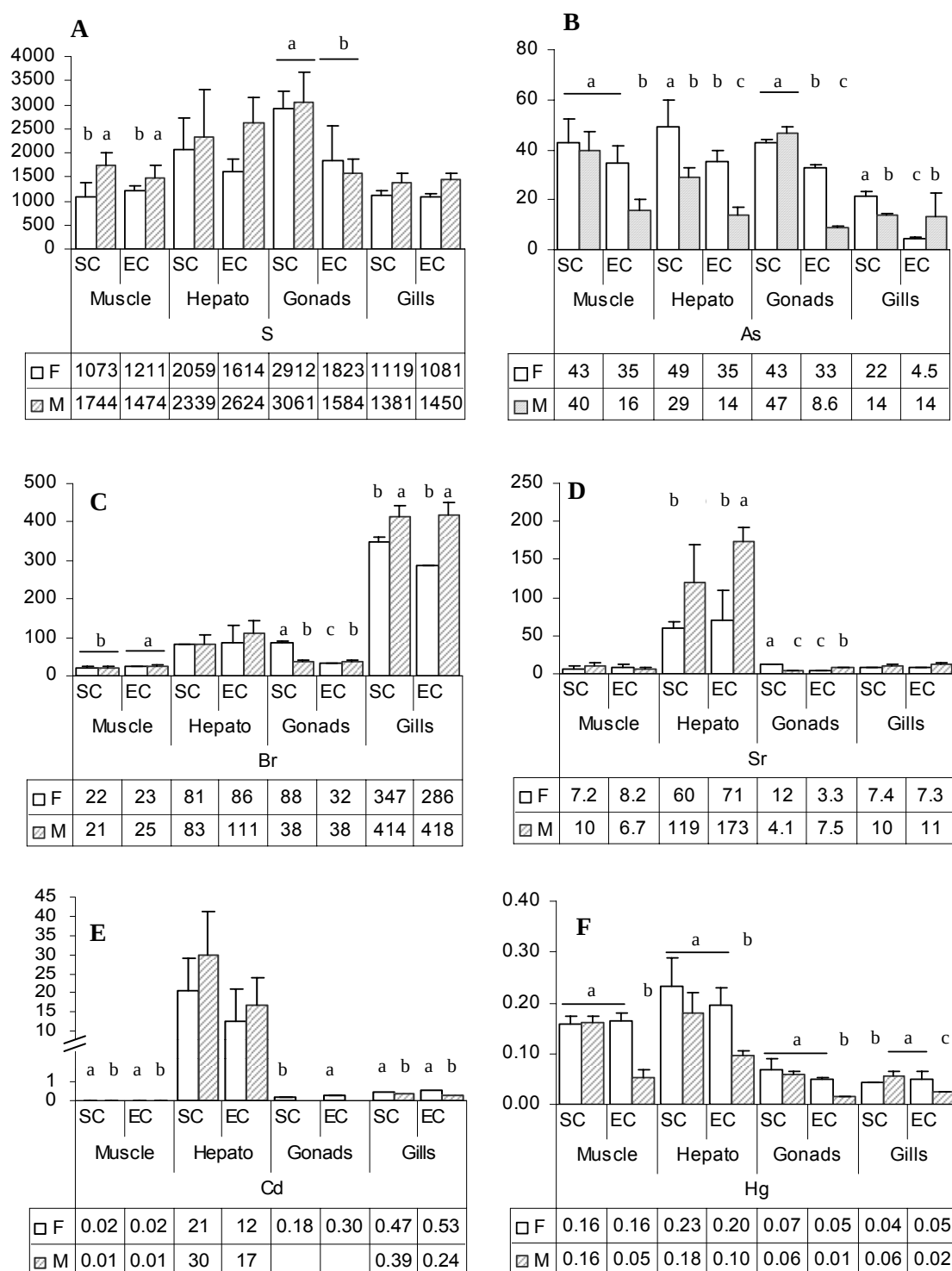


Figure 4.5.1 Mean elemental composition ($\mu\text{g g}^{-1}$) in the edible tissues of female (F, blank bars) and male (M, striped bars) *C. pagurus* from the Scottish Coast (SC) and English Channel (EC). (A) sulphur; (B) arsenic; (C) bromine; (D) strontium; (E) cadmium; (F) mercury. Different letters correspond to statistical differences ($p < 0.05$) for each tissue.

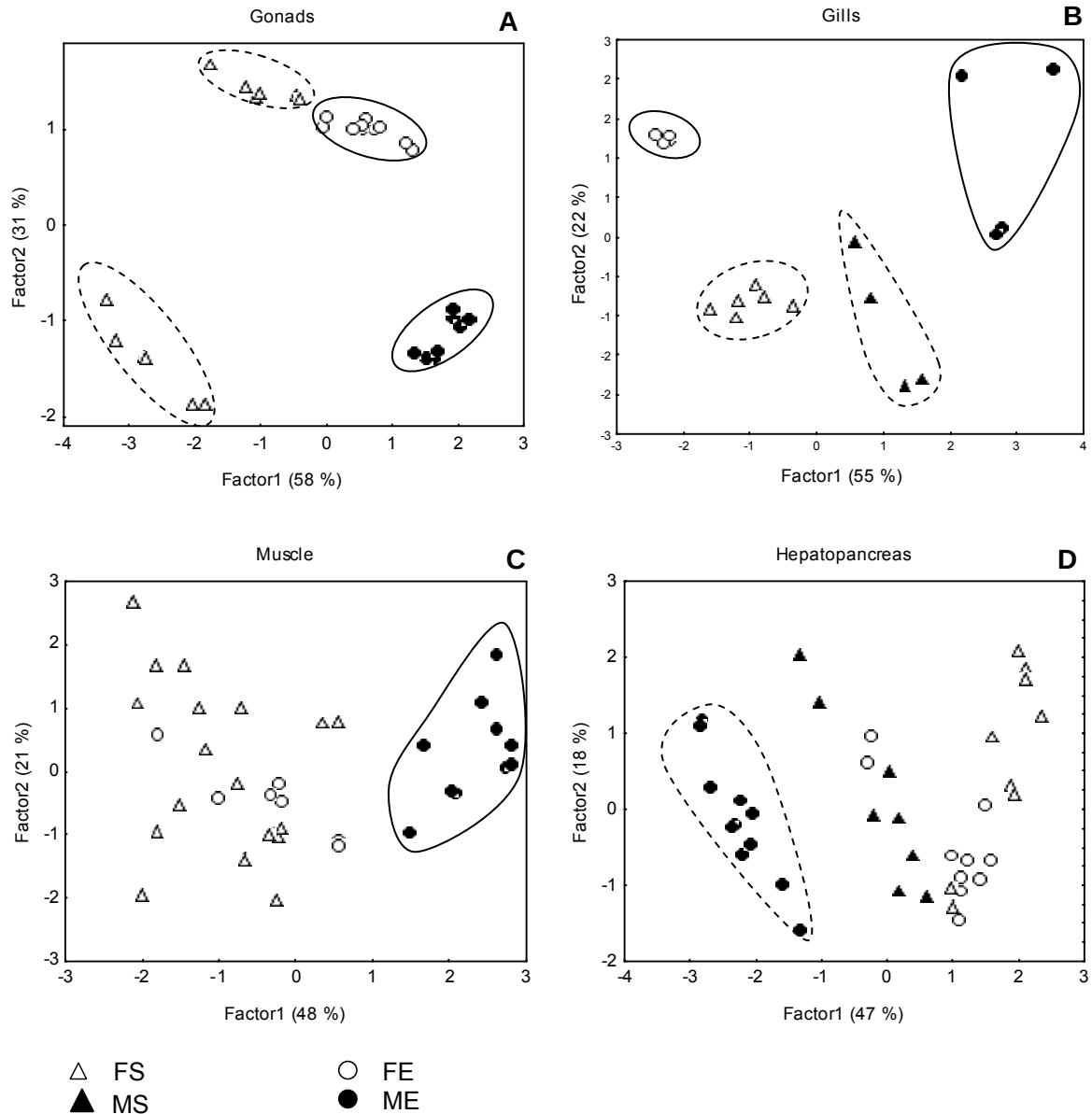


Figure 4.5.2 Result of PCA analyses using the concentrations of all elements. (A) gonads; (B) gills; (C) muscle; and, (D) hepatopancreas. Abbreviations: (FS) female crabs from the Scottish Coast; (MS) male crabs from the Scottish Coast; (FE) female crabs from the English Channel; (ME) male crabs from the English Channel. Solid lines indicate clusters of crabs from the English Channel, while dot lines indicate clusters of crabs from the Scottish Coast. Cadmium and Pb were not considered in gonads PCA analysis since both elements were not determined in testis due to insufficient amount of sample. Lead was not considered in muscle and hepatopancreas PCA analyses since values were below the detection limit.

Potential hazards to consumers

The concentrations of contaminants in each edible tissue and in whole crab were compared to the maximum level (ML) and action level (AL) and the results are presented in table 4.5.2. Cadmium concentration in hepatopancreas of male and female crabs of both capture origins was the only contaminant above the level set by both regulating organizations (EC and USFDA). Even Cd content in a mixed portion (total edible tissues) was well above the ML and AL, despite Cd values in gonads and muscle were relatively low compared to the limits imposed by these regulating agencies.

Table 4.5.2 Ratio between the content of As, Cd and Hg in the edible tissues (muscle, hepatopancreas, gonads and whole crab) of *C. pagurus* and the maximum level (ML) set by the European Commission for crustaceans' muscle, or the action level (AL) set by the USFDA for the food commodity Crustacea, data labelled with*.

Elements	ML/AL	Muscle (%)				Hepatopancreas (%)				Gonads (%)				Total edible tissues (%)			
		SF	SM	EF	EM	SF	SM	EF	EM	SF	SM	EF	EM	SF	SM	EF	EM
Arsenium (As)	76 ppm*	57	53	46	21	64	38	46	18	57	50	43	11	60	46	46	19
Cadmium (Cd)	0.5 ppm	4.0	2.0	4.0	2.0	4120	6004	2400	3400	36	-	60	-	1915	2601	1049	1460
Cadmium (Cd)	3 ppm*	0.67	0.33	0.67	0.33	687	1001	400	567	6.0	-	10	-	319	433	175	243
Mercury (Hg)	0.5 ppm	32	32	33	11	46	36	39	19	14	12	10	3.0	36	33	32	14
Mercury (Hg)	1 ppm*	16	16	16	5.3	23	18	20	10	6.8	6.0	5.0	1.5	18	16	16	6.9

Values are expressed as percentage. Abbreviations: (SF) female crabs from the Scottish coast; (SM) male crabs from the Scottish coast; (EF) female crabs from the English Channel; (EM) male crabs from the English Channel.

Discussion

Element composition in crabs from the English Channel and Scottish Coast

The elemental accumulation in aquatic animals depends on the intake route, which can be: (1) food, via hepatopancreas in crustaceans; or (2) seawater, over the permeable part of the exoskeleton, namely the gills in crustaceans (Rainbow, 2002; Silvestre *et al.*, 2004). The proportion of each intake route is related to the crabs' physiological needs/endogenous factors (e.g. sex, age, condition and tissue) and to the elements bioavailability in seawater and diet, which is influenced by external environmental factors (e.g. season, location, substrate, depth, salinity, temperature and anthropogenic pressure; Rainbow, 2002). In the present study, two endogenous factors (tissue and sex) and one external factor (geographical location) were analysed.

Sulphur had the highest concentration in crabs of both origins compared to the other elements (Figure 4.5.1). Sulfur is a major component commonly found in seawater (~28 mM), and an essential macroelement present in the body of animal tissues (in levels of mg g⁻¹ dry weight) as a constituent of amino acids (e.g. methionine, cysteine and taurine), proteins, enzymes, vitamins and other biomolecules (Komarnisky *et al.*, 2003; Hannelore *et al.*, 2008). The S concentrations in the edible tissues of brown crab is in the same magnitude of those found in clawed lobsters, *Homarus gammarus* and *H. americanus* (1695-1716 ppm in muscle, 1955-2029 ppm in hepatopancreas and 1320-1696 ppm in gonads; Sub-chapter 4.1), thus indicating that this element is ubiquitous in the crustaceans tissues. In addition, S might also be implicated in the sequestering of toxic elements, like Cd, by S-donating groups on ligands located in hepatopancreas (Lyon *et al.*, 1984).

Apart from S, all remaining elements analysed are not considered to be essential to brown crabs and most probably do not have any physiological role. The accumulation pattern of Br, Sr, Cd, Hg and Pb in brown crab from both locations seemed to be mostly influenced by the type of tissue and sex, whereas the accumulation of S and As was mainly influenced by the geographical location and sex. Most aquatic animals absorb elements via gills and intestines, which are transferred to blood and other parts of the body (Wu and Chen, 2005). Gills are the major entry site of elements in crustaceans and act as transient store for few (Soegianto *et al.*, 1999). In the present study, Br and Pb were more concentrated in gills and not heavily accumulated in the remaining tissues (Figure 4.5.1). To our knowledge, so far only one study analysed Br in the muscle of crabs, *Scylla serrata* and *S. tranquebarica* (Mohapatra *et al.*, 2007), and detected higher values (152–295 ppm) than those found in *C. pagurus* (21-25 ppm).

Lead is a contaminant and non essential element, usually detected at low concentrations in crustaceans (Sivaperumal *et al.*, 2007). The low Pb level in crustaceans' tissues is likely due to its accumulation in the crabs' exoskeleton via the same processes as Ca (Barwick and Maher, 2003). Mercury was found preferentially in hepatopancreas and muscle of *C. pagurus*. The only study performed so far evaluating Hg among crab tissues reported higher Hg content in the muscle of *Pseudocarcinus gigas* than in hepatopancreas (Turoczy *et al.*, 2001). On the other hand, Sr and Cd were mostly accumulated in the brown crab hepatopancreas, an organ that regulates cations and heavy metals by sequestering these elements and thereby reducing their load in haemolymph and

other tissues (Chavez-Crooker *et al.*, 2003). To our knowledge, there are no studies reporting Sr concentration in crabs' hepatopancreas. Studies performed with muscle of *S. serrata* and *S. tranquebarica* (Mohapatra *et al.*, 2007) revealed Sr values in the same range of concentrations obtained for *C. pagurus* in the present study (7-10 ppm). As to Cd concentration, several studies identified an accumulation pattern in crabs' hepatopancreas, like *Carcinus maenas* and *C. pagurus*, (Davies *et al.*, 1981; Legras *et al.*, 2000; Bjerregaard *et al.*, 2005; Karouna-Renier *et al.*, 2007).

Considering each crab tissue, striking differences were observed between sexes. Females had higher concentrations of As (hepatopancreas) and Cd (muscle and gills), but lower levels of S (muscle), Sr (hepatopancreas), Br and Pb (gills). So far, few studies have considered differences in the elemental composition of crabs according to sex: e.g. no major differences were found in Cd, Hg and Pb for *Callinectes spp.* and *Tachypleus tridentatus* (Kannan *et al.*, 1995; Sastre *et al.*, 1999); in *S. serrata* and *S. tranquebarica* females had higher Pb content than males, while no major differences were detected in Br and Sr (Mohapatra *et al.*, 2007). Differences in the elemental concentration between male and female crabs can be due to several factors, such as growth rates, as the growth rate of adult male crabs is lower than adult females (Davies *et al.*, 1981), metabolism and feeding habits (Woll *et al.*, 2006; Mohapatra *et al.*, 2007).

Regarding locations, brown crabs caught off the EC had higher Cd (gonads) and Br (muscle), while crabs from the SC had higher S and As in gonads. Particularly, male crabs from the SC had more As and Hg than male crabs from the EC. Such differences likely reflect distinct availability of contaminants in both locations. Changes of As and Hg levels in crustaceans according to capture origin have been reported elsewhere as a consequence of anthropogenic input of these contaminants (Fabris *et al.*, 2006; Karouna-Renier *et al.*, 2007). However, high As levels in crabs from uncontaminated areas have also been reported (Barwick and Maher, 2003), particularly those exhibiting benthic feeding behaviour, like *C. pagurus*, while pelagic crab species usually have lower As content (Rattanachongkiat *et al.*, 2004). The reason for such pattern is still unknown.

Potential hazards to consumers

The concentration of As ($16\text{--}49\ \mu\text{g g}^{-1}$), Cd ($10\text{--}20\ \mu\text{g g}^{-1}$), Hg ($0.6\text{--}0.16\ \mu\text{g g}^{-1}$) and Pb ($<0.1\ \mu\text{g g}^{-1}$) obtained in the muscle was in the range of the typical concentrations found in crustaceans (Francesconi, 2007). Despite crabs' hepatopancreas and gonads are widely consumed all over the world, the European Commission has only set ML for crustaceans' muscle. This study indicates that Cd concentration in the hepatopancreas of *C. pagurus* was much above the ML and AL, which can represent a concern for heavy consumers. Cadmium exerts a variety of toxic effects, including nephrotoxicity, osteoporosis, neurotoxicity, carcinogenicity, genotoxicity, teratogenicity, endocrine disruptions and reproductive effects (EFSA, 2004). The high hepatopancreas yield and its high Cd content certainly advocate that future studies concerning health risks to heavy consumers should analyse Cd in all edible tissues of brown crab. In addition, risk assessment analyses should be performed prior to the extensive use of processed crustaceans' hepatopancreas in food products. Generally, total As and Hg concentrations were higher in all tissues of female crabs compared to

males, though always below the ML and AL. Arsenic from seafood is mainly available in the organic form as the non-toxic arsenobetaine (97-99 %), while As in the inorganic form (the most toxic) can damage DNA and cause cancer (EFSA, 2004). On the other hand, mercury in seafood is usually accessible in the organic form as methylmercury (above 90 %) which is the most toxic form, and can affect the brain and nervous system (WHO, 2000; Francesconi, 2007).

Few studies have focused on concentrations of S, Br and Sr in seafood. However, the scientific interest in these elements is growing. All living specimens need S as this element is essential to amino acids, proteins and enzymes (Walker *et al.*, 2001). However, high intake of S compounds can represent a potential risk to human health, causing bronchitis, bronchoconstriction, and increased pulmonary resistance (Komarnisky *et al.*, 2003; Calace *et al.*, 2005). Bromine exists exclusively as Br salts that are accumulated in sea water (85 ppm) due to leaching from rocks and has been found in lipids of marine organisms (Tinsley and Lowry, 1980), however its essentiality or toxicity has not yet been tested. Strontium has an important role in bone cells and bone metabolism (Delannoy *et al.*, 2002). Considering that there is still a lack of information regarding the essentiality and toxicity of S, Br and Sr, and considering that these elements are commonly found in marine organisms, there is an urgent need to characterize these elements in seafood and to evaluate their impact to human consumption.

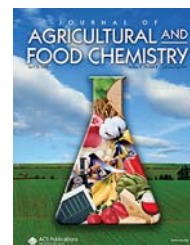
Conclusions

The present study highlights differences in the elemental composition of crabs according to capture origin, sex and tissue. Such differences are likely related with the elemental bioavailability and crabs' physiological needs. *C. pagurus* muscle and gonads were considered to be safe food items. Yet, Cd content in hepatopancreas was above the ML and AL, which can be a concern for heavy consumers. Therefore, future health risk assessment studies for heavy consumers of crustaceans should consider all edible tissues and the extensive use of processed crustaceans' hepatopancreas in food products. Additionally, there is an urgent need to evaluate essentiality and toxicity of S, Br and Sr in aquatic organisms due to the lack of information.

Sub-chapter 4.6

C. pagurus biochemical composition: seasonal changes

Barrento, S., Marques, A., Teixeira, B., Anacleto, P., Vaz-Pires, P., and Nunes, M. L. 2009. Effect of season on the chemical composition and nutritional quality of the edible crab *Cancer pagurus*. Journal of Agricultural and Food Chemistry, 57: 10814-10824.



Cancer pagurus is most appreciated in southern Europe for its muscle and brown meat content. In Portugal, consumption occurs mostly in summer and at Christmas. In this study the seasonal nutritional quality of edible tissues of female and male *C. pagurus* was determined. Tissue composition was affected by season and sex. All tissues had a well-balanced essential amino acid composition. Muscle and gonads of females had n-3/n-6 ratios in the range of the usual recommended values, and all tissues had PUFA/SFA above the recommended level. Autumn was the season with the highest brown meat yield, total essential amino acids (muscle), taurine (all tissues), EPA (male gonads), and n-3/n-6 ratio (gonads). However, it was also the season with the highest fat content and cholesterol concentration in ovaries. Therefore, people with restricted diets should moderate the consumption of ovaries in autumn. The remaining tissues pose no risks with respect to their proximate chemical composition.

Keywords: hepatopancreas, gonads, cholesterol, index of atherogenicity, index of thrombogenicity, seafood, crustaceans.

Introduction

In recent years, there has been a notable promotion of seafood consumption, based on its importance as part of a healthy diet, mainly due to its content of high-quality protein, low saturated fat, and high omega-3 fatty acids (Simopoulos, 1986). Among seafood, crustaceans are nutritionally valuable sources of protein and minerals, but they have often been rejected as a healthy food due to their presumably high levels of cholesterol (Mathew *et al.*, 1999). Nevertheless, crustaceans are widely appreciated and, in particular, the European edible crab, *Cancer pagurus*, is one of the most important commercial crabs targeted by fishing fleets throughout Britain (31,079 t), Ireland (11,525 t), Norway (8,510 t), and France (5,724 t) (Tully *et al.*, 2006; Eurostat, 2009). The major markets are located in France, Spain, Portugal, and Italy, where the muscle (white meat from claws, legs, and abdomen) and brown meat (hepatopancreas and reproductive organs) are much appreciated. In Portugal, *C. pagurus* is the most marketed live crustacean, crabs are consumed all year round but with consumption peaks during the summer holidays and Christmas festivities (Sub-chapter 2.1). Also, larger males are traditionally more expensive than smaller males and females (Brown and Bennett, 1980). In their habitats, crustaceans are exposed to a large number of environmental variables, which follow annual and daily cycles according to the geographical region and that affect their behaviour, migration patterns, feeding, and metabolism. Therefore, biological responses to seasonal variations may have a significant effect on the crustaceans' biochemical composition, particularly in hepatopancreas and gonads (Buckup *et al.*, 2008).

Because the nutritional data of this species is still incomplete, the purpose of this study was to determine the seasonal nutritional quality of *C. pagurus* by determining the proximate chemical composition, amino acid and fatty acid profiles, and cholesterol content of males and females. Moreover, considering the controversy of the benefits related to shellfish consumption and the potential impact in consumer's diet regarding the different edible tissues and consumption seasonality, samples were taken during the four seasons. Data was also analyzed to evaluate the nutritional quality in human consumption by calculating the index of atherogenicity (IA) and of thrombogenicity (IT), the essential amino acid scores (AS), and two ratios related with fatty acid content, namely the n-3/n-6 and PUFA/SFA (polyunsaturated fatty acids/saturated fatty acids).

Material and methods

Ethical statement

All live animals utilized in the experiments have been treated with proper care, minimizing discomfort and distress, and were painlessly killed. Also, the number of animals was kept to the minimum necessary to obtain scientific results, considering that the gain in knowledge and long-term benefit to the subject species are high.

Biological material

C. pagurus caught in the Scottish coast was sampled during spring (May, $n = 24$; 12 females, 12 males), summer (August, $n = 20$; 10 females, 10 males) and autumn (October, $n = 20$; 10 females, 10 males) of 2007, and winter (March, $n=20$; 10 males, 10 females) of 2008. Every season, intermoult crabs were live-transported to the laboratory. Animals were kept under refrigerated conditions ($\sim 4^{\circ}\text{C}$) during 1 h to decrease their metabolism and stunned before being euthanized by piercing the two nerve centers by means of a stainless steel rod. The rod was inserted through one of the eyes and through the vent as recommended in Codex Alimentarius, (FAO/WHO, 1983). For each crab several parameters were recorded: sex, total weight, gonad and hepatopancreas weight, carapace width and length, and maturity stage (only for females). The ovarian maturity scale was adapted from Edwards, (1979). Females were classified as follows: stage 1 (S1), immature, that is, no ovarian tissue was observed; stage 2 (S2), initial ovary development, that is, ovaries with a small spot of light orange found near the posterior part of the carapace; stage 3 (S3), developing orange ovaries extending into carapace; and stage 4 (S4), ripe ovaries, that is, carapace full of bright red ovary material. For both sexes, gonadosomatic (GSI) and hepatosomatic (HI) indices were calculated, as well as the claw muscle meat yield (MY) and the total meat yield (TMY). The GSI, HI, and MY were calculated as follows: $(\text{tissue wet weight} / \text{body wet weight}) \times 100$. The TMY was calculated according to the formula $(\text{gonad wet weight} + \text{hepatopancreas wet weight} + \text{claw muscle wet weight})/(\text{body wet weight}) \times 100$. For the biochemical analyses pools of two to three individual crabs were performed (depending on the amount of sample) taking into account sex, season, and maturity stage. These pools were homogenized with a grinder (Retasch Grindomix GM200; 5000 rpm; material: PP cup and stainless steel knives); a portion of this homogenized sample was vacuum packed and stored at -20°C , whereas the remaining was frozen at -80°C and subsequently freeze-dried for 48 h at -50°C and low pressure (approximately 10^{-1} atm). Samples were powdered and stored at -20°C under controlled moisture conditions (vacuum packed) until further analyses.

Proximate chemical composition and energy content

Moisture, ash, protein, and lipid contents were determined in each tissue according to the AOAC methodologies (AOAC, 2005). Briefly, the moisture content was obtained by drying the sample overnight at 105°C (laboratory heater, P-Selecta 207); ash was quantified after combustion for 16 h at 550°C (muffle furnace, Heraeus Hanau, TYPMR170); crude protein content was determined according to the Kjeldahl method using an automatic distillation and titration unit (VELP Scientifica, UDK152) with a conversion factor of 6.25; and total lipid was determined with the Soxhlet extraction method using ethyl ether. The energy content was estimated as protein, 4.27 kcal g^{-1} of wet weight; lipids, 9.02 kcal g^{-1} of wet weight ($1 \text{ kcal} = 4.184 \text{ kJ}$).

Cholesterol

The quantification of cholesterol content was based on the modified procedure of Naeemi *et al.* (1995). Each sample (300 mg of dry weight) was combined with 260 μL of the internal standard solution (5 α -cholestane Sigma; 5mg mL^{-1} cyclohexane, Merck), 3mL of saturated methanolic potassium hydroxide solution (2 M, Merck), and 3 mL of methanol pro analysis (Merck). Following heating (80 $^{\circ}\text{C}$; 30 min water bath), samples were cooled and supplied with 250 μL of magnesium chloride solution (1 M, Merck) and 1.5 mL of cyclohexane pro analysis (Merck). Samples were shaken and centrifuged (1500g; 4 min, centrifuge Sigma 2K15) until phase separation. The moisture content of the upper phase was removed with anhydrous sodium sulfate (Panreac). The cholesterol in the upper phase (2 μL) was separated by gas chromatography (Varian Star 3400 Cx) using helium as carrier gas at a flow rate of 1 mL min^{-1} in a flame ionization detector and a fused silica capillary CP-Sil 8 CB column (30 m length $\times$ 0.25 mm internal diameter, 0.25 μm film thickness; J&W Scientific). The temperatures of the oven, injector, and detector were 280, 285, and 300 $^{\circ}\text{C}$, respectively. Cholesterol was identified and quantified by comparison with the calibration curve of a pure cholesterol standard with 5 α -cholestane (Sigma). Cholesterol/cholestane peak area ratios obtained with the Varian software were plotted with cholesterol concentrations, and a straight line was fitted to data points by linear regression.

Fatty acid analysis

The percent distribution of fatty acids methyl esters (FAME) of nonpolar and polar lipids was based on the experimental procedure of Lepage and Roy (1986) as modified by Cohen *et al.*, (1988). Each sample (300 mg of dry weight) was dissolved in 5 mL of acetyl chloride/methanol (1:19 v/v; Merck), shaken, and heated (80 $^{\circ}\text{C}$; 1 h). After cooling, 1 mL of Milli-Q distilled water and 2 mL of n-heptane pro analysis (Merck) were added, and samples were shaken and centrifuged (2000g; 5min, Sigma 2k15) until phase separation. The moisture content of the upper phase was removed with anhydrous sodium sulfate (Panreac). An aliquot (2 μL) of the upper phase was then injected onto a gas chromatograph (Varian Star 3800 Cp) equipped with an autosampler and fitted with a flame ionization detector at 250 $^{\circ}\text{C}$ for FAME analysis. The separation was carried out with helium as carrier gas at a flow rate of 1 mL min^{-1} , in a capillary column DB-WAX (30 m length $\times$ 0.32 mm internal diameter; 0.25 μm film thickness; Hewlett-Packard) programmed at 180 $^{\circ}\text{C}$ for 5 min, raised to 220 at 4 $^{\circ}\text{C min}^{-1}$, and maintained at 220 $^{\circ}\text{C}$ for 25 min, with the injector at 250 $^{\circ}\text{C}$. FAME were identified by comparing retention times with those of Sigma standards. Quantitative data was calculated using the peak area ratio (percent of total fatty acids) and the Varian software.

Amino acids

To extract total amino acids (protein bound + free), 40-90 mg of sample was placed in 10 mL ampoules with 3 mL of 6 M HCl (Merck) with 0.1 % phenol (Merck), according to the method described by AOAC (2005). Ampoules were vacuum-sealed, and samples were hydrolyzed at 110 $^{\circ}\text{C}$ for 24 h; hydrolysates were filtered (0.45 μm pore size, Life sciences) and dissolved with Milli-Q distilled water to 20 mL. Samples were frozen at -80 $^{\circ}\text{C}$ and freeze-dried during 48 h at -50 $^{\circ}\text{C}$

under low pressure (approximately 10^{-1} atm). Samples were dissolved in 5 mL of 0.1 M HCl (Merck) and stored at -80°C until amino acid separation. After thawing samples were filtered (0.2 μm pore size Life sciences) and separation was performed with high-performance liquid chromatography (Agilent 1100 HPLC) using precolumn o-phthalaldehyde and 3-mercaptopropionic acid in borate buffer (OPA, Agilent Technologies) and 9-fluorenylmethyl chloroformate in acetonitrile (FMOC; Agilent Technologies) derivatization, a Phenomenex Gemini ODS C18 guard column ($4 \times 3\text{mm}$), and a Phenomenex Gemini ODS C18 110A column ($4.6 \times 150\text{ mm}$, $5\text{ }\mu\text{m}$). The solvents and gradient conditions were those described by Henderson *et al.* (2000). Detection wavelengths were set at UV 338 and 262 nm and fluorescence 340/450 and 266/305 nm. The identity and quantity of the amino acids were assessed by comparison with the retention times and peak areas of standard amino acids (Sigma- Aldrich) using norvaline as internal standard.

Nutritional quality

To measure the propensity of each edible tissue to influence the incidence of coronary heart disease, two indices: the Index of Atherogenicity (IA) and the Index of Thrombogenicity (IT) were calculated according to the Ulbricht and Southgate (1991) equations:

$$\text{IA} = [12:0 + (4 \times 14:0) + 16:0] / [(n-6 + n-3) \text{ PUFA} + \text{MUFA}];$$

$$\text{IT} = (14:0 + 16:0 + 18:0) / [(0.5 \times \text{MUFA}) + (0.5 \times (n-6) + 3 \times (n-3) + (n-3)/n-6)]$$

Where MUFA means monounsaturated fatty acids and PUFA, polyunsaturated fatty acids. Two ratios related with fatty acid content were also calculated n-3/n-6 and PUFA/SFA, in order to allow comparisons with the United Kingdom Department of Health recommendations (UKDH, 1994). Essential amino acid scores (AS) were also computed with respect to reference amino acid requirements for adults (FAO/WHO/UNU, 2007). This score compares the content of each essential amino acid in the protein/diet with its content in the requirement pattern: $\text{AS} (\%) = (\text{mg of amino acid in 1 g of test protein} / \text{mg of amino acid requirement}) \times 100$. The ratio of essential to non-essential amino acids was also calculated.

Statistical analysis

All analyses were carried out in duplicate. Results were expressed as mean values $\pm$ standard deviation (SD). Data was analyzed by one-way ANOVA followed by multiple-comparison test (Tukey HSD). Whenever necessary, data was transformed to satisfy normal distribution and homoscedasticity requirements. If transformed data could not meet these assumptions, differences were analyzed with nonparametric analysis of variance (Kruskall-Wallis) followed by a nonparametric multiple-comparison test (Mann-Whitney). Principal component analysis (PCA) was also employed to reduce the multidimensional data sets of several elements to lower dimensions, thus simplifying the presentation and interpretation of data. All statistical analyses were tested at the 0.05 level of probability with the software STATISTICATM 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Results

Proximate chemical composition and energy content

The biometric data of animals sampled in this study is presented in table 4.6.1. The meat yields are represented in table 4.6.2. In general, males had more muscle meat yield (10.3-13.6 %) than females (7.8-8.5 %) but less gonad meat yield (males, 0.9-1.9 %; females, 1.8-7.1 %), whereas in hepatopancreas the only difference was observed in winter females (7.8 %).

Table 4.6.1 Biometric data of female and male *C. pagurus* sampled in each season.

Sex	Season	Weight (g)	CW (mm)	CL (mm)	TMY (%)
Females	Spring	770±33	163.9±4.1	108.4±0.4	21.6±2.8
	Summer	748±25	163.3±2.3	107.4±1.8	22.5±3.8
	Autumn	770±153	167.5±18.0	107.7±7.7	27.6±6.4
	Winter	693±59	159.8±7.0	99.4±3.7	17.5±2.1
Males	Spring	828±44	160.9±4.7	100.2±2.1	25.6±2.4
	Summer	751±27	156.3±3.0	98.4±1.9	23.5±2.2
	Autumn	869±230	167.7±18.0	103.6±8.5	30.1±8.2
	Winter	650±75	153.7±6.3	93.4±3.4	21.1±3.5

Abbreviations: (CW) carapace width; (CL) carapace length; (TMY) total meat yield.

The meat yield patterns in gonads during the four seasons were similar for males and females, with increasing values from spring to autumn followed by a decrease in winter. The dominant maturity stage in autumn was S4 (100 % females), in winter S2 and S3 were found in equal proportions (50 %), and S3 was predominant in summer (40 %; remaining females were in S2 and S4 with 30 % each) and also in spring (58 %; remaining females were S2).

Significant differences were detected in the proximate chemical composition of edible tissues between tissues, seasons, and sexes (Table 4.6.2). In general, female gonads and muscle were rich in protein, whereas hepatopancreas had more fat. Gonads and hepatopancreas had higher energy and cholesterol contents compared to muscle. Female gonads showed significantly lower ash and moisture than male gonads, whereas no major differences were found in hepatopancreas and gonads. The protein content of muscle and hepatopancreas did not strongly vary through seasons (except in summer, muscle), whereas testis had a lower amount of protein than ovaries (except in winter). The fat content of muscle was significantly lower in winter, males (0.1 %) and in hepatopancreas it decreased in winter (both sexes). In ovaries, the fat content (3.1-5.6 %) was always higher than in testis (0.9-1.3 %), with maximum values obtained in winter in both sexes. The cholesterol content in hepatopancreas was constant throughout seasons, whereas muscle of crabs sampled in spring showed the highest values. The cholesterol content increased in ovaries from spring (120.5 mg 100 g⁻¹) to autumn (200.8 mg 100 g⁻¹) followed by a strong decrease in winter (93.7 mg 100 g⁻¹), whereas in testis there was a decrease in winter (65.0 mg 100 g⁻¹). Overall, ovaries always had more cholesterol than testis throughout the seasons. Statistical variations in the energy content were observed only in gonads, with ovaries showing higher energy content than testis regardless of season.

Table 4.6.2 Tissue indices (%): muscle meat yield, hepatossomatic, and gonadossomatic indices; proximate chemical composition (%); cholesterol content (mg g⁻¹ wet weight); and energy content (kJ 100 g⁻¹ wet weight) in muscle, hepatopancreas, and gonads of female and male crabs.

Sex	Season	Indices	Moisture	Ash	Proteins	Fat	Cholesterol	Energy
Muscle								
Female	Spring	8.1±0.5b	77.8±0.8a	2.0±0.2a	18.2±1.4ab	0.3±0.0a	46.0±3.6a	365.7±15.3a
	Summer	8.5±1.0b	77.8±2.1a	2.1±0.2a	16.4±4.2b	0.3±0.1a	37.3±4.7b	359.5±42.5a
	Autumn	7.8±1.4b	76.2±1.6a	2.1±0.2a	19.1±1.1ab	0.3±0.1a	41.3±1.7ab	370.6±73.8a
	Winter	7.9±1.1b	77.8±1.7a	2.1±0.1a	18.6±1.4ab	0.3±0.1a	35.7±5.5b	364.4±32.8a
Male	Spring	12.0±2.1a	76.0±2.0a	1.9±0.1a	19.2±1.8ab	0.3±0.1a	47.3±2.2a	398.9±39.4a
	Summer	11.9±2.2a	74.6±2.2a	1.9±0.1a	20.5±1.7a	0.2±0.1a	37.0±9.1b	399.0±43.0a
	Autumn	13.6±3.0a	77.4±2.6a	1.9±0.1a	17.3±2.9ab	0.3±0.2a	40.0±4.3ab	365.9±59.9a
	Winter	10.3±1.7b	76.6±2.0a	2.0±0.2a	18.7±1.9ab	0.1±0.1b	36.7±9.3ab	382.1±36.5a
p		<0.001	0.052	0.052	0.001	<0.001	<0.001	0.807
Hepatopancreas								
Female	Spring	11.1±2.4a	64.1±4.7a	3.2±1.1bc	11.6±1.2b	16.6±5.1a	110.6±33.8a	936.7±193.5a
	Summer	10.4±1.8a	67.9±7.0a	3.0±0.4c	12.2±1.6b	10.2±3.6ab	116.6±27.3a	700.1±220.5a
	Autumn	11.9±1.3a	64.3±3.7a	3.2±0.3b	14.5±1.8ab	10.8±2.0a	93.4±12.9a	790.9±88.1a
	Winter	7.8±1.1b	64.0±6.2a	3.5±0.3b	12.9±1.4ab	7.2±3.2b	104.3±11.7a	714.9±167.2a
Male	Spring	12.5±2.1a	59.3±4.4a	4.4±0.7b	14.5±0.9ab	14.3±3.0a	92.5±16.6a	926.4±130.9a
	Summer	10.2±2.2a	61.8±7.3a	5.0±1.2a	13.3±1.9ab	12.2±3.4a	112.4±10.1a	818.8±196.0a
	Autumn	10.5±2.6a	67.6±2.6a	3.8±1.8b	13.2±3.0ab	10.2±2.3a	104.0±27.1a	690.6±167.1a
	Winter	10.0±1.7a	60.3±3.8a	6.4±0.7a	15.8±2.1a	2.9±0.6c	94.1±12.5a	642.0±56.2a
p		<0.001	0.055	<0.001	0.002	<0.001	0.100	0.073
Gonads								
Female	Spring	2.4±1.0b	61.9±6.0c	1.5±0.0c	23.1±3.7a	3.8±1.5ab	120.5±7.7b	721.7±161.3a
	Summer	3.6±2.0ab	56.8±1.6c	1.6±0.0c	25.5±0.0a	3.1±0.5b	170.5±5.2a	804.6±196.3a
	Autumn	7.1±2.1a	56.5±2.5c	1.7±0.1c	24.8±5.9a	3.3±0.1b	200.8±15.3a	815.5±57.1a
	Winter	1.8±0.5b	68.2±5.5c	1.6±0.1c	19.2±3.4ab	5.6±2.4a	93.7±26.7c	640.4±128.6a
Male	Spring	1.1±0.5b	79.4±0.0a	2.9±0.0a	14.7±0.4c	0.9±0.1d	89.4±9.5c	331.7±42.2b
	Summer	1.4±0.7b	79.9±0.6a	3.0±0.0a	13.1±0.0c	0.9±0.0d	84.6±4.3c	268.0±22.7b
	Autumn	1.9±1.0b	80.4±0.1a	2.7±0.0b	13.9±0.1c	0.9±0.1b	83.4±0.9c	319.5±15.5b
	Winter	0.9±0.3c	77.0±0.5b	2.7±0.0b	14.7±1.9bc	1.3±0.0c	65.0±0.5d	387.1±59.9b
p		<0.001	0.001	<0.001	0.002	0.004	<0.001	0.003

In each column different letters indicate significant differences per tissue

Fatty acids

The fatty acid profiles were statistically different between sexes and seasons of the same tissue and between different tissues (Tables 4.6.3 and 4.6.4, respectively). Muscle and gonads had a similar pattern dominated by PUFA, followed by MUFA and SFA; in contrast, hepatopancreas had proportionally more MUFA, followed by PUFA and SFA. Muscle had the most homogeneous fatty acid composition with fewer differences between sexes and seasons compared to gonads and hepatopancreas (Table 4.6.3). In muscle the lowest and highest levels of SFA were obtained for females in winter and summer, respectively; MUFA values were generally lower in autumn and winter and higher in females during spring, and PUFA levels were constant throughout the year.

Table 4.6.3 Fatty acid profile (%) and nutrition quality parameters of edible tissues of female and male crabs during the four seasons.

Fatty acid	Females				Males				p
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	
Muscle									
14:00	1.2a	1.2a	1.2a	0.7b	1.2a	1.1a	0.8b	0.6b	<0.001
15:00	0.5a	0.5a	0.5a	0.5a	0.5a	0.6a	0.6a	0.6a	0.060
16:00	9.8a	9.5a	9.8a	7.9b	9.7a	9.6a	8.9a	9.6a	0.005
18:00	4.5ab	5.1a	4.8ab	3.8c	4.0bc	4.4ab	4.1b	3.6c	<0.001
SFA	16.6ab	17.4a	17.1ab	13.9c	16.2b	16.7ab	15.3b	15.4b	<0.001
16:1n-7	3.6c	4.2c	6.2b	3.7c	4.1c	4.6bc	5.1b	6.9a	<0.001
16:1n-5	0.5b	0.5ab	0.8ab	0.9a	0.5ab	0.6ab	1.0a	0.6ab	0.003
18:1n-9	17.6a	17.0ab	15.1bc	13.7c	17.2ab	16.8b	14.7c	14.7c	<0.001
18:1n-7	5.1ab	5.1ab	4.9ab	4.5ab	5.0ab	5.3a	4.5ab	4.4b	0.004
20:1n-9	2.0a	2.3a	1.0b	1.1b	1.8ab	2.0a	1.1b	0.8b	<0.001
MUFA	30.7a	31.9ab	30.4ab	26.6b	30.8ab	30.9ab	27.6b	28.4b	<0.001
16:3n-4	1.1ab	1.1ab	1.4ab	1.1ab	1.2ab	1.4a	1.0b	1.0ab	0.004
16:3-n3	2.7a	2.6a	0.8b	0.9b	3.0a	2.8a	1.1b	0.8b	<0.001
16:4n-3	0.4ab	0.2ab	0.5a	0.9a	0.5ab	0.2b	0.8a	1.0a	<0.001
18:2n-6	1.0ab	1.0ab	0.8ab	0.8b	1.0ab	1.1a	1.0ab	0.8b	<0.001
20:2n-6	1.0ab	1.3a	0.7b	0.9ab	0.8b	1.1ab	1.1ab	0.5b	<0.001
20:4n-6	6.7ab	7.0a	4.5b	5.9ab	7.2ab	7.5ab	6.4ab	7.3a	0.002
20:5n-3	21.0a	21.3a	20.6a	22.4a	19.1a	19.5a	20.6a	19.4a	0.062
22:5n-3	0.9a	1.1a	1.1a	1.3a	1.2a	1.2a	1.2a	1.2a	0.288
22:6n-3	11.2b	10.9b	12.2ab	13.8a	11.7ab	11.5ab	12.6ab	12.9ab	0.012
PUFA	47.8a	47.0a	43.5a	49.2a	47.9a	48.5a	47.8a	47.2a	0.054
n-3	36.4b	36.6ab	35.5ab	39.1a	35.6b	36.1b	36.1b	34.9b	0.007
n-6	9.2a	9.6a	6.9b	8.4a	9.9a	10.3a	9.4a	9.9a	<0.001
(n-3/n-6)	4.0b	3.8b	5.2a	4.7a	3.6b	3.8b	3.9b	3.5b	<0.001
PUFA/SFA	2.9b	2.7b	2.6b	3.5a	3.0b	2.9b	3.1ab	3.1ab	<0.001
IA	0.19a	0.18a	0.20a	0.14b	0.19a	0.19a	0.16ab	0.17ab	<0.001
IT	0.12a	0.12a	0.12a	0.09b	0.11a	0.12a	0.11ab	0.11ab	<0.001
Hepatopancreas									
14:00	3.7a	4.2a	4.6a	2.3b	3.2ab	3.3ab	3.3ab	1.7b	<0.001
15:00	0.7b	0.7ab	0.8ab	0.5b	0.8	0.8ab	1.1a	0.8ab	0.004
16:00	11.5bc	12.6bc	17.4a	9.9c	13.0b	12.8b	16.5a	11.3bc	<0.01
18:00	2.2b	3.2ab	3.3ab	2.6ab	2.6ab	2.7ab	3.5a	2.8ab	0.003
SFA	18.8b	21.8ab	27.0a	16.3c	20.4b	20.5bc	25.5a	17.5b	0.000
16:1n-7	7.0b	9.0ab	15.6a	7.9ab	8.5ab	8.8ab	13.3a	12.5a	0.001
16:1n-5	0.2a	0.3a	0.3a	0.1b	0.2a	0.3a	0.2ab	0.5a	0.006
18:1n-9	17.5a	17.8a	13.5b	13.5b	17.0a	15.7ab	14.1b	14.0b	<0.001
18:1n-7	4.5b	5.7b	8.3a	5.6b	5.1b	5.2b	7.2a	6.3ab	<0.001
20:1n-9	6.4a	6.7a	3.2b	6.3a	5.1ab	4.6b	4.7ab	4.1b	<0.001
MUFA	42.9b	46.2a	45.7ab	38.2ab	40.8ab	38.8b	44.5ab	42.3ab	0.006
16:3n-4	0.9a	1.0a	0.8a	0.8a	0.9a	1.0a	1.1a	1.0a	0.060
16:3-n3	0.4b	0.5ab	0.6ab	0.5ab	0.5ab	0.6ab	0.8a	0.4b	0.007

Abbreviations: (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity; (IT) index of thrombogenicity. In each row different letters indicate significant differences between sexes and seasons ($p < 0.05$).

Table 4.6.3 (cont.)

Fatty acid	Females				Males				p
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	
Hepatopancreas									
16:4n-3	0.9a	1.2a	1.1a	0.1b	1.4a	1.7a	0.1b	0.3b	<0.001
18:2n-6	1.2a	0.9b	0.7b	0.8b	1.1ab	0.9b	0.7b	0.5c	<0.001
20:2n-6	1.2ab	1.6a	0.8ab	1.2ab	1.1ab	1.6ab	1.0ab	0.7b	<0.001
20:4n-6	2.7b	2.6b	1.5b	3.0a	3.3a	3.7a	1.7b	3.8a	<0.001
20:5n-3	7.0a	5.5b	7.7a	8.5a	7.9a	7.4a	4.6b	9.0a	<0.001
22:5n-3	1.3a	0.8b	0.4b	1.6a	1.3a	1.3a	0.6b	1.4a	<0.001
22:6n-3	12.0ab	6.5c	4.1c	14.6a	11.3ab	10.4b	5.5c	12.6ab	<0.001
PUFA	31.3a	24.2b	20.9b	34.9a	31.9a	32.8a	18.8b	33.5a	<0.001
n-3	23.3b	15.4c	14.3c	27.2a	22.6b	21.5b	12.5c	24.7ab	<0.001
n-6	5.8b	5.8b	3.5c	6.0a	6.1b	6.9ab	4.0bc	7.1a	<0.001
(n-3/n-6)	4.1ab	2.6b	4.2ab	4.6a	3.7ab	3.5ab	3.1ab	3.5ab	<0.001
PUFA/SFA	1.7b	1.1c	0.8c	2.2a	1.6b	1.6b	0.8c	1.9a	<0.001
IA	0.37ab	0.44ab	0.57a	0.27b	0.38ab	0.39ab	0.49a	0.24b	<0.001
IT	0.18b	0.27ab	0.36a	0.14c	0.20b	0.21b	0.37a	0.15b	<0.001
Gonads									
14:00	1.8a	1.9a	2.1a	2.2a	1.1b	0.6c	0.6c	0.8c	0.001
15:00	0.8ab	0.9ab	0.9a	0.9a	0.5ab	0.4b	0.6ab	1.1a	0.002
16:00	11.9b	11.7ab	13.8a	16.2a	8.5c	8.7c	10.5b	12.7ab	0.002
18:00	2.2c	2.5c	3.1b	2.4c	4.3b	5.5a	6.3a	5.7a	<0.001
SFA	17.2b	17.6ab	20.1a	21.9a	15.3b	16.6b	18.9ab	21.1a	0.010
16:1n-7	0.1ab	0.2ab	0.1ab	0.1ab	0.1ab	0.0b	0.5a	0.3ab	0.006
16:1n-5	0.5ab	0.6ab	0.7ab	1.0ab	0.6ab	0.6b	1.1a	0.7ab	0.001
18:1n-9	19.4a	17.3ab	15.0ab	18.0a	13.2ab	13.0ab	12.7b	14.9ab	0.001
18:1n-7	4.9ab	4.8b	5.4ab	5.9ab	4.9ab	5.2ab	6.0ab	6.3a	0.004
20:1n-9	2.3ab	2.4ab	1.5ab	1.3ab	3.7a	2.4ab	1.4ab	1.1b	0.001
MUFA	35.5a	34.3a	35.3a	36.9a	39.4a	32.2a	33.0a	38.7a	0.060
16:3n-4	1.0a	1.0ab	0.5ab	0.7ab	0.6ab	0.4b	0.8ab	0.8ab	0.002
16:3n-3	0.5b	0.5b	0.5b	0.7ab	1.0a	1.2a	0.9a	1.4a	0.001
16:4n-3	1.6b	2.4a	1.1b	1.5b	1.6b	1.5b	1.3b	1.1b	0.002
18:2n-6	1.0a	0.9a	0.7b	0.8b	0.7b	0.6b	0.6b	0.5c	0.001
20:2n-6	0.7ab	0.7ab	0.5b	0.5b	1.0a	1.3a	0.9ab	0.5b	0.001
20:4n-6	4.7c	4.9c	2.6d	3.2d	6.7b	9.2a	7.5b	7.4b	<0.001
20:5n-3	14.2b	13.9b	13.1b	13.5b	17.1ab	19.7a	20.9a	16.8ab	0.001
22:5n-3	1.7a	1.5a	1.3a	0.9b	1.1b	1.1b	0.6c	0.5c	0.002
22:6n-3	16.3a	14.2ab	13.6ab	10.7ab	13.4ab	15.5ab	10.8ab	8.7b	0.012
PUFA	44.3a	43.2ab	37.8ab	35.4b	40.3ab	48.6a	43.3ab	36.6b	0.009
n-3	33.9ab	31.6ab	30.4ab	27.2b	33.6ab	38.3a	34.0ab	27.9b	0.005
n-6	7.4c	7.6c	5.2d	5.4d	8.7b	11.9a	9.7b	9.0b	<0.001
(n-3/n-6)	4.6b	4.2c	5.8a	5.0a	3.9c	3.2d	3.5d	3.1d	<0.001
PUFA/SFA	2.6b	2.5ab	1.9c	1.7c	2.6ab	2.9a	2.3b	1.7c	<0.001
IA	0.25ab	0.26a	0.31a	0.36a	0.16b	0.14b	0.17b	0.21b	<0.001
IT	0.12b	0.14b	0.16a	0.20a	0.11b	0.11b	0.14b	0.17a	<0.001

Abbreviations: (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity; (IT) index of thrombogenicity. In each row different letters indicate significant differences between sexes and seasons ($p < 0.05$).

Hepatopancreas had proportionally more SFA in autumn (both sexes) and less in winter (females); MUFA was lower in spring (females) and summer (males); and PUFA levels were lower during summer (females) and autumn (both sexes; Table 4.6.3). Concerning gonads, in both sexes SFA values were lower in spring and summer; no seasonal differences were detected in the proportion of MUFA levels, and PUFA content was lower in winter (both sexes; Table 4.6.3).

Table 4.6.4 Fatty acids statistical differences between tissues with the respective p values.

Fatty acid	Tissue	p
14:00	hepatopancreas > (muscle = gonads)	<0.01
15:00	(hepatopancreas = gonads) > muscle	<0.01
16:00	(hepatopancreas = gonads) > muscle	<0.01
18:00	muscle > gonads > hepatopancreas	<0.01
16:1n-7	hepatopancreas > muscle > gonads	<0.01
16:1n-5	(muscle = gonads) > hepatopancreas	<0.01
18:1n-9	ND	0.68
18:1n-7	(hepatopancreas = gonads) > muscle	<0.01
20:1n-9	hepatopancreas > (muscle = gonads)	<0.01
16:3n-4	muscle > hepatopancreas > gonads	<0.01
16:3n-3	hepatopancreas > gonads > muscle	<0.01
16:4n-3	gonads > hepatopancreas > muscle	<0.01
18:2n-6	(muscle = hepatopancreas) > gonads	<0.01
20:2n-6	hepatopancreas > (muscle = gonads)	<0.01
20:4n-6	(muscle = gonads) > hepatopancreas	<0.01
20:5n-3	muscle > gonads > hepatopancreas	<0.01
22:5n-3	ND	0.98
22:6n-3	(muscle = gonads) > hepatopancreas	<0.01
SFA	(hepatopancreas = gonads) > muscle	<0.01
MUFA	hepatopancreas > gonads > muscle	<0.01
PUFA	muscle > gonads > hepatopancreas	<0.01
n-3	muscle > gonads > hepatopancreas	<0.01
n-6	muscle > gonads > hepatopancreas	<0.01
(n-3/n-6)	muscle = gonads > hepatopancreas	<0.01
PUFA/SFA	muscle > gonads > hepatopancreas	<0.01
IA	hepatopancreas > gonads > muscle	<0.01
IT	hepatopancreas = gonads > muscle	<0.01

Each fatty acid was tested independently of sex and season. Abbreviations: (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity index; (IT) index of thrombogenicity; (ND) no differences.

PCA considering all fatty acids, seasons, and sexes were applied to all edible tissues, and factors 1, 2, and 3 yielded a total of 65 % of explainable results (Figure 4.6.1A). Most fatty acids loaded heavily on factor 1 except 18:1n-9, 18:2n-6, 20:2n-6, and 22:5n-3, which loaded on factor 2, and 16:3n-4, 16:3n-3; 16:4n-3, and 22:6n-3, which loaded on factor 3. Clear cluster separation is evidenced between muscle and hepatopancreas, whereas gonads overlap both tissues, although predominantly overlapping the muscle.

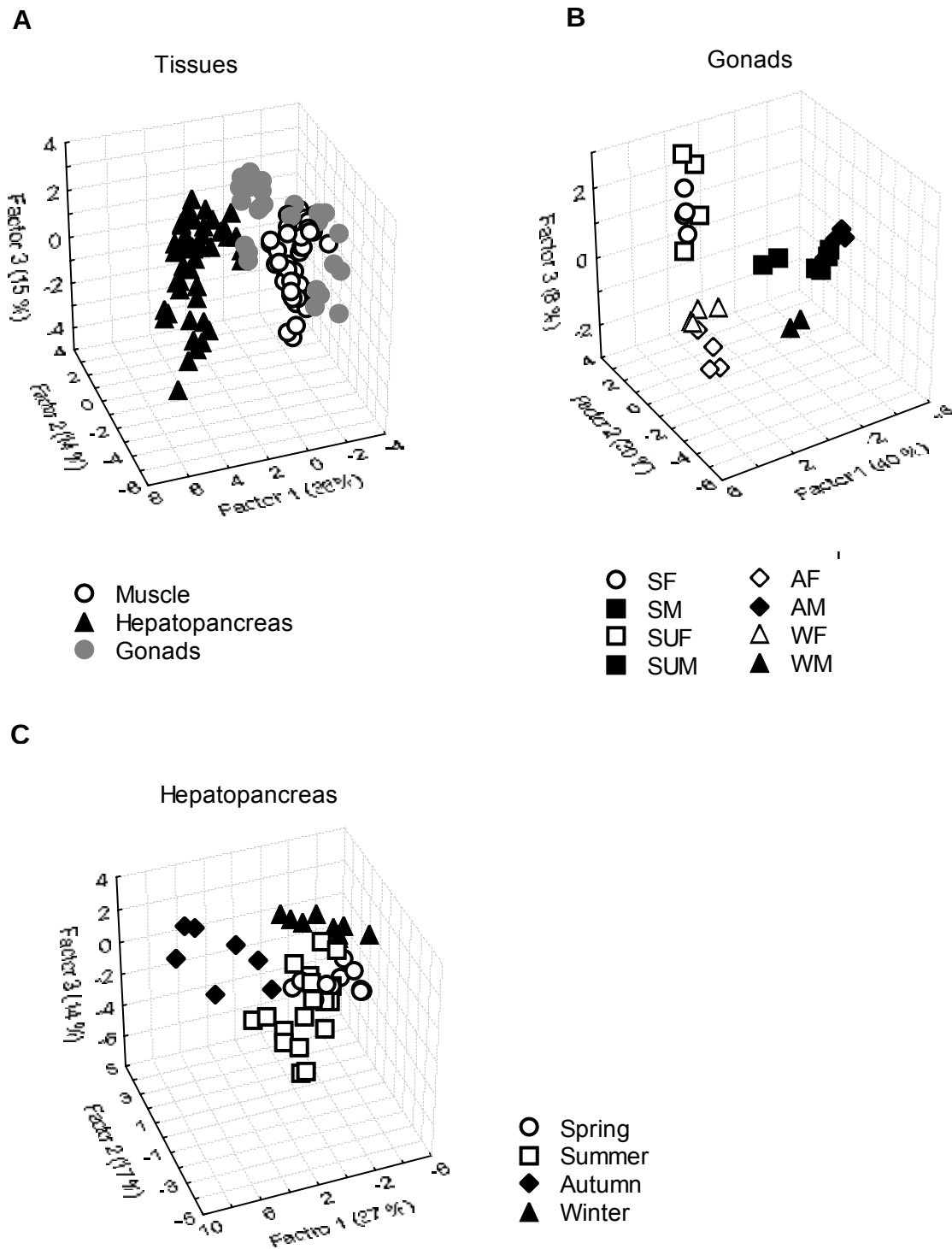


Figure 4.6.1 Results of PCA considering the fatty acids profile: (A) in all tissues; (B) in gonads depending on sex and season; (C) in hepatopancreas depending on season. Abbreviations: (SF) spring females; (SM) spring males; (SUF) summer females; (SUM) summer males; (AF) autumn females; (AM) autumn males; (WF) winter females; (WM) winter males.

This cluster pattern is in accordance with the statistical results obtained with one-way ANOVA in which differences between tissues were tested for each fatty acid independently of sex and season (Table 4.6.4). The content of several fatty acids did not differ between gonads and hepatopancreas (15:0, 16:0, and 18:1n-7) and between gonads and muscle (16:1n-5, 20:4n-6, and 22:6n-3). In contrast, hepatopancreas had proportionally more 14:0, 20:1n-9, and 20:2n-6, whereas 18:0 and 20:5n-3 had higher proportion in the muscle.

The main saturated fatty acid in all tissues was palmitic acid (16:0), which was fairly constant in the muscle during the four seasons, whereas in hepatopancreas and gonads it predominated during autumn and winter, respectively. Among MUFA, oleic acid (18:1n-9) was the prevailing fatty acid in all tissues, with particularly higher values found in spring (all tissues), summer (hepatopancreas), and winter (gonads).

The main n-3 PUFA was EPA (20:5n-3; muscle and gonads) and DHA (22:6n-3; hepatopancreas) (Table 4.6.4). In muscle the proportion of EPA was fairly constant during the four seasons independent of sex, whereas in hepatopancreas lower values were detected in summer (females) and autumn (males) and in testis there was a higher proportion during summer and autumn than ovaries. DHA in the muscle of females had higher proportion during winter than in spring and summer, in hepatopancreas DHA was predominant in winter and spring in both sexes, whereas in gonads there was a tendency of decreasing DHA levels from spring to winter in both sexes. The n-3 PUFA were higher in muscle (34.9-39.1 %) and gonads (27.2-38.3 %) than in hepatopancreas (14.3-27.2 %) (Tables 4.6.3 and 4.6.4). Higher levels of these fatty acids occurred during spring, summer, and autumn in gonads and during winter in hepatopancreas.

The major n-6 PUFA was arachidonic acid (AA, 20:4n-6), mostly in muscle and gonads. The AA values were always higher in testis than in ovaries. The n-6 PUFA were also dominant in muscle (6.9-10.3 %) and gonads (5.2-11.9 %) in opposition to hepatopancreas (3.5-7.1 %) (Tables 4.6.3 and 4.6.4).

Overall, major differences in fatty acid composition of gonads were observed between sexes and seasons, which are evidenced in the PCA representation considering all fatty acids, seasons, and sexes and where the three factors yielded a total of 78 % of explainable results (Figure 4.6.1B). Most fatty acids loaded heavily on factor 1, except 13:1n-5, 18:1n-7, 20:1n-9, 18:2n-6, 22:5n-3, and 22:6n-3, which loaded on factor 2, and 16:1n-7, 16:3n-4, and 16:4n-3, loaded on factor 3.

In hepatopancreas, PCA indicates a separation between seasons with the three factors having a total of 68 % of explainable results (Figure 4.6.1C). Most fatty acids loaded in factor 1, but 16:1n-5, 18:1n-9, 20:1n-9, and 18:2n-6 loaded on factor 2 and 16:3n-4, 16:3n-3, 20:2n-6, and 20:4n-6 loaded on factor 3. The ratio n-3/n-6 was lower in hepatopancreas and generally higher in ovaries than in testis. The PUFA/SFA ratio was higher in muscle followed by gonads and hepatopancreas (Table 4.6.4). This ratio was higher in winter in the muscle of females and in the hepatopancreas of both sexes, whereas in gonads higher values were found in the spring and summer for both sexes (Table 4.6.3). The indexes of atherogenicity and thrombogenicity were usually higher in hepatopancreas followed by gonads and muscle (Table 4.6.4).

Amino Acids

The amino acid composition of the edible tissues is given in table 4.6.5. In general, crab protein in all tissues contained high amounts of leucine, arginine, aspartic acid, glutamic acid and glycine.

Table 4.6.5 Amino acid profile (g 100 g⁻¹ of wet weight) of edible tissues of female and male crabs in spring, summer, autumn, and winter.

Amino acid	Females				Males				p
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	
Muscle									
Thr	0.89b	0.99ab	1.10a	1.09a	0.95ab	1.06a	1.02ab	1.12a	<0.001
Val	0.67c	0.69b	0.79a	0.73ab	0.76b	0.77a	0.77a	0.79a	0.001
Met	0.27b	0.18c	0.23b	0.16c	0.32a	0.26b	0.31a	0.20b	<0.001
Ile	0.65b	0.67b	0.76ab	0.71ab	0.75ab	0.75ab	0.75a	0.79a	0.004
Leu	1.09b	1.11b	1.26ab	1.17ab	1.22ab	1.25a	1.24a	1.27ab	0.004
Phe	0.61b	0.63ab	0.70ab	0.65ab	0.70a	0.70a	0.68a	0.71a	0.008
His	0.33b	0.36b	0.37ab	0.36b	0.37ab	0.40a	0.33b	0.42a	<0.001
Lys	0.77b	0.89ab	0.81ab	0.96a	0.87ab	1.00a	1.06a	1.01a	0.001
Arg	1.68b	1.64b	1.93a	1.77a	1.50c	1.79a	1.92a	1.85a	<0.001
Asp	1.50b	1.49b	1.74a	1.59a	1.65a	1.73a	1.66a	1.68a	0.003
Ser	0.65b	0.67ab	0.75ab	0.71a	0.69ab	0.74a	0.74a	0.74a	0.022
Glu	2.40b	2.50b	2.88a	2.46b	2.63a	2.79a	2.83a	2.74a	0.016
Gly	1.36a	1.34a	1.23a	1.29a	1.27a	1.24a	1.28a	1.26a	0.090
Ala	0.90b	0.91b	1.04ab	0.93b	0.94ab	0.98ab	1.03a	0.99ab	0.004
Tyr	0.63b	0.62b	0.72ab	0.69a	0.70ab	0.71a	0.72a	0.76a	0.001
Pro	0.61b	0.73a	0.63ab	0.60b	0.69a	0.75a	0.71a	0.69a	0.016
Hyp	0.44b	0.39b	0.53a	0.54a	0.55a	0.45ab	0.52a	0.50a	<0.001
Tau	0.35b	0.33b	0.49ab	0.51ab	0.41ab	0.43ab	0.61a	0.57a	<0.001
EAA/NEAA	0.79a	0.80a	0.79a	0.82a	0.78a	0.81a	0.80a	0.82a	0.058
TAA	15.79b	16.16b	17.96ab	16.92b	16.99b	17.78ab	18.16a	18.11ab	0.043
Hepatopancreas									
Thr	0.59d	0.75c	0.96ab	0.83b	0.78c	0.75c	0.74c	10.3a	0.001
Val	0.49d	0.55c	0.69b	0.63b	0.63c	0.60c	0.57c	0.75a	0.004
Met	0.11a	0.03c	0.01c	0.05b	0.13a	0.12a	0.06b	0.04b	0.016
Ile	0.42c	0.47b	0.59ab	0.55b	0.56b	0.52b	0.49c	0.67a	0.004
Leu	0.67d	0.75b	0.96b	0.86b	0.87b	0.81b	0.78c	1.03a	0.008
Phe	0.40d	0.46b	0.60b	0.5b2	0.53b	0.50b	0.48c	0.64a	0.011
His	0.21c	0.29bc	0.38ab	0.34b	0.33b	0.35b	0.29c	0.48a	0.003
Lys	0.34d	0.54bc	0.72a	0.61b	0.60b	0.60b	0.47c	0.70ab	<0.001
Arg	0.78c	0.92bc	1.25b	1.06b	1.06b	1.08b	0.95b	1.49a	0.002
Asp	0.82c	0.98bc	1.26ab	1.09b	1.13b	1.08b	1.02b	1.38a	0.005
Ser	0.40d	0.47bc	0.60a	0.52b	0.50b	0.48bc	0.45c	0.60a	0.003
Glu	1.10d	1.30bcd	1.63ab	1.43b	1.46b	1.39b	1.31c	1.75a	0.009
Gly	0.49d	0.58bc	0.67b	0.64c	0.61c	0.61c	0.54c	0.84a	0.001
Ala	0.52c	0.57bc	0.70a	0.56c	0.61b	0.60b	0.55c	0.70a	0.001

Abbreviations: (THR) threonine; (VAL) valine; (MET) methionine; (ILE) isoleucine; (LEU) leucine; (PHE) phenylalanine; (HIS) histidine; (LYS) lysine; (ARG) arginine; (ASP) aspartic acid; (SER) serine; (GLU) glutamic acid; (GLY) glycine; (ALA) alanine; (TYR) tyrosine; (PRO) proline; (HYP) hydroxyproline; (TAU) taurine; (EAA/NEAA) essential amino acids/nonessential amino acids; (total AA) total amino acids. In each row different letters indicate significant differences per tissue ($p < 0.05$).

Table 4.6.5 (cont.)

Amino acid	Females				Males				p
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	
Hepatopancreas									
Tyr	0.49d	0.52bc	0.71b	0.66b	0.63b	0.58c	0.55c	0.81a	<0.001
Pro	0.30c	0.35ab	0.45a	0.31c	0.37b	0.39ab n 283	0.28c	0.44a	0.002
Hyp	0.23c	0.33b	0.41a	0.35b	0.34bc	0.28c	0.34bc	0.41a	0.006
Tau	0.43c	0.54b	0.71a	0.64a	0.46c	0.58ab	0.58ab	0.57ab	<0.001
EAA/NEAA	0.84b	0.84b	0.86b	0.88b	0.89b	0.89b	0.86b	0.92a	0.002
TAA	8.79d	10.39c	13.30b	11.67bc	11.53c	11.32bc	10.46c	14.42a	0.007
Gonads									
Thr	1.69ab	2.03a	1.87a	1.49b	0.89d	0.81d	0.86d	1.07c	<0.001
Val	1.34a	1.55a	1.55a	1.17ab	0.76b	0.68b	0.70b	0.88b	<0.001
Met	0.32b	0.43b	0.53a	0.28b	0.13c	0.09c	0.08c	0.12c	<0.001
Ile	1.07a	1.32a	1.35a	0.92a	0.53c	0.49c	0.49c	0.66b	<0.001
Leu	1.67a	2.04a	2.04a	1.46b	0.74d	0.75d	0.73d	0.95c	<0.001
Phe	1.03a	1.23a	1.19a	0.92a	0.56b	0.51c	0.49c	0.64b	<0.001
His	0.59a	0.72a	0.71a	0.62a	0.25c	0.29c	0.26c	0.39b	<0.001
Lys	0.95a	1.13a	1.15a	1.01a	0.52c	0.81b	0.76b	1.01a	<0.001
Arg	1.73a	2.13a	2.12a	1.56b	0.92c	0.96c	1.00c	1.30c	<0.001
Asp	1.94ab	2.37a	2.23a	1.77b	1.25d	1.13e	1.16e	1.50c	<0.001
Ser	1.31ab	1.64a	1.66a	1.12ab	0.78b	0.66c	0.68c	0.81b	<0.001
Glu	2.95ab	3.60a	3.57a	2.66b	1.71c	1.64c	1.66c	2.09b	<0.001
Gly	1.25a	1.30a	1.20a	1.21a	0.77c	0.87b	0.84b	0.92b	<0.001
Ala	1.14a	1.28a	1.26a	1.04b	0.56d	0.67c	0.64c	0.69c	<0.001
Tyr	1.16b	1.41a	1.39a	1.11b	0.62d	0.55d	0.54d	0.76c	<0.001
Pro	1.10ab	1.30a	1.16ab	0.85b	0.70c	0.70c	0.69c	0.83b	<0.001
Hyp	0.55b	0.69b	0.84a	0.56b	0.33c	0.34c	0.34c	0.43b	<0.001
Tau	0.73b	0.73b	0.80a	0.77a	0.24d	0.49c	0.50c	0.46c	<0.001
EAA/NEAA	0.85a	0.88a	0.90a	0.85a	0.76b	0.77b	0.76b	0.83ab	0.008
TAA	22.52ab	26.90a	26.76a	20.51b	12.26d	12.44d	12.42d	15.50c	0.003

Abbreviations: (THR) threonine; (VAL) valine; (MET) methionine; (ILE) isoleucine; (LEU) leucine; (PHE) phenylalanine; (HIS) histidine; (LYS) lysine; (ARG) arginine; (ASP) aspartic acid; (SER) serine; (GLU) glutamic acid; (GLY) glycine; (ALA) alanine; (TYR) tyrosine; (PRO) proline; (HYP) hydroxyproline; (TAU) taurine; (EAA/NEAA) essential amino acids/nonessential amino acids; (total AA) total amino acids. In each row different letters indicate significant differences per tissue ($p < 0.05$).

Generally, hepatopancreas had the lowest amounts of amino acids compared to muscle and gonads (Table 4.6.6). The only exception was taurine (lower in muscle than in the remaining tissues). Major differences were observed regarding sex and season, which were more pronounced in gonads and hepatopancreas than in muscle (Table 4.6.5). These results are corroborated by the PCA presented in figure 4.6.2, where factors 1 and 2 gave a total of 91 % of explainable results. There is a clear cluster separation of the three tissues and of both sexes in gonads. All amino acids loaded on factor 1 except taurine.

Table 4.6.6 Amino acid statistical differences between tissues with the respective p values

Amino acid	Tissue	p
Thr	(muscle = gonads) > hepatopancreas	<0.01
Val	(muscle = gonads) > hepatopancreas	<0.01
Met	(muscle = gonads) > hepatopancreas	<0.01
Ile	(muscle = gonads) > hepatopancreas	<0.01
Leu	(muscle = gonads) > hepatopancreas	<0.01
Phe	(muscle = gonads) > hepatopancreas	<0.01
His	muscle = gonads > hepatopancreas	0.01
Lys	(muscle = gonads) > hepatopancreas	<0.01
Arg	gonads > muscle > hepatopancreas	<0.01
Asp	(muscle = gonads) > hepatopancreas	<0.01
Ser	(muscle = gonads) > hepatopancreas	<0.01
Glu	(muscle = gonads) > hepatopancreas	<0.01
Gly	muscle > gonads > hepatopancreas	<0.01
Ala	(muscle = gonads) > hepatopancreas	<0.01
Tyr	(muscle = gonads) > hepatopancreas	<0.01
Pro	gonads > muscle > hepatopancreas	<0.01
Hyp	muscle = gonads > hepatopancreas	<0.01
Tau	(gonads = hepatopancreas) > muscle	<0.01
EAA/NEAA	(gonads = hepatopancreas) > muscle	<0.01
TAA	(muscle = gonads) > hepatopancreas	<0.01

Each amino acid was tested independently of season and sex. Abbreviations as in table 4.6.5.

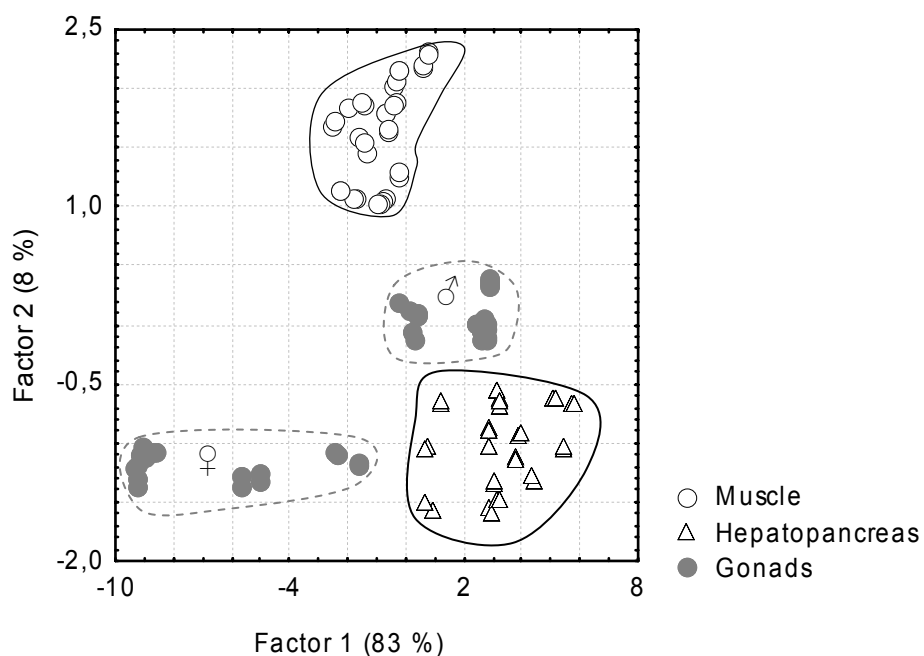


Figure 4.6.2 PCA considering the content of all amino acids in edible tissues. Solid lines represent clusters related to tissue, whereas dashed lines represent clusters related to sex. Symbols: ♂ (males); ♀ (females).

Female gonads had higher amounts of most amino acids during all seasons, compared to males. Ovaries had the lowest amino acid values in winter (leucine, arginine, and alanine) compared to the remaining seasons, whereas testis had some of the highest values in this season (threonine, isoleucine, leucine, histidine, lysine, aspartic acid, glutamic acid, tyrosine, proline, and hydroxyproline).

Hepatopancreas of male crabs had higher concentration of amino acids during winter, but particularly of threonine, valine, isoleucine, leucine, phenylalanine, histidine, arginine, aspartic acid, glutamic acid, glycine, and tyrosine. On the other hand, during autumn female hepatopancreas had higher concentration of lysine, whereas in spring the lowest amino acid content was detected for threonine, valine, leucine, phenylalanine, lysine, serine, glutamic acid, glycine, and taurine (both sexes).

With regard to the muscle, generally no significant differences were detected between seasons and sexes. The ratio of essential amino acids to nonessential amino acids (EAA/NEAA) did not vary in muscle; in hepatopancreas it was fairly constant throughout the year with a peak in winter male crabs, and ovaries had generally higher ratios than testis except in winter.

The amino acid scores are presented in figure 4.6.3; the highest scores were obtained for threonine, phenylalanine + tyrosine in all tissues but with the highest values in gonads. Values below 100 % were obtained for methionine: muscle, all seasons in females, summer and winter in males; hepatopancreas, all seasons in both sexes; ovaries, spring and winter, testis all seasons; lysine: muscle, spring and autumn in females; hepatopancreas, spring in females, spring and autumn in males; ovaries and testis, spring; valine: muscle, spring in females and summer in males) and leucine (testis, spring).

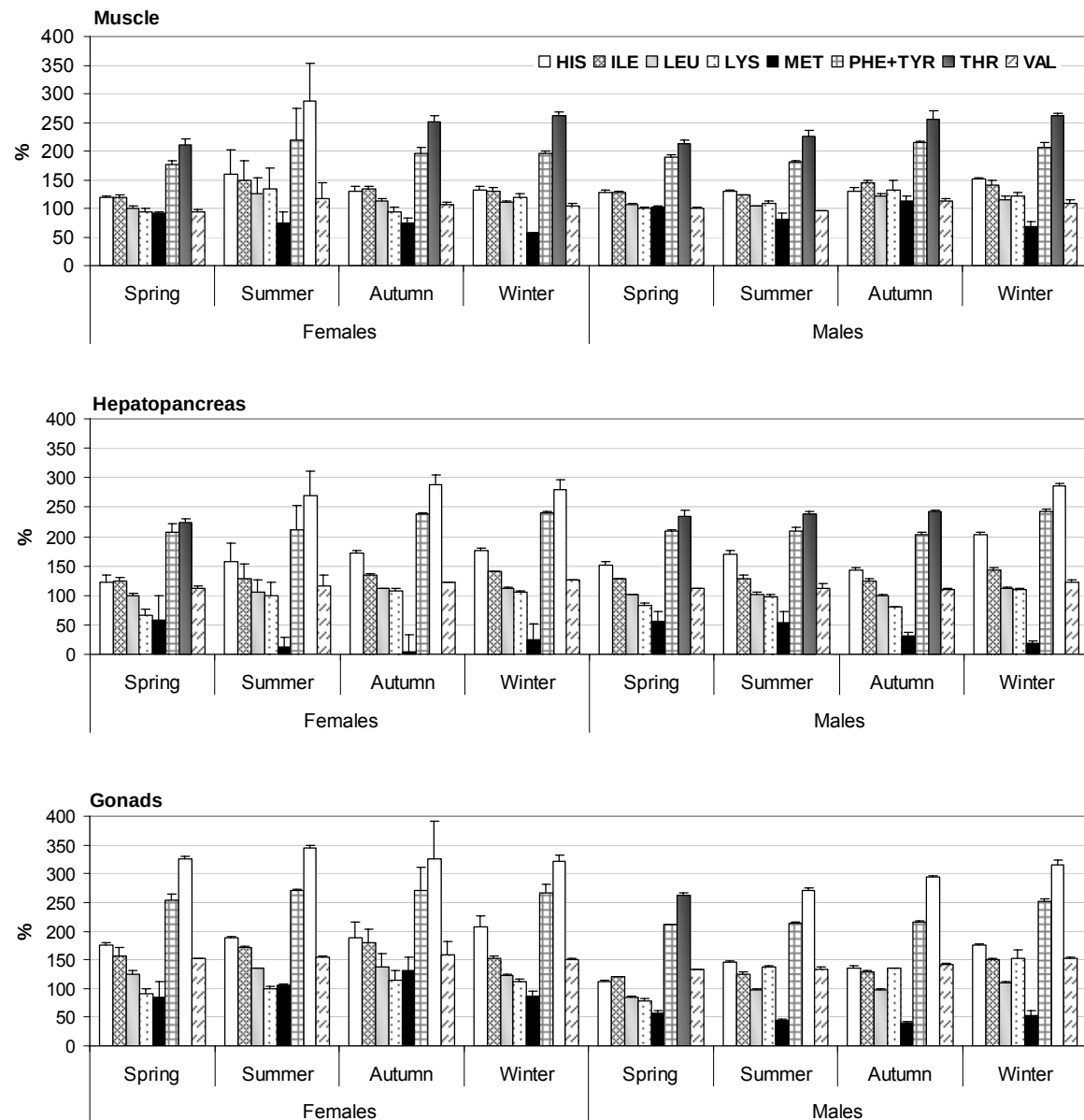


Figure 4.6.3 Essential amino acid score (%) of edible tissues of female and male *C. pagurus* during spring, summer, autumn, and winter. Abbreviations as in table 4.6.5.

Discussion

Meat yield and chemical composition

The results indicate that the meat yield of edible crab varied throughout the seasons in relation to moulting and breeding cycle as previously described by Edwards (1979). The variation is most significant in female crabs due to changes in the brown meat content following the development of gonads. The gonad development generally occurs in autumn, after moulting and mating took place during summer; spawning occurs in late autumn and winter (Edwards *et al.*, 1979; Tallack, 2007). After spawning, there is a period of egg incubation that can last up to 8 months before the broods hatch throughout spring, summer, and early autumn, depending on latitude and temperature (Edwards, 1979; Tallack, 2007). The GSI obtained in the present study is in agreement with the reproductive cycle of edible crab, as the highest values in autumn corresponded to the greater

proportion of S4 (ripe ovaries). The gonadosomatic index as a function of gonad development of marine invertebrates was described by several authors, and in general an increase in GSI of female ovaries as maturation approaches was observed (Harrison, 1990).

The proximate chemical composition and fatty acid and amino acid contents varied between tissues according to season and sex. The white meat from claws of males and females showed fewer seasonal variations than other tissues. Muscle is a structural tissue, the major site for protein storage, and its fat content is mainly composed by structural lipids with up to 72-74 % of phospholipids (Sriket, *et al.*, 2007), which are important components of cellular membranes. Therefore, this tissue has a more stable composition less exposed to seasonal variations (Ackman, 1995). Levels of muscle lipids were comparatively low and remained nearly constant throughout the seasons, demonstrating the absence of lipid mobilization to other tissues. Sex differences were more pronounced in the muscle protein and amino acid contents; higher values were quantified in males than in females, particularly in summer. This might be due to an increase in the biosynthesis of hormones and enzymes involved in mating that occurs during this season. Major differences between sexes were also observed in the muscle meat yield, as the claws of mature males are larger and with higher meat content than those of mature females of similar size (Edwards, 1979).

Marked sex and seasonal variations in the proximate chemical composition and fatty acid, cholesterol, and amino acid contents were observed in gonads, which can be related to food availability, temperature, migration pattern, sexual maturity, and gametogenesis, which are variables that are influenced by seasons (Oliveira *et al.*, 2007). This was evidenced by the higher amounts of protein, amino acids, fat, and cholesterol found in ovaries compared to testis. Also, the higher cholesterol and amino acid contents in summer and autumn and fat in winter (females) correspond to mating seasons and subsequent gonad development. Although in decapod crustaceans muscle is the major site of protein storage and hepatopancreas that of fat and cholesterol, these results indicate that ovaries become an additional centre for protein, fat, and cholesterol metabolism during maturation (Oliveira *et al.*, 2007). Protein and amino acid variations may indicate an increase in the biosynthesis of various proteins, including hormones, enzymes, and lipoproteins involved in mating, fertilization, gonad maturation, spawning, and normal development of embryo in decapods (Rosa and Nunes, 2003b). An optimum protein to energy ratio is essential for efficient nutrient deposition, where available protein is used primarily for gonad development, with energy requirements being satisfied by lipids and, to a lesser extent, by carbohydrates (Cortes-Jacinto *et al.*, 2004).

Cholesterol is an essential nutrient for crustaceans because they are not capable of *de novo* synthesis of steroid rings, and therefore cholesterol stores are derived from the diet (Harrison, 1990). Hepatopancreas is the major site of cholesterol metabolism, which is consistent with the constant cholesterol values obtained throughout the seasons. Souchet and Laplante (2007) have reported no seasonal variations in cholesterol content of hepatopancreas, but argued that this could be due to the proximity of their sampling periods (spring and summer). On the other hand, ovaries showed the highest cholesterol values in summer and autumn, whereas in muscle the

lowest values were attained in summer. These seasonal differences might be due to the mobilization of cholesterol from muscle to gonads, as previously reported by Teshima *et al.* (1989).

In the current study significant increases in the content of SFA in hepatopancreas and maturing ovaries suggest that these fatty acids might be important at this stage. Previous studies reported that SFA and MUFA are the major sources of energy during embryonic early larval development of the shrimp *Macrobrachium rosenbergii* (Roustaian *et al.*, 1999). Also, PUFA from the n-3 series, especially EPA and DHA, have been identified in recent decades as essential nutrients for marine invertebrates in general (Sargent *et al.*, 1999). In crabs, these fatty acids are essential for maturation and reproduction and might be implicated in the moulting process (Wu *et al.*, 2007). EPA is an important structural component of cell membranes which corroborates the present study considering that EPA levels were kept constant in muscle (Lilly and Bottino, 1981). EPA is also a precursor of prostaglandins, which might explain the differences observed between sexes in gonads, with males having higher EPA proportion during summer and autumn (main mating period) than females (Lilly and Bottino, 1981).

Nutritional quality

The present research evidenced the wide variation in the nutritional quality of edible crab tissues. Moreover, in each tissue the nutritional quality was affected by season and sex at different levels. Muscle composition was less variable than the remaining edible tissues, in hepatopancreas the chemical composition was mostly affected by season, and in gonads both season and sex contributed to the differences observed.

The Western diet is based on the consumption of domesticated animals and cultured vegetables and fruits. Most food items are standardized and cultured under restricted conditions, those that are best for rapid growth. Wild seafood, on the other hand, faces the effect of different variables throughout the year, which are interlinked combinations of factors. Season is crucial and affects the life cycle, behaviour, migration pattern, and type of food available. Season also affects water temperature, salinity, and light exposure, to name only a few. Wild crabs, such as *C. pagurus*, are affected by these factors, and their nutritional compositions reflect these influences.

Muscle, being a structural tissue, did not suffer as many changes as hepatopancreas and gonads. *C. pagurus* is mainly appreciated in southern Europe because of its gonads and hepatopancreas. From a consumer's perspective, autumn is the best season to eat crab, especially due to the characteristic high brown meat yield. Nevertheless, sex is certainly also important in autumn, as females have higher brown meat yields and muscle is the predominant tissue in males. From a nutritional point of view it is also important to stress that the cholesterol and fat contents were higher in female gonads than in males, particularly in autumn. Considering that the current dietary recommendations suggest a total cholesterol intake of <200 mg per day, people under a cholesterol-restricted diet should moderate the consumption of female gonads during autumn. Nevertheless, the remaining tissues of *C. pagurus* pose no risks during the four seasons even to a

restricted cholesterol diet. The fat content was also higher in autumn and spring in female hepatopancreas, mainly composed of SFA and MUFA.

On the other hand, taurine concentration in autumn and winter was higher in muscle (males), hepatopancreas (females), and gonads (females) than in remaining seasons. Beneficial effects of dietary taurine have been observed in animal and human studies, and there are indications of reduced cardiovascular disease risk and cholesterol absorption (Elvevoll *et al.*, 2008). The capacity for taurine biosynthesis varies remarkably among species. Humans have only limited ability for its biosynthesis; consequently, taurine needs to be acquired with the diet, but still there is no recommended dietary intake value. In general, muscle and female gonads were better sources of protein and amino acids than hepatopancreas. Particularly in autumn, muscle had the highest amino acid concentration.

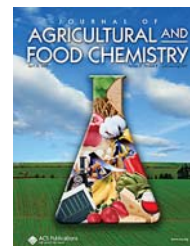
According to the amino acid score, protein in the edible tissues of *C. pagurus* was well balanced in the essential amino acid composition. Threonine and, phenylalanine + tyrosine had the highest scores in all tissues but especially in female gonads. The limiting amino acids were methionine and lysine in the three tissues, but particularly in hepatopancreas and male gonads. Similar results were reported in the muscle of shrimps and Norway lobster (Rosa and Nunes, 2003a). Tryptophan and the sulfur-containing amino acid cysteine are lost during the acid hydrolysis of meat products and therefore were not quantified, but they are typically available in low concentration in seafood products. The tissues are also well balanced with respect to EAA and show a favourable ratio and may be considered as a food source of high-quality protein.

The ratio of n-3/n-6 fatty acids has been cited as an excellent index to compare the relative nutritional value of lipids, where high values correspond to better quality foods. In this case, the highest values were obtained in autumn, particularly in female gonads. Compared to other crustacean species, like the crabs *Callinectes sapidus* (muscle, 2.32; hepatopancreas, 1.57) and *Carcinus mediterraneus* (muscle, 1.4), all tissues of *C. pagurus* during all seasons had higher nutritional quality with respect to this ratio (Çelik *et al.*, 2004, Cherif *et al.*, 2008). The U.K. Department of Health stipulated an ideal n-6/n-3 ratio of < 4 in the human diet and a minimum value of PUFA/SFA ratio recommended of 0.45 (UKDH, 1994). In this regard, muscle and gonads of females had n-3/n-6 ratios in the range of the recommended values, and all tissues of both sexes in the four seasons had PUFA/SFA ratios above 0.45. In conclusion, the edible tissues of *C. pagurus* have distinct nutritional composition that varies between sexes and seasons, but overall it was a well balanced nutritious food.

Sub-chapter 4.7

C. pagurus macro and trace elements: seasonal changes

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Cancer pagurus is much appreciated in Southern Europe, where the muscle, hepatopancreas, and gonads are consumed regularly with peaks in summer and December. The elemental contents of *C. pagurus* edible tissues were analyzed in this study during the four seasons. Results indicate that the content varied with tissue, season, and sex. The hepatopancreas had more S, Cl, Ca, Br, Sr, Fe, Cu, Cd, and Pb, the gonads had a higher concentration of Na, and the muscle was richer in Zn. Autumn and winter corresponded to a high Mg, S, Cl, K, Ca, Fe, and Zn content in both the muscle and hepatopancreas. Female gonads had more Fe, Zn, As, and Se than males but less Ca, Cl, Br, and Sr. Regarding toxic elements for human consumption, the levels of As, Hg, and Pb found in all edible tissues pose minimal risks to consumers. However, Cd concentration in the hepatopancreas was always above the action level. Therefore, we recommend moderate hepatopancreas consumption.

Keywords: *Cancer pagurus*, muscle, hepatopancreas, gonads, toxic and essential elements, FAAS, EDXRF, risk assessment.

Introduction

In the last decades, much attention has been paid to the study of macro and trace element content in foodstuffs, as a result of a growing concern about health benefits and risk of food consumption. Health benefits are related to the essential elements content in food (e.g., Ca, Fe, and Se), which by definition are required for the maintenance of normal physiological functions. In opposition, risk assessment of inorganic elements has examined two ends of the toxicity spectrum: (a) those related with intakes that are too high and the resulting toxicity and, (b) those associated with intakes that are too low and resulting in nutritional deficiencies (Goldhaber, 2003). Food safety authorities around the world, namely, the U.S. Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), and the World Health Organization (WHO), have set limits for several chemicals, including essential and toxic elements. In particular, the evaluation of risks and benefits of seafood consumption has been controversial, and several studies have focused on the characterization of macro and trace element content of fish, molluscs, and crustaceans (Chien *et al.*, 2003; Goldhaber, 2003; Sivaperumal *et al.*, 2007; Uluozlu *et al.*, 2007; Sub-chapter 4.2, 4.4 and 4.5).

Crustaceans are known to be an important supply of essential elements, including antioxidants such as Se and Zn, but also contaminants such as Cd (Sub-chapter 4.2; 4.4 and 4.5). However, most wild marine animals have in general great variability in elemental content, mainly due to differences between populations and environments to which they are exposed to (Silva-Castiglioni *et al.*, 2007). The elemental accumulation in aquatic animals is affected by endogenous (e.g., sex, age, condition, moulting, and tissue) and exogenous factors (e.g., elemental bioavailability in seawater and diet; Rainbow, 2002). The exogenous factors are influenced by environmental changes (e.g., season, location, substrate, depth, salinity, temperature, and anthropogenic sources; Rainbow, 2002). The edible crab *Cancer pagurus* is subjected to a large number of environmental variables following their annual and daily cycles (e.g., migration and habitats) that influence behaviour, feeding, metabolism, and ultimately the elemental composition.

This species is much appreciated in Southern European countries, being imported mostly from Britain, Ireland, Norway, Sweden, and France (Tully *et al.*, 2006). In Spain, France, Portugal, and Italy, *C. pagurus* white meat (muscle) and brown meat (hepatopancreas and gonads) are consumed separately or as a mixture all year round, but with peak consumption usually occurring during summer holidays and Christmas festivities (Sub-chapter 2.1). In addition, larger males are usually more expensive than smaller males and females due to the larger size of claws and consequent meat yield (Brown and Bennet, 1980). Considering consumers' habits and the potential variability in the elemental content of crabs, it is important to characterize and understand these variations in order to establish the benefits and risks of *C. pagurus* to human consumption. Therefore, the aims of this study were: (a) to quantify the Na, Mg, S, Cl, K, Ca, Mn, Fe, Cu, Zn, As, Se, Br, Sr, Cd, Hg, and Pb content in the edible tissues of female and male *C. pagurus* during the four annual seasons and (b) compare the concentrations of essential elements with the recommended intake values as well as with the limits set by authorities for contaminants.

Material and methods

Ethical statement

All live animals utilized in the experiment have been treated with proper care, minimizing discomfort and distress, and were painlessly killed. Also, the number of animals was kept to the minimum necessary to obtain scientific results, considering that the gain in knowledge and long-term benefit to the subject species is high.

Biological material

Cancer pagurus harvested in the Scottish coast were sampled during spring (May), summer (August), and autumn (October) of 2007, and winter (March) of 2008. Every season, 20 intermoult crabs (10 females and 10 males) were purchased from a local importer and transported live to the laboratory. Animals were kept under refrigerated conditions ($\sim 4^{\circ}\text{C}$) during 1 h to decrease their metabolism and to stun them before being euthanized by piercing the two nerve centers by means of a stainless steel rod. The rod was inserted through one of the eyes and through the vent. Precautionary measures to prevent contamination during collection, dissection, and analyses were taken. Muscle from the claws, hepatopancreas, and gonads of every animal were individually separated by using sterilized stainless steel scalpels and forceps; disposable plastic containers were used for collection and plastic tubes for preservation and analytical purposes. Samples were pooled when there was insufficient amount of tissue to perform all analyses (e.g., male gonads). All tissues were weighted and individually homogenized with a grinder (Retasch Grindomix GM200; 5000 rpm; material, PP cup and stainless steel knives), vacuum packed, and stored at -20°C . A portion of each frozen sample was freeze-dried for 48 h at -50°C and low pressure (approximately 10^{-1} atm). Samples were powdered and stored at -20°C under controlled moisture conditions (vacuum packed) until further analyses.

Element analyses

Energy dispersive X-ray fluorescence (EDXRF) was used to quantify the elements S, Cl, K, Ca, Fe, Cu, Zn, As, Se, Br, and Sr. The EDXRF technique consists of an X-ray tube equipped with a changeable secondary target, normally molybdenum (EXTRA II A, Atomika Instruments, Temple). The characteristic radiations emitted by the elements in the sample were detected by a Lithium drifted Silicon [Si (Li)], with a 30 mm^2 active area and an $8\text{ }\mu\text{m}$ beryllium window. The energy resolution was 135 eV at 5.9 keV, and the acquisition system was a Nucleus PCA card. Quantitative calculations were made with the fundamental parameters method (Custódio *et al.*, 2003). The X-ray generator was operated at 50 kV and 20 mA, and an acquisition time of 1000 s. Each sample powder ($\sim 1\text{ g}$) was pressed into cylindrical pellets of 2 cm diameter without any chemical treatment. A minimum of three pellets (replicates) per sample were glued onto Mylar films, on sample holders, and placed directly in the X-ray beam. Flame atomic-absorption spectrometry (FAAS), through the spectrometer Varian (Australia) Spectr AA 20 with deuterium background correction (Varian), was employed to quantify Na, Mg, Mn, Cd, and Pb (Jorhem, 2000). Each sample (5 g wet weight to quantify Na, Mg, and Mn, and 10 g wet weight to quantify Cd and

Pb) were dry-ashed at 450 °C under a gradual temperature increase (50 °C per hour). Ash was dissolved in concentrated nitric acid, and the solution obtained was evaporated to dryness. The final residue was dissolved with 12 or 5 mL of 15 % nitric acid (v/v) and transferred to 25 or 10 mL volumetric flasks (10 mL for Pb and Cd and 25 mL for the other elements); final volumes were adjusted with Ultrapure water. A minimum of three replicate analyses were performed per sample. Concentrations were calculated from linear calibration plots obtained by measurement of standard solutions absorbance: NaNO₃ (Merck) dissolved in HNO₃ (0.5 M); Mg (NO₃)₂ (Merck) dissolved in HNO₃ (0.5 M); and Mn (NO₃)₂ (Merck) dissolved in HNO₃ (0.5 M), Cd(NO₃)₂ (Merck) dissolved in HNO₃ (0.5 M), and Pb(NO₃)₂ (Merck) dissolved in HNO₃ (0.5 M). All glassware was cleaned with HNO₃ (10 %) or HCl (20 %) for 24-48 h and rinsed with Ultrapure water (18.2MX cm) to avoid contamination. Chemical reagents were pro analysis or superior. Total Hg was measured in triplicate with an AMA 254 Mercury Analyzer spectrometer that uses the mercury vapor generation technique. The procedure is based on dry sample decomposition (hepatopancreas, 3 mg; muscle, 6 mg; gonads, 10 mg), by combustion, preconcentration of mercury by amalgamation with gold, and atomic absorption spectrometry. Concentrations were calculated from linear calibration plots obtained by measurement of the absorbance of a Hg standard solution (Hg diluted in HNO₃; 0.5 M) supplied by Merck.

Accuracy tests

Accuracy was checked by analyses of certified biological reference material. The elemental concentrations obtained for canned matrix meat (SMRD-2000; Swedish Meats R & D and Scan Foods/National Food Administration, Sweden), nondefatted lobster hepatopancreas (LUTS-1; National Research Council of Canada), oyster tissue (SRM 1566; National Bureau of Standards), freeze-dried animal blood (IAEA-A-13; International Atomic Energy Agency), and lobster hepatopancreas (TORT-2; National Research Council of Canada) were compared with certified values. The detection limits (DL) of each element (Table 4.7.1) were determined by two means: (a) EDXRF with the signal-to-noise approach, where the equipment compares the signal of each element with blank samples and establishes the minimum concentration at which the element is reliably detected and (b) FAAS with the residual standard deviation (RSD) of the response and the slope (S) of the calibration curve of each standard solution used ($DL=3.3 \times RSD/S$).

Nutritional quality and potential hazards to consumers

To evaluate the elemental nutritional quality and potential consumption hazards of *C. pagurus* during spring, summer, autumn, and winter, the concentration of elements per 100 g serving portion were calculated in the edible tissues and compared with the recommended intake and limits set by international authorities. The concentrations of Na, K, Ca, and Mn were compared with the daily adequate intakes (AI); Mg, Cl, Fe, Cu, Zn, and Se were balanced considering the recommended dietary allowances (RDA); Na, Ca, Cu, and Se were also compared with the daily tolerable upper intake levels (UL). AI, RDA, and UL were set by the USA Food and Nutrition Board of the Institute of Medicine for individual adults aged between 19 and 50 years old (IOM, 1997;

2001; 2004 and 2008). The contaminants Cd, Hg, and Pb were compared with the maximum level (ML) set by the European Commission, while As was compared with the action level (AL) set by the United States Food and Drug Administration (USFDA). Since Cd and Hg maximum limits are only set for crustacean muscle, the levels of these contaminants were also compared with the AL set for the food commodity named Crustacea. Recommendations regarding As, S, Br, and Sr have not been set so far by food authorities, and therefore, these elements were not considered in the evaluation.

Statistical analysis

Main difference among the tissues, sexes, and seasons were tested with analysis of variance (ANOVA). Whenever necessary, data were transformed to satisfy normal distribution and homoscedasticity requirements, followed by nonparametric analysis of variance (Kruskal-Wallis), if transformed data could not meet these assumptions. Student's t-test and the equivalent nonparametric analysis' Mann-Whitney were also applied as appropriate. Principal component analysis (PCA) was also employed to reduce the multidimensional data sets of several elements to lower dimensions, thus simplifying the presentation and interpretation of data. All statistical analyses were tested at the 0.05 level of probability with the software STATISTICA™ 6.1. (Statsoft, Inc., Tulsa, OK 74104, USA).

Table 4.7.1 Elemental concentration ($\mu\text{g g}^{-1}$ DW; n=4) and detection limits ($\mu\text{g g}^{-1}$ DL) of certified reference material ($\pm$ standard deviation) analyzed by FAAS and EDXRF.

Elements	Technique	D.L.	Certified reference material	Certified value	Present work
Na	FAAS	0.37	Canned matrix meat (SMRD-2000)	8533 $\pm$ 281	8346 $\pm$ 280
Mg	FAAS	0.05	Non defatted lobster hepatopancreas (LUTS-1)	90 $\pm$ 4	91 $\pm$ 2
S	EDXRF	100	Oyster tissue (SRM 1566)	7600*	8200 $\pm$ 500
Cl	EDXRF	100	Oyster tissue (SRM 1566)	10000*	10200 $\pm$ 500
K	EDXRF	50	Oyster tissue (SRM 1566)	9690 $\pm$ 50	10000 $\pm$ 80
Ca	EDXRF	20	Oyster tissue (SRM 1566)	1500 $\pm$ 200	1350 $\pm$ 50
Mn	FAAS	0.04	Non defatted lobster hepatopancreas (LUTS-1)	1.20 $\pm$ 0.13	1.28 $\pm$ 0.03
Fe	EDXRF	3.1	Oyster tissues (SRM 1566)	195 $\pm$ 34	210 $\pm$ 15
Cu	EDXRF	0.7	Oyster tissues (SRM 1566)	63 $\pm$ 4	64 $\pm$ 4
Zn	EDXRF	1.1	Oyster tissues (SRM 1566)	852 $\pm$ 14	830 $\pm$ 40
As	EDXRF	0.7	Oyster tissues (SRM 1566)	13 $\pm$ 2	13 $\pm$ 1
Se	EDXRF	0.5	Oyster tissues (SRM 1566)	2.1 $\pm$ 0.5	2.3 $\pm$ 0.5
Br	EDXRF	0.8	Freeze dried animal blood (IAEA-A-13)	22 $\pm$ 3	22 $\pm$ 2
Sr	EDXRF	0.5	Oyster tissue (SRM 1566)	10 $\pm$ 1	9.9 $\pm$ 0.8
Cd	FAAS	0.01	Lobster hepatopancreas (TORT-2)	27 $\pm$ 1	27 $\pm$ 0
Hg	FAAS	0.02	Lobster hepatopancreas (TORT)	0.27 $\pm$ 0.06	0.28 $\pm$ 0.00
Pb	FAAS	0.02	Lobster hepatopancreas (TORT)	0.35 $\pm$ 0.13	0.35 $\pm$ 0.06

*non-certified values provided by the United States National Bureau of Standards.

Results and discussion

Influence of tissue, sex, and season in elemental composition

This study evaluated the content of elements in the edible tissues of *C. pagurus* considering the effect of tissue and sex (endogenous factors), and season (exogenous factor). Biometric data of all specimens analyzed are shown in table 4.7.2.

Table 4.7.2 *Cancer pagurus* biometric data and muscle, hepatopancreas and gonads wet weight of female and male crabs sampled during spring, summer, autumn and winter (average $\pm$ standard deviation).

Season	Sex	Weight (g)	CW (mm)	CL (mm)	Muscle (g)	Hepat. (g)	Gonads (g)
spring	F	770 $\pm$ 33	163.9 $\pm$ 4.1	108.4 $\pm$ 0.4	62.8 $\pm$ 5.7	85.3 $\pm$ 19.9	18.5 $\pm$ 7.7
	M	828 $\pm$ 44	160.9 $\pm$ 4.7	100.2 $\pm$ 2.1	99.1 $\pm$ 10.0	104.0 $\pm$ 19.3	9.0 $\pm$ 4.8
summer	F	748 $\pm$ 25	163.3 $\pm$ 2.3	107.4 $\pm$ 1.8	63.6 $\pm$ 7.8	77.5 $\pm$ 12.6	27.0 $\pm$ 14.8
	M	751 $\pm$ 27	156.3 $\pm$ 3.0	98.4 $\pm$ 1.9	89.2 $\pm$ 10.0	76.8 $\pm$ 16.3	10.4 $\pm$ 5.3
autumn	F	770 $\pm$ 153	167.5 $\pm$ 18.2	107.7 $\pm$ 7.7	60.2 $\pm$ 17.8	91.5 $\pm$ 21.5	54.0 $\pm$ 14.7
	M	869 $\pm$ 230	167.7 $\pm$ 18.0	103.6 $\pm$ 8.5	121.5 $\pm$ 42.1	88.3 $\pm$ 23.5	16.7 $\pm$ 8.5
winter	F	694 $\pm$ 59	159.8 $\pm$ 7.0	99.4 $\pm$ 3.7	55.2 $\pm$ 9.0	78.1 $\pm$ 14.8	12.6 $\pm$ 4.3
	M	650 $\pm$ 75	153.7 $\pm$ 6.3	93.4 $\pm$ 3.4	66.9 $\pm$ 14.5	85.9 $\pm$ 15.1	5.6 $\pm$ 1.7

Abbreviations: (CW) carapace width; (CL) carapace length; hepat., hepatopancreas.

Statistical differences in the concentration of elements were found between the edible tissues (4.7.3). Overall, the hepatopancreas had more S, Cl, Ca, Br, Sr, Fe, Cu, Cd, and Pb ($p < 0.01$), the gonads had higher concentration of Na ($p < 0.01$), while the muscle was richer in Zn ($p < 0.01$). No statistical differences were found between tissues in the concentration of K and Sr (Table 4.7.3).

Table 4.7.3 Statistical differences between tissues considering a one way ANOVA or Kruskal Wallis test with the respective p values. Each element was tested independently of season and sex.

Elements	Tissue	p
Na	Gonads	<0.01
Mg	Hepatopancreas/Gonads	<0.01
S	Hepatopancreas	<0.01
Cl	Hepatopancreas	<0.01
K	ND	0.814
Ca	Hepatopancreas	<0.01
Zn	Muscle	<0.01
Br	Hepatopancreas	<0.01
Sr	Hepatopancreas	<0.01
As	ND	0.13
Mn	Hepatopancreas/Gonads	<0.01
Fe	Hepatopancreas	<0.01
Cu	Hepatopancreas	<0.01
Se	Hepatopancreas/Gonads	<0.01
Cd	Hepatopancreas	<0.01
Hg	Muscle/Hepatopancreas	<0.01
Pb	Hepatopancreas	<0.01

For each element the corresponding tissue with the greatest concentration is shown. (ND) no differences.

PCA analysis considering sex, season, and all elements was applied to differentiate tissues; factors one, two, and three yielded a total of 65 % of explainable results (Figure 4.7.1A). Clear cluster separation is evidenced by the muscle, while the hepatopancreas and gonads overlap. Most elements loaded heavily on factor one, except for Na, K, As, and Se, which loaded on factor three, while Hg and Pb loaded on factor two, which is in agreement with the differences found with ANOVA (Table 4.7.3).

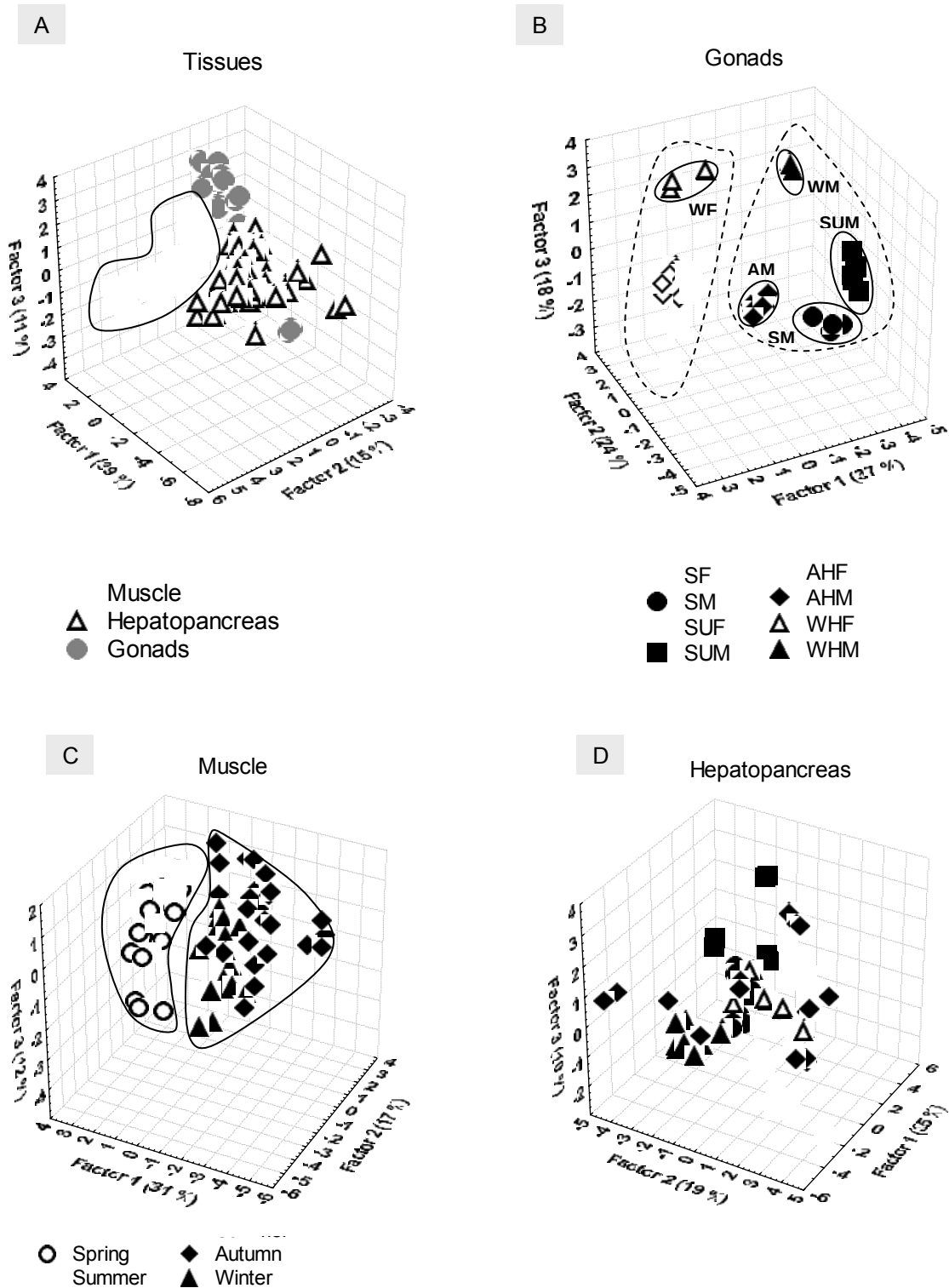


Figure 4.7.1 Result of four PCA analyses considering the concentration of all elements in: (A) all tissues, muscle, hepatopancreas and gonads; (B) in gonads considering sexes and seasons; (C) in muscle considering only seasons and, (D) in hepatopancreas considering sexes and seasons. Abbreviations: (SF) spring females; (SM) spring males; (SUF) summer females; (SUM) summer males; (AF) autumn females; (AM) autumn males; (WF) winter females; (WM) winter males.

The accumulation pattern of Fe, Cu, and Cd in the hepatopancreas is similar to that of other species of crabs, namely, *Portunus pelagicus*, *Pseudocarcinus gigas*, and *Eriocheir sinensis* (Falconer *et al.*, 1986; Berge and Brevik, 1996; Al-Mohanna *et al.*, 2001; Turoczy *et al.*, 2001). The hepatopancreas is a multifunctional organ that among several functions act as a temporary reservoir for minerals, regulating physiologically important cations and detoxifying dietary contaminants such as Cd (Zanotto and Wheatly, 2002; Chavez-Crooker *et al.*, 2003). Calcium and Cu are extremely important to crustaceans' homeostasis. Calcium takes part in the biomineralization process of the rigid exoskeleton by precipitation of calcium carbonate (Luquet and Marin, 2004), while Cu is incorporated into the respiratory pigment haemocyanin that is responsible for gas transport in the haemolymph (Lee and Shiau, 2002).

The importance of other elements, such as K, Zn, Fe, Sr, Br, and Se, to the physiology of crabs is not well studied. Clearly, the pattern of elemental accumulation in tissues is complex, and it is difficult to arrive at generalizations that universally apply. Generally, season and sex affected the concentration of most elements in the gonads (Figure 4.7.1B). In this PCA, a clear cluster separation is shown between sexes and seasons, with factors one, two, and three yielding 79 % of explainable results, with Mg, Cl, K, Ca, Zn, Br, Sr, Fe, and Se loading heavily on factor 1, Na, As, Cd, Hg, and Pb loading on factor two, and S, Mn, and Cu on factor three.

The concentration of Br and Sr (all seasons), Cl (spring, summer and autumn) and K (all seasons), and Mg (winter and spring) and Ca (winter) in male gonads was statistically higher than that in females (Tables 4.7.4 and 4.7.5). In contrast, females had statistically higher Fe, Zn, As, and Se levels than males during all seasons (Tables 4.7.4 - 4.7.6). Earlier studies with the semaphore crab also reported higher Zn accumulation in female gonads compared to that in males (MacFarlane *et al.*, 2002).

The concentration of elements in the muscle did not vary significantly and was mostly affected by seasons: Mg, S, Cl, K, Ca, and Hg were more concentrated in autumn and winter (Tables 4.7.4 - 4.7.6). These results are also evidenced in the PCA analysis, where separation between summer and spring is clear from the autumn and winter cluster (Figure 4.7.1C). The three factors yielded 60 % of explainable results, in which K, Zn, Mn, Fe, Hg, and Pb loaded on factor 1, Sr, As, and Se on factor 2, and Ca, Br, Cu, and Cd on factor 3.

In contrast, the hepatopancreas was affected either by sex and season: higher concentrations of Cu (autumn, females), Zn (autumn and winter, both sexes), As (spring, females), Ca (winter, males), Mg, and Sr (winter, males) (Tables 4.7.4 and 4.7.5). The PCA analysis for the hepatopancreas considering all elements, sex, and season yielded 64 % of explainable results and did not evidence any clear cluster separation between season and sex, which is in accordance with the results obtained (Figure 4.7.1D). Magnesium, S, Cl, K, Ca, Zn, Sr, As, and Mn loaded on factor 1, Fe, Cu, Se, Hg, and Pb loaded on factor 2, while Na, Br, and Cd loaded on factor 3. The usually higher Ca content in the hepatopancreas of males is likely due to the sexual dimorphism of crabs, in which males have bigger claws and harder exoskeletons (composed by calcium phosphate; Woll, 2006).

Table 4.7.4 Mean macro elemental composition (mg per 100 g⁻¹ wet weight; ± standard deviation) in female and male *C. pagurus* edible tissues during the four seasons.

Tissue	Season	Sex	Na AI=1500 UL=2300	Mg RDA = 400	S	Cl RDA=2300	K RDA=4700	Ca RDA=1000 UL=2500
Muscle	spring	F	351±46 a	36±4 ab	117±20 b	243±25 b	164±26 b	48±16 a
		M	241±54 a	25±7 b	162±38 ab	244±30 b	172±24 b	47±11 a
	summer	F	275±54 a	30±7 b	107±31 b	283±40 b	165±19 b	20±6 b
		M	278±98 a	21±10 b	135±63 b	244±33 b	179±21 b	24±5 b
	autumn	F	301±54 a	39±5 a	247±51 a	430±48 a	305±25 a	38±1 a
		M	354±56 a	39±4 a	251±45 a	465±96 a	286±30 a	35±4 a
	winter	F	306±78 a	31±6 b	240±21 a	515±56 a	294±21 a	38±8 a
		M	272±11 a	29±1 b	224±61 a	508±61 a	303±12 a	35±3 a
Hepato	spring	F	223± 75 a	30±2 c	164±34 b	417±64 c	162±8 b	285±58 c
		M	224±67 a	50±10 b	181±45 b	326±68 c	167±12 b	631±157 b
	summer	F	307±79 a	33±7 bc	206±68 b	567±64 b	185±10 b	260±79 c
		M	334±118 a	42±17 bc	234±37 b	469±64 b	201±10 b	943±79 b
	autumn	F	253±27 a	53±5 b	387±81 a	600±55 b	287±36 a	642±165 b
		M	279±68 a	57±23 ab	340±81 a	668±83 b	281±48 a	919±717 abc
	winter	F	281±77 a	51±7 b	345±49 a	780±34 a	288±23 a	781±214 b
		M	228±53 a	79±5 a	392±92 a	664±54 b	318±25 a	1926±368 a
Gonads	spring	F	309±155 c	72±0.6 b	126±51 b	154±67 d	108±49 d	8.5±3.4 e
		M	110±2 d	56±1 b	152±128 ab	673±19b	377±34 b	59±6 b
	summer	F	220±22 c	17±2 c	306±61 a	380±11 c	187±7 d	20±1 d
		M	281±1 c	12±1 d	291±37 a	1114±39 a	488±18 a	57±3 b
	autumn	F	167±56 cd	23±4 c	366±69 a	333±72 c	179±16 d	21±2 d
		M	502±118 b	26±4 c	161±35 b	601±60 b	293±32 bc	33±4 c
	winter	F	754±246 b	65±14 b	293±61 ab	490±53 b	274±12 c	21±1 d
		M	1879±7 a	90±1 a	210±16 b	673±19 b	348±2 b	85±2 a

In each column different letters indicate significant differences in the elemental concentration per tissue ($p < 0.05$). Abbreviations: (F) female; (M) male; (AI) adequate intakes; (RDA) recommended dietary allowances; (UL) tolerable upper intake levels set by authorities.

Table 4.7.5 Mean trace elemental composition (mg per 100 g⁻¹ wet weight; $\pm$ standard deviation) in female and male *C. pagurus* edible tissues during the four seasons.

Tissue	Season	Sex	Fe RDA = 8	Cu RDA = 0.9 UL = 10	Zn RDA = 11	As AL=7.6	Br	Sr
Muscle	spring	F	0.37 $\pm$ 0.06 b	0.86 $\pm$ 0.13 a	5.5 $\pm$ 0.2 b	4.2 $\pm$ 1.1 ab	2.0 $\pm$ 0.3 b	1.6 $\pm$ 0.3 a
		M	0.48 $\pm$ 0.14 ab	0.93 $\pm$ 0.28 a	6.0 $\pm$ 1.0 ab	3.6 $\pm$ 1.1 b	2.3 $\pm$ 0.3 b	1.1 $\pm$ 0.3 ab
	summer	F	0.48 $\pm$ 0.30 ab	0.93 $\pm$ 0.21 a	5.5 $\pm$ 0.4 b	4.3 $\pm$ 0.9 a	2.2 $\pm$ 0.2 b	0.72 $\pm$ 0.29 b
		M	BDL	0.86 $\pm$ 0.25 a	5.9 $\pm$ 0.3 ab	4.0 $\pm$ 0.8 a	2.1 $\pm$ 0.3 b	1.0 $\pm$ 0.5 ab
	autumn	F	0.68 $\pm$ 0.10 a	1.0 $\pm$ 0.2 a	7.0 $\pm$ 0.9 a	2.4 $\pm$ 0.3 c	2.2 $\pm$ 0.1 b	0.82 $\pm$ 0.30 b
		M	0.38 $\pm$ 0.15 b	0.67 $\pm$ 0.25 a	7.4 $\pm$ 1.5 ab	3.5 $\pm$ 2.1 b	2.4 $\pm$ 0.2 b	0.73 $\pm$ 0.37 b
	winter	F	0.44 $\pm$ 0.09 b	0.90 $\pm$ 0.18 a	6.6 $\pm$ 0.4 a	2.7 $\pm$ 0.5 c	2.6 $\pm$ 0.2 ab	1.1 $\pm$ 0.4 ab
		M	0.32 $\pm$ 0.04 b	1.1 $\pm$ 0.2 a	6.1 $\pm$ 0.4 ab	1.8 $\pm$ 0.4 d	2.9 $\pm$ 0.3 a	0.59 $\pm$ 0.10 b
Hepato	spring	F	1.6 $\pm$ 0.3 b	3.1 $\pm$ 0.2 b	2.2 $\pm$ 0.2 b	4.3 $\pm$ 0.4 a	5.5 $\pm$ 0.8 b	6.8 $\pm$ 1.1 c
		M	1.7 $\pm$ 0.1 b	1.8 $\pm$ 1.1 b	1.7 $\pm$ 0.2 b	2.4 $\pm$ 0.6 bc	6.4 $\pm$ 1.2 b	10 $\pm$ 2 b
	summer	F	1.8 $\pm$ 0.2 b	4.7 $\pm$ 1.9 ab	2.6 $\pm$ 0.5 a	4.9 $\pm$ 1.1 ab	8.1 $\pm$ 0.3 a	6.0 $\pm$ 0.9 c
		M	2.3 $\pm$ 1.0 ab	1.7 $\pm$ 0.6 b	2.0 $\pm$ 0.2 b	2.9 $\pm$ 0.4 b	8.3 $\pm$ 2.4 ab	12 $\pm$ 5 ab
	autumn	F	4.5 $\pm$ 1.1 a	10 $\pm$ 4 a	2.7 $\pm$ 0.5 a	2.6 $\pm$ 0.2 b	7.0 $\pm$ 0.5 b	9.1 $\pm$ 1.3 b
		M	3.2 $\pm$ 0.8 a	3.3 $\pm$ 3.9 ab	2.5 $\pm$ 0.8 a	2.0 $\pm$ 0.2 c	11 $\pm$ 4 a	11 $\pm$ 8 ab
	winter	F	3.0 $\pm$ 0.7 a	2.9 $\pm$ 2.0 b	2.6 $\pm$ 0.3 a	2.6 $\pm$ 0.5 bc	6.5 $\pm$ 1.0 b	8.9 $\pm$ 1.2 b
		M	4.3 $\pm$ 0.5 a	3.9 $\pm$ 1.7 b	2.7 $\pm$ 0.4 a	2.0 $\pm$ 0.3 c	7.7 $\pm$ 0.8 b	17 $\pm$ 2 a
Gonads	spring	F	1.2 $\pm$ 0.4 a	0.56 $\pm$ 0.28 bc	4.7 $\pm$ 0.8 b	3.0 $\pm$ 1.8 ab	1.9 $\pm$ 0.9 c	0.25 $\pm$ 0.11 d
		M	BDL	0.75 $\pm$ 0.03 b	2.8 $\pm$ 0.1 c	2.1 $\pm$ 0.0 c	8.2 $\pm$ 0.2 a	1.6 $\pm$ 0.0 a
	summer	F	1.9 $\pm$ 0.1 a	1.2 $\pm$ 0.1 a	9.0 $\pm$ 0.4 a	4.7 $\pm$ 0.2 a	3.8 $\pm$ 0.2 b	0.41 $\pm$ 0.03 d
		M	0.45 $\pm$ 0.06 b	1.4 $\pm$ 0.0 a	3.3 $\pm$ 0.1 c	4.3 $\pm$ 0.1 b	8.8 $\pm$ 0.2 a	1.2 $\pm$ 0.0 b
	autumn	F	1.9 $\pm$ 0.2 a	0.96 $\pm$ 0.15 b	9.0 $\pm$ 1.3 a	1.8 $\pm$ 0.2 c	2.7 $\pm$ 0.3 c	0.31 $\pm$ 0.04 d
		M	BDL	0.44 $\pm$ 0.02 c	1.8 $\pm$ 0.1 d	1.5 $\pm$ 0.0 d	4.2 $\pm$ 0.1 b	0.61 $\pm$ 0.01 c
	winter	F	2.7 $\pm$ 0.5 a	0.83 $\pm$ 0.07 b	5.7 $\pm$ 1.3 b	2.5 $\pm$ 0.2 c	3.3 $\pm$ 0.3 c	0.39 $\pm$ 0.04 d
		M	0.32 $\pm$ 0.03 b	0.70 $\pm$ 0.02 b	2.0 $\pm$ 0.1 d	1.1 $\pm$ 0.0 d	4.1 $\pm$ 0.1 b	0.78 $\pm$ 0.01 c

In each column different letters indicate significant differences in the elemental concentration per tissue ($p < 0.05$). Abbreviations: (F) female; (M) male; (BDL) below detection limit; (AI) adequate intakes; (RDA) recommended dietary allowances; (UL) tolerable upper intake levels; (AL) action levels set per day by authorities; (BDL) below detection limit.

Table 4.7.6 Mean ultra-trace elemental composition (mg per 100 g⁻¹ wet weight; ± standard deviation) in female and male *C. pagurus* edible tissues during the four seasons.

Tissue	Season	Sex	Mn AI = 2.3	Se RDA=0.055 UL=0.4	Cd MPC=0.05 AL=0.3	Hg MPC=0.05 AL=0.1	Pb MPC=0.05 AL=0.15
Muscle	spring	F	0.022±0.003 b	0.11±0.02 ab	0.003±0.002 a	0.018±0.001 b	BDL
		M	0.027±0.005 b	0.09±0.03 ab	0.002±0.001 a	0.016±0.004 b	0.002±0.000 a
	summer	F	0.027±0.003 b	0.09±0.02 ab	0.002±0.001 a	0.016±0.001 b	0.002±0.000 a
		M	0.024±0.004 b	0.09±0.02 ab	BDL	0.016±0.001 b	0.002±0.000 a
	autumn	F	0.062±0.012 a	0.13±0.01 a	BDL	0.061±0.018 a	BDL
		M	0.026±0.006 b	0.10±0.06 ab	BDL	0.049±0.015 a	BDL
	winter	F	0.024±0.001 b	0.083±0.17 b	0.002±0.001 a	0.038±0.008 a	0.003±0.001 a
		M	0.029±0.006 b	0.11±0.02 ab	0.003±0.003 a	0.021±0.004 b	0.002±0.000 a
Hepato	spring	F	0.11±0.01 c	0.19±0.04 a	0.80±0.70 b	0.021±0.003 b	0.002±0.001 b
		M	0.21±0.02 b	0.15±0.04 a	0.60±0.30 b	0.015±0.001 b	0.002±0.001 b
	summer	F	0.20±0.13 bc	0.16±0.05 a	2.0±1.2 ab	0.023±0.006 b	0.003±0.002 b
		M	0.17±0.11 bc	0.11±0.04 a	2.8±1.1 a	0.018±0.004 b	0.003±0.002 b
	autumn	F	0.31±0.05 b	0.20±0.06 a	0.70±0.30 b	0.051±0.013 a	0.010±0.004 a
		M	0.33±0.32 abc	0.14±0.07 a	1.6±1.4 ab	0.039±0.014 a	0.003±0.002 b
	winter	F	0.22±0.06 b	0.18±0.04 a	2.7±1.6 ab	0.033±0.011 a	0.004±0.002 b
		M	0.47±0.06 a	0.17±0.02a	0.80±0.30 b	0.030±0.008 a	0.006±0.003 b
Gonads	spring	F	0.10±0.00 d	0.20±0.10 ab	0.013±0.003 e	0.006±0.004 b	BDL
		M	0.11±0.00 d	0.13±0.01 b	0.006±0.000 f	0.006±0.000 b	BDL
	summer	F	0.24±0.01 b	0.26±0.02 a	0.018±0.001 d	0.006±0.001 b	0.002±0.000 a
		M	0.11±0.03 d	0.14±0.01 b	0.072±0.020 c	0.007±0.002 b	0.002±0.000 a
	autumn	F	0.24±0.06 b	0.29±0.06 a	0.004±0.002 e	0.007±0.002 b	0.004±0.002 a
		M	0.17±0.02 c	BDL	0.032±0.001 d	0.014±0.000 a	0.002±0.000 a
	winter	F	0.41±0.07 b	0.34±0.06 a	0.20±0.11 b	0.017±0.004 a	BDL
		M	0.58±0.00 a	0.12±0.00 b	0.43±0.01 a	0.009±0.001 b	BDL

In each column different letters indicate significant differences in the elemental concentration per tissue ($p < 0.05$). Abbreviations: (F) female; (M) male; (hepat.) hepatopancreas; (AI) adequate intakes; (RDA) recommended dietary allowances; (UL) tolerable upper intake levels; (ML) maximum permissible concentrations; (AL) action levels set per day by authorities; (BDL), below detection limit.

Previous studies with several crustacean species, such as *Dardanus arrosor*, *Carcinus mediterraneus*, *Homarus gammarus*, and *H. americanus*, also reported that most important changes in the concentration of elements are found in tissues involved in regulatory processes such as the hepatopancreas in opposition to structural tissues such as muscle (Cuadras *et al.*, 1981; Devescovi and Lucu, 1995; Sub-chapter 4.2). The seasonal and sex differences observed are most probably related with the spawning cycle and metabolism that influence feeding, reproductive state, and weight (Legras *et al.*, 2000).

Cancer pagurus mating season coincides with moulting periods and occurs during summer and early autumn; spawning occurs in late autumn and winter (Tallack, 2007). After spawning, there is a period of incubation of the eggs that can last up to eight months before the broods hatch throughout spring, summer, and early autumn, depending on latitude and temperature (Tallack, 2007). During the premoult period of *C. pagurus* (winter to spring), Ca is accumulated in the hepatopancreas probably to be used in exoskeleton calcification (Luquet and Marin, 2004). Throughout spawning, large females tend to migrate long distances, thus diversifying their feeding grounds, while males are more stationary, staying within the same habitats (Woll, 2006). During the incubation period (winter/spring), females hardly eat (Woll, 2006). Feeding deprivation probably explains the acute low levels of K, Cl, and Br in female gonads and of Mg, Ca, and Sr in both female gonads and the hepatopancreas during spring compared to the high levels in males. In contrast, autumn is a high feeding period for both males and females, resulting in high concentrations of Mg, S, Cl, K, and Zn content in the muscle and hepatopancreas. The high concentration of all macro elements and Fe during winter might be a consequence of the intense feeding during autumn.

No seasonal changes in the content of some elements were observed in the muscle (Na, Fe, Cu, and Se), hepatopancreas (Cu, Se, and Hg) and gonads (K, Ca, Zn, Br, Fe, and Se; Tables 4.7.5-4.7.6). It is likely that these elements are vital for edible tissues all year long, while others such as contaminants are bioaccumulated in edible tissues. As far as contaminants are concerned, As concentration was higher in summer (all tissues), while Cd and Hg (muscle and gonads) were more concentrated in autumn and winter (Table 4.7.6). Lead concentration did not vary in muscle and gonads but had a peak in female hepatopancreas during autumn.

The bioavailability and uptake of nonessential elements by crustaceans depends on environmental factors such as temperature, salinity, and chelating agents (Berge and Brevik, 1996; Rainbow and Black, 2001). In this study, the higher concentrations of Cd and Hg in gonads coincided with winter, a period of lower seawater temperatures and salinities, factors that have been reported to be inversely related with Cd uptake rate by crabs (Legras *et al.*, 2000; Rainbow and Black, 2001). Additionally, adult crabs have annual migrations, moving to deeper waters during winter and shallower waters during summer, thus diversifying their feeding grounds; the females bearing eggs that do not eat during the incubation period are the exception (Woll, 2006).

Comparison with intake recommendations and limits

Worldwide, authorities have set recommendations and limits for elements in food to guarantee its safety and quality. Results presented in tables 4.7.3-4.7.6 for *C. pagurus* tissues, indicate that the average elemental intake is rather variable depending on the tissue, season, and sex. Regarding the nutritional quality of *C. pagurus*, the muscle is an excellent source of Cu and Se (as values can reach the RDA/AI and are below the UL) and a good source of Na and Zn in both sexes all year long (as values are higher than 20 % of the recommended values). The hepatopancreas is an excellent source of Ca (males all year long and females in winter), Cu, Zn, and Se (all year long, both sexes), Mg and Mn (winter males), Cl (all seasons but especially winter), and Fe (autumn and winter). Gonads are excellent sources of Na (winter), Cu (summer), and Se (all year long), and good sources of Mg (winter), Cl (summer males), Fe (summer/autumn/winter females), Zn (females all year long and spring/summer males), and Mn (winter males).

Concerning the potential hazards of *C. pagurus* consumption, only Cu, Cd, and Hg levels were above the limits set by authorities. Copper was above the UL in the hepatopancreas of autumn females. In the hepatopancreas, Cd was above the AL and ML in every season and in male gonads it was above both limits in winter. Cadmium was also above the ML in female gonads during winter. Mercury was above the ML in the muscle and hepatopancreas of most autumn males but always below the AL limit. It is important to remember that ML is only set for muscle and not for other tissues.

Several authors have reported high Cd values in the hepatopancreas of crabs such as *Cancer pagurus* (4.9 mg per 100 g wet weight; Davies *et al.*, 1981), *Pseudocarcinus gigas* (2.24 mg per 100 g dry weight; Turoczy *et al.*, 2001), *Carcinus maenas* (0.3 mg per 100 g dry weight; Bjerregarrd *et al.*, 2005), and *Calinectes sapidus* (0.4 mg per 100 g wet weight; Karouna-Renier *et al.*, 2007), while there are no reports of high Cd values in crabs' gonads so far.

Exceeding the UL, ML, and AL does not necessarily mean that the item is unfit for consumption, as the levels assume the worst case scenarios of food items constituting a major part of the total diet (Turoczy *et al.*, 2001), which is unlikely the case in *C. pagurus*. A more meaningful hazard assessment may be obtained by calculating the amount of toxic elements expected in a typical diet and comparing this to the provisional maximum tolerable daily intake (PMTDI) set by the Joint Expert Committee on Food Additives of the Food and Agriculture Organization of the United Nations (Herrman and Younes, 1999).

Assuming the consumption of three crabs per year (equivalent to 1.5 g portion per day) by a 60 kg adult, the daily Cd intake due to *C. pagurus* hepatopancreas (0.002-0.036 mg) does not exceed this element PMTDI (0.06 mg). However, considering this scenario, Cd can be a concern for regular consumers of *C. pagurus*. Yet, several authors reported that Se has a protective action, especially selenite ($\text{Se}_2\text{O}_3^{2-}$), on many toxicological effects of Cd and Hg (Skowerski *et al.*, 1997a, b). Since the hepatopancreas of *C. pagurus* is rich in Se, this element might counteract Cd and Hg effects. Nevertheless, it is recommended to avoid the regular consumption of *C. pagurus* hepatopancreas in every season since Cd is known to exert a variety of toxic effects, including

nephrotoxicity, osteoporosis, neurotoxicity, carcinogenicity, genotoxicity, teratogenicity, endocrine disruptions, and reproductive disfunctions (EFSA, 2004). Moreover, in autumn female crabs should be avoided considering the high Hg content in muscle.

As far as S, Br, and Sr are concerned, there is still a lack of information regarding their essentiality or toxicity. Considering that these elements are commonly found in marine organisms, there is an urgent need to evaluate their impact on human consumption.

Chapter 5

General discussion

2



3



4



2) *Pulling the lobster traps*
Sue Lynn Cotton (private collection)

3) *Two crabs, 1889*
Vincent van Gogh (The National Gallery, London, UK)

4) *Still life with cat and lobster, 1962*
Pablo Picasso (private collection)

5 General discussion

Today we live in a globalized world where our daily food travels by air, land and sea from the vastness of all continents and oceans to our table. Worldwide, live animals for consumption whether vertebrates or invertebrates, wild or cultured are traded everyday, and crustaceans are no exception. In Portugal, the commercialization of live crustaceans is not a recent trend, as this activity is mentioned in literature dating from the 15th century. Yet, there is a lack of knowledge regarding the trade of live crustacean in the 21st century and its implications to consumers. This thesis is an endeavour to fill this knowledge gap.

5.1 Crustaceans live trade: major problems

The national survey report, presented in sub-chapter 2.1, was a first approach to characterize the live trade of crustaceans from capture to consumption. Several problems were pointed out and are mainly related to the fact that this sector is dependent on import and particularly susceptible to crustaceans' mortality and increasing transport costs owing to petrol prices.

Generally, crab species had greater rates of mortality and shorter duration of storage than lobsters. In particular, *Cancer pagurus* was the most imported species but with one of the highest mortality rates (up to 50 %). The survey was an important tool to alert for the major problems affecting this sector, its critical points in a context similar to a HACCP, and to develop codes of conduct. The *in situ* experiment with *C. pagurus* described in sub-chapter 3.1 was essential to examine in detail specific stressors causing physiological measurable responses during import transport from England and, storage in Portugal. For clarity, the main factors identified along the trade chain as causing stress and mortality are summarized in table 5.1.

Capture is known to be a major stressor but different magnitudes of stress are promoted depending on fishing gear, capture depth and duration; e.g. creel is less stressful than trawling (Ridgway *et al.*, 2006). Typically, after hauling the catch and through the entire trade chain, more careful handling is used in more expensive species, such as spiny and clawed lobsters, in opposition to crabs. For instance, immediately after capture edible crabs are nicked to immobilize the claws, while in homarids, plastic bandages are used.

Following capture, the unloading process at the pier caused different magnitudes of stress and mortality to the same consignment of *C. pagurus* (Sub-chapter 3.1). Prior to transport, crabs that were conveyed in the top of the vivier lorry were exposed to air longer, developed more lactate and an acidic pH, than those transported at the bottom.

Transport is also a major stressor in the live trade of crustaceans. *H. gammarus*, *C. pagurus*, and other crabs are mainly imported from the UK to Portugal in immersed conditions at too high densities (Sub-chapter 2.1). As a consequence, seawater can become hypoxic and acidic during transport ultimately leading to death (Wyman *et al.*, 1985).

Table 5.1 Factors identified along the trade chain that cause stress and promote mortality.

Trade chain	Factor	Result	
1) Capture (English Channel) From fishing ground to harbour	Gear, duration, depth Poor handling Mechanical damage (e.g. nicking, see photo) High density Air exposure Temperature variations	Mortality Missing legs and claws Tips of legs broken Bleeding/infection Metabolic rate variation Anaerobic metabolism Dehydration	
2) Unloading (Weymouth) from fishing vessel to vivier lorry	Poor handling Air exposure Temperature variations Mechanical damage	Missing legs and claws Anaerobic metabolism Dehydration Bleeding/infection	
3) International transport (~58 h) in vivier lorries	High density Mechanical damage Deterioration of seawater quality: low dissolved oxygen and pH, high ammonia and nitrites	Mortality Condition deterioration Missing legs and claws Bleeding/infection Anaerobic metabolism	
4) Reception (Setúbal) at importers premises	Poor handling Air exposure Temperature changes High densities Deterioration of reception water	Missing legs and claws Bleeding/infection Dehydration Anaerobic metabolism	
5) Storage at importers (Setúbal)	Poor handling Density fluctuation Hypoxia/aerial exposure Pump vibration/noise Starvation	Mortality Condition deterioration Missing legs and claws Bleeding/infection Variation of metabolic rate Catabolism	
6) National transport (Portugal)	Poor handling Mechanical damage Hypoxia/aerial exposure Temperature changes	Mortality Condition deterioration Missing legs and claws Bleeding/infection Anaerobic metabolism	
7) Storage at wholesalers and retailers (Portugal)	Poor handling Density fluctuation Hypoxia/aerial exposure Pump vibration/noise Starvation	Mortality Condition deterioration Missing legs and claws Bleeding/infection Variation of metabolic rate Catabolism	

Photo 1, adapted from the EU Collective Research CRUSTASEA project; photos 2 to 7 by Sara Barrento ©

During the live trade of *C. pagurus* from England to Portugal (Sub-chapter 3.1) deterioration of seawater was evidenced, as dissolved oxygen (2.8 mg L^{-1}) and pH (7.41) were lower than normal environmental values of 6 mg L^{-1} and 7.8-8.2, respectively (Chartois *et al.*, 1994). Similar results were reported for *N. puber* after 57 h of transport at 12.7°C ; seawater pH was 6.87 and oxygen saturation fell from 84 to 50 % (Wymann *et al.*, 1985). The high densities also caused more missing legs and claws to bottom crabs than to top ones, and in particular bottom males had more missing claws than females.

Unloading to the reception tanks, after long duration transport is a critical procedure for crustaceans that have just experienced prolonged stress. At this phase animals have little tolerance to sudden temperature changes, aerial exposure, and physical damages promoted by handlers, *i.e.* stressors that otherwise crabs could withstand, can become lethal after a long journey (Ugnow, 1997a).

On the other hand, the lower transport densities of lobsters enables the individual grading (vigour check) prior to transport and careful loading in cardboard under moist and refrigerated conditions, or in tanks with refrigerated seawater. After transport, the reception is again most careful as lobsters are individually checked and revitalised in tanks with low animal density (Sub-chapter 2.1).

In sub-chapter 3.1 vigour evaluation also proved to be an efficient grading tool for *C. pagurus*, as 67 % of crabs categorized as moribund died against only 8 % of crabs classified as weak. However, further experiments are still required especially regarding sex differences and seasonality. In sub-chapter 3.1 it was pointed out that males were more vigorous after transport than females and, in the end, 26 % of females died against 16 % of males. Ugnow *et al.* (1986) stressed the importance of life cycle especially because spawning females were found to be more susceptibility to transport stress.

Storage conditions were different mainly because traders either use open, semi-open or closed systems and also due to the different activity types. Importer trade more crustaceans per week than retailers. Nevertheless, the daily routine is similar in most traders and, is stressful. The routine includes animal checking twice a day to discard dead animals. However, due to the poorly designed holding tanks, which are basically concrete swimming pools, the regular stock quality control assessment is most adverse as seawater is almost completely drained, while one handler is inside the tank with a stick to remove dead animals. The same procedure is done whenever a buyer needs a fair amount of crustaceans. Therefore, at least twice a day, all animals suffer periods of hypoxia and air exposure of at least 2 h. Though this is apparently a short duration period, Webster (1999) reported that even only a 30 minute of aerial exposure is sufficient to increase lactate concentration from nearly 0 to 2.5 mM in the haemolymph of *C. pagurus*. A further 2 h exposure period increased this value to 10 mM . Starvation was also a factor pointed out as most lobsters are fed with live mussels, while *C. pagurus* face periods of starvation thus increasing the rate of haemolymph protein losses (Ugnow *et al.*, 1986). Most crustaceans use their protein reserves, including haemocyanine as a food source during starvation. In sub-chapter 3.3, haemocyanine concentration decreased in crabs that were recovering from simulated 48 h

transport after at least 11 days starving. This process, also known as catabolism can lower total protein by 22 % and haemocyanine by 26 % after 20 days without feeding which limits the recovery process of crabs that should occur at the holding facilities (Ugnow *et al.*, 1986). Also during storage, the management of water quality is not properly performed as most personnel are not aware of the importance of water quality parameters and trained to implement correct procedures if a problem arises. Even when these parameters are measured, surely they are not monitored on a regular basis.

Distribution of live crustaceans across Portugal uses a wide variety of baskets or crates, which are poorly designed, e.g. with holes where animals insert their limbs, resulting in appendages fractures (Figure 5.1).



Figure 5.1 From left to right, boxes used to transport *N. puber*, *Maja* spp., and *C. pagurus* (Photos by Sara Barrento ©).

This is an additional stressor already identified by Ugnow (1997a) in respect to the trade of live brown crabs in Spain, that could be avoided with an integrate crate system allowing for easy handling and utilization for both holding and transporting animals from shipboard to the final destination.

Though the survey did not reveal significant differences in mortality storage data in respect to activity type (importer, wholesaler and retailer) or to water supply system (open, closed or semi open) there was a tendency for mortality to increase along the trade chain (Figure 5.2). The longer the trade chain, more stressors are inflicted to crustaceans, and the magnitude becomes more variable.

Stress is cumulative, and mortality is usually postponed. For instance, transport is an important stressor, but major mortality occurs at the reception facility in the following 96 h (Sub-chapter 2.1 and 3.1). Several variables contribute to the mortality rate, these can be environmental (e.g. season, food availability), biological (e.g. life cycle) and further triggered by handling procedures. Clearly, no single variable can be identified as causing high mortality but it is rather the result of the sum of events. Therefore, the fact that crabs die more than lobsters is probably the result of poorer handling of crabs, together with a lower biological resistance.

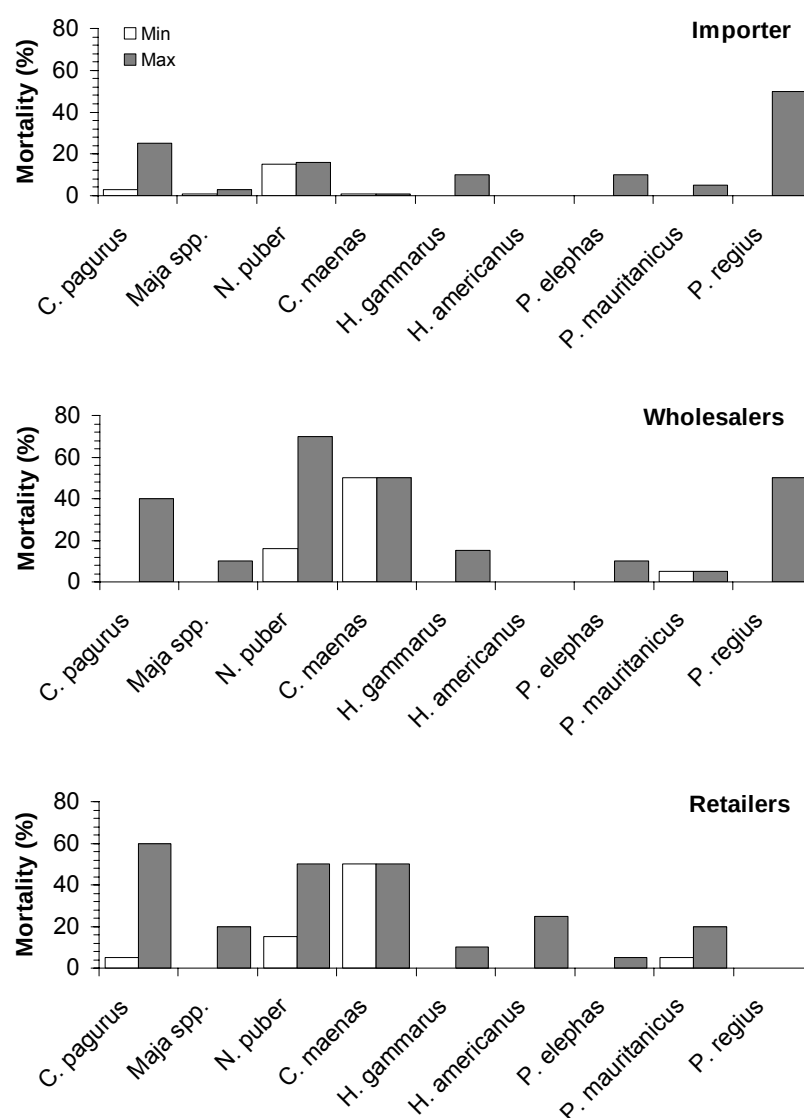


Figure 5.2 Mortality of live crustaceans traded in Portugal at importers, wholesalers and retailers. Minimum values are represented as white bars, and maximum values as grey bars (data from Sub-chapter 2.1).

Understanding the influence of live trade on crabs' mortality is fundamental to envisage alternative solutions. Poor handling was a continuous factor in all steps of the trade chain of live *C. pagurus*. It was also observed that immersed transport does not avoid hypoxia and that mechanical damage can be inflicted almost unwittingly. Though damage can promote immediate economical losses mainly due to cripple males, *i.e.* crabs missing one or both claws, traders still act as if crabs are unbreakable.

Overall, seven main events were identified along the trade chain of live crustaceans that are critical and promote mortality. The national distribution of *C. pagurus* is one of the last events and therefore, crabs that suffered cumulative stressors must face an additional transport in severe conditions thus promoting further mortality at wholesalers and retailers. This information prompted the experiments simulating the national transport conditions described in sub-chapters 3.2 and 3.3.

The aim was to compare different transport systems, including immersed and semi-dry conditions. Semi-dry transport is particularly wanted by traders not only because transport is independent of available seawater, but it also reduces transport costs by increasing the loading factor of crabs in refrigerated lorries or even in air freight.

5.1.1 Simulated national distribution of *C. pagurus*

Though transport in semi-dry conditions is a common and efficient practice for long transport of live homarid and palinurid lobsters, it is much less used for crabs, mostly because mortality is usually higher and profits are lower. However, as demand for crabs increase worldwide mainly in Asian countries, research has focused in testing different semi-dry transport systems for crabs (Ottwell and Webb, 1977; Wyman *et al.*, 1985; Lorenzon *et al.*, 2008).

C. pagurus exposed to air at 15 °C has proven to elicit lower mortality (17.0 %) after 24 h than those reported for *N. puber* (76.9 %), but similar to *Callinectes sapidus* (16.1 %) at 10 °C (Ottwell and Webb, 1977; Wyman *et al.*, 1985; Table 5.2). Yet, 17 % mortality is too high and thus semi-dry transport conditions must be further improved.

Table 5.2 Cumulative percent mortality of different crab species during aerial exposure at several temperatures.

Species	Conditions	Time intervals (h)					Reference
		12	24	48	72	96	
<i>C. sapidus</i>	5 °C _{n=24}	6.7	27.3	50.6	72.6	89.5	Ottwell and Webb (1977)
	10 °C _{n=24}	6.1	16.1	32.8	56.3	76.9	
	20 °C _{n=24}	11.7	32.5	61.3	77.4	79.1	
<i>N. puber</i>	15 °C _{n=13}	-	76.9	-	-	-	Wyman <i>et al.</i> (1985)
<i>C. pagurus</i>	15 °C _{n=13}	-	17.0	-	-	-	

In this context, several relevant issues to the trade of live *C. pagurus* in Portugal were taken into consideration in the design of the experiments tested in sub-chapters 3.2 and 3.3, including:

1. The evidence that transport is efficient in semi-dry conditions and is employed for other crustacean species with lower mortality than those reported by Portuguese traders of *C. pagurus* for national distribution in air exposure conditions (40 %).
2. The variable refrigerating temperatures in most Portuguese holding systems.
3. The traditional resistant attitude of traders to procedure changes lead a cautious design of these experiments and therefore some common practices were also simulated and the transport period was extended to 48 h.
4. Also, recommendations regarding national transport took in consideration the least expensive system type and, implementation easiness.

The current national transport is usually done at 12 °C either in dry or immersed conditions with piled crabs. Immersed conditions were simulated, but instead of dry transport, semi-dry conditions were tested in boxes without holes, and that avoid direct contact with fluid excretions. Animals were not piled; instead they were tightly packed in a single row. In order to test the effectiveness of different transport, both conditions, immersed and semi-dry transport, were compared in sub-chapter 3.2 at different temperatures (8, 12 and 16 °C) with and without an anaesthetic (AQUI-S®). Since mortality is often postponed to later stages of the trade chain, another experiment tested the viability of crabs subjected to transport and during recovery at different acclimation temperatures (sub-chapter 3.3). An overview of findings obtained in both experiments is presented in table 5.3, highlighting the best transport systems tested (0 %; mortality), as well as not recommended (0 % mortality but costly and laborious), acceptable (1-5 % mortality), and not acceptable (> 5 % mortality).

Table 5.3 Transport systems tested that are recommended, in opposition to those that should be avoided according to transport duration (8 and 24 h; acceptable, not recommended, or not acceptable).

Transport system		4 °C	8 °C	12 °C	16 °C
8 h	WT	Not acceptable M=14.0	Not recommended		Not acceptable M=12.5
	WQS	-	Not recommended		
	AIR	Recommended (autumn)	Recommended (summer)	Acceptable	
	AQS	-	Not acceptable M=25.0	Not recommended	
24 h	WT	-	Not acceptable M=11.1	Not recommended	Not acceptable M=75.0
	WQS	-	Not recommended		Not acceptable M=12.5
	AIR	Acceptable (autumn)	Recommended (summer)	Not acceptable M=28.6	Not acceptable M=25.0
	AQS	-	Not acceptable M=25.0	Not recommended	Not acceptable M=25.0

The criteria included mortality rate, physiological stress and implementation easiness. Abbreviations: (AIR) crabs exposed to air in semi-dry conditions; (WT) immersed crabs in seawater with aeration; (AQS) air exposed crabs in semi-dry conditions pre-treated with AQUI-S®; (WQS) immersed crabs in seawater dissolved with AQUI-S®; (M) mortality %.

The results allow concluding that higher temperatures and long transport duration are critical factors that promoted mortality. Similar observations were previously mentioned regarding *C. sapidus*, *N. puber* and also *C. pagurus* (Otwell and Webb, 1977; Wyman *et al.* 1985). *C. pagurus* survival at different temperatures also seemed to be affected by season, as during summer immersed crabs at 12 °C had no mortality, but at 8 °C mortality reached 22.2 % (sub-chapter 3.1). In contrast, during autumn, crabs transported at 12 °C displayed 12.5 % mortality (sub-chapter 3.2). According to these evidences the best transport temperature is season dependent. Consequently, for transport durations of less than 8 h, the best recommended transport systems were semi-dry at 4 and 8 °C during autumn and summer, respectively. For longer periods (up to 24 h) the best recommended option was semi-dry transport at 8 °C in summer. During autumn, at 4 °C mortality was still 4 %, this might mean that crabs during this season are less resistant or that transport temperature must be rechecked. Cuculescu *et al.*, (1998), concluded that the upper critical temperature limit for *C. pagurus* is season dependent as during summer the upper limit is higher than during winter. Most probably the same is valid for the lower critical temperature, during winter being lower than during summer. Though transport in seawater at 12 °C without anaesthetic and at 8 °C with AQUI-S® had no mortality after 24 h, both systems are not recommended because of their higher costs and labour than the semi-dry system.

The results obtained in sub-chapters 3.2 and 3.3 were much better than the 17 % mortality reported by Wyman *et al.* (1985) and definitely much lower than the 40 % reported by national traders. Apparently, the resting period after international import transport, the use of proper boxes without holes that avoid direct contact with excretion fluids, the moist conditions given by the wet cloth, the single layer of tight animals and appropriate transport temperature are simple procedures that contributed to make the difference.

As far as haemolymph parameters are concerned, L-lactate, pH and to a less extend D-glucose concentration were the most reactive haemolymph parameters to the development of anaerobic metabolism. Though the development of anaerobiosis was a common factor in all treatments, the magnitudes were different and the values presented in table 5.4 corroborate that the transport treatments promoting mild responses were also those with lower cumulative mortality. Such transport treatments were semi-dry at 4 °C in autumn and 8 °C in summer, and immersed at 12 °C (summer) in opposition to higher temperatures.

Temperature is in fact able to govern the severity of stress. Crabs are cold-blooded animals whose metabolic rates are largely determined by external temperatures. Within limits the higher the temperature the higher the metabolic rate and the oxygen consumption. If the environmental conditions during transport become hypoxic, temperature raise will be more detrimental; the animals will become more stressed by inadequate oxygen supply and, will eventually die (Otwell and Webb, 1977). However, the range of temperature choice varies with the species ecological tolerance that depends on the geographical location of the animals, season and sex. For instance, Otwell and Webb (1977) reported that the seawater temperature in the area where *C. sapidus* was captured varied from 22 to 32 °C, and the best temperature to transport these crabs was 10 °C.

Table 5.4 Haemolymph pH and L-lactate concentration in *C. pagurus* during *in situ* simulated transport considering the best and worst treatments for cumulative mortality presented in sub-chapters 3.1, 3.2 and 3.3, and by Lorenzon *et al.* (2008).

	Transport system	Simulated transport duration				Reference
		24 h	36 h	48 h	58 h	
Lactate mM	AIR 4 °C	5.60 ± 2.41	-	5.90 ± 3.35	-	Sub- chapter 3.3
	AIR 8 °C	2.93 ± 1.75	-	1.75 ± 0.28	-	
	WT 12 °C	4.16 ± 1.61	-	2.13 ± 0.24	-	Sub- chapter 3.2
	WT 16 °C	21.19 ± 0.05	-	D	-	
	AQS 16 °C	24.07 ± 2.92	-	D	-	
	Immersed 13°C	-	-	-	2.19 ± 0.19	Sub- chapter 3.1
	Immersed 10-13°C	-	9.44 ± 0.72	-	-	Lorenzon <i>et al.</i> (2008)
	AIR 10-13°C	-	10.81 ± 1.12	-	-	
pH	AIR 4 °C	7.41 ± 0.13	-	7.47 ± 0.08	-	Sub- chapter 3.3
	AIR 8 °C	7.63 ± 0.08	-	7.63 ± 0.08	-	
	WT 12 °C	7.60 ± 0.03	-	7.65 ± 0.03	-	Sub- chapter 3.2
	WT 16 °C	6.91 ± 0.12	-	D	-	
	AQS 16 °C	7.03 ± 0.12	-	D	-	
	Immersed 13°C	-	-	-	7.16 ± 0.12	Sub- chapter 3.1
	Immersed 10-13 °C	-	8.01 ± 0.02	-	-	Lorenzon <i>et al.</i> (2008)
	AIR 10-13°C	-	7.86 ± 0.07	-	-	
Glucose mM	AIR 4 °C	0.50 ± 0.25	-	0.42 ± 0.17	-	Sub- chapter 3.3
	AIR 8 °C	0.41 ± 0.10	-	0.26 ± 0.08	-	
	WT 12 °C	0.47 ± 0.26	-	0.82 ± 0.74	-	Sub- chapter 3.2
	WT 16 °C	0.39 ± 0.33	-	D	-	
	AQS 16 °C	1.65 ± 0.42	-	D	-	
	Immersed 13°C	-	-	-	0.26 ± 0.10	Sub- chapter 3.1
	Immersed 10-13 °C	-	1.63 ± 0.15	-	-	Lorenzon <i>et al.</i> (2008)
	AIR 10-13°C	-	1.03 ± 0.15	-	-	

Abbreviations: (AIR) crabs exposed to air in semi-dry conditions; (WT) immersed crabs in seawater with aeration; (AQS) air exposed crabs in semi-dry conditions pre-treated with AQUI-S®; (D) all crabs died.

Temperature also influences the efficiency of anaesthetics, as low temperature can have an anaesthesia-inducing effect *per se*. Therefore, the combination of low temperature with the use of anaesthetic can be unfavourable as observed in the air exposed crabs at 8 °C pre-anaesthetised with AQUI-S®. In opposition, the use of anaesthetic at higher temperatures in seawater seems to counteract the detrimental effect of temperature.

During transport, progressive increase in L-lactate content was observed as the duration of hypoxia increased up to 48 h, being more pronounced at higher temperatures. This is a common trend reported in lobsters, crabs and shrimps (Taylor and Spicer, 1987; Spicer *et al.*, 1990; Durand *et al.*, 2000; Ridgway *et al.*, 2006). In sub-chapters 3.2 and 3.3 this trend was accompanied at higher temperatures by an extreme acidification of the haemolymph and by severe mortalities (up to 100 %; Table 5.4). The acidosis that develops is promoted by the increased L-lactate levels and accumulation of CO₂ during anaerobiosis, which is sufficient to overwhelm the buffering capacity (Taylor and Whiteley, 1989). Therefore, the initial industrial procedure of keeping crabs exposed to air at ambient temperature, waiting to be loaded is a much negative handling practice, especially in warmer months.

On the other hand, glucose seems to be less affected by external variables and according to Durand *et al.* (2000) the increase in blood glucose content of *M. squinado* is not dependent either on seasonal conditions, or sex or length of the emersion period and apparently a glucose threshold fully develops within the first 12 h of emersion. Ridgway *et al.* (2006) reported changes in haemolymph glucose concentration of *N. norvegicus* in relation to exposure temperature. In sub-chapters 3.2 and 3.3 few correlations were obtained between glucose, temperature and transport duration. Glucose maximum concentration registered for *C. pagurus* during short term emersion was 2.05 mM (1 h; Sub-chapter 3.1); 1.35 mM (4 h; Uglow *et al.*, 1986) and 2.22 mM (4 h; Webster, 1996). Apparently there is a threshold (~2 mM) that is obtained few hours after hypoxia is experienced and which is maintained under these conditions as long as tissues are enabled to re-supply glucose to haemolymph from their glycogen reserves. Glucose is mainly re-supplied to the plasma via tissue based glycogenolysis occurring in the hepatopancreas, muscle and haemocytes. Once exhausted of glycogen, tissues are unable to re-supply glucose to the haemolymph and maintain carbohydrate homeostasis (Ridgway *et al.*, 2006).

In ideal conditions, immersion would not have promoted anaerobic metabolism, but during transport animal density is too high, and dissolved oxygen is too low. As a consequence stress response was not much different between immersed and semi-dry transport conditions at the same temperature (Table 5.4). Apparently, crabs transported in semi-dry conditions did not show more physiological stress than that measured immediately after the immersed import transport over 58 h (Sub-chapter 3.1) and 36 h (Lorenzon *et al.*, 2008). It can also be speculated that crabs not submitted to air exposure at ambient temperature before transport, or if crabs were dipped in colder seawater for a couple of minutes before transport (probably at 6 °C in autumn), similarly to the transport practice of *J. edwardsii*, anaerobic metabolism could be delayed and survival further improved. In the case of *J. edwardsii* pre chilling significant delays anaerobiosis for up to 24 h (Morris and Oliver, 1999). However, such changes must be tested to evaluate their cost-benefit for *C. pagurus*.

Crustaceans are exposed to a large number of environmental variables, which follow annual and daily cycles according to their geographical region that affect their behaviour, life cycle, feeding and metabolism (Buckup *et al.*, 2008). Reports from importers obtained in sub-chapter 2.1 and by

Uglow *et al.* (1986) suggest that the greatest mortalities occur during summer months. Most traders, based on their empirical experience are also of the firm opinion that Scottish crabs are generally of less quality than crabs captured in the English Channel, which influences the final price as observed in sub-chapter 4.3 to 4.5. Therefore, additional research is still required to understand the seasonal and geographical influence on *C. pagurus* physiological condition that may predispose some crabs to premature mortality.

5.1.2 The importance of acclimation and recovery

Transport mortality *per se* is not sufficient to evaluate the real benefit of a transport system as mortality is postponed (Durand *et al.*, 2000). Therefore, mortality and physiological parameters were measured immediately after transport and during recovery from international *in situ* transport (Sub-chapter 3.1) and simulated national transport (Sub-chapter 3.3; Table 5.5). Comparatively to import transport, the simulated conditions of national transport elicited *per se* a much higher mortality immediately after transport. Such difference can be due to the smaller sample size in simulated national transport. Yet, during the recovery period, mortality of semi-dry transported and acclimated crabs from 6 to 16 °C was similar or even lower than import transport (Table 5.5). In fact, acclimated crabs, not subjected to drastic temperature changes had less mortalities and stronger vigour.

Table 5.5 Cumulative mortality (%) of *C. pagurus* during recovery from live import and national transport.

Transport Type	D (h)	T (° C)	Recovery conditions	Recovery time intervals (h)								Reference
				AT	6	8	12	24	48	72	96	
Import immersed <i>in situ</i> (S)	58	13	13-15 °C n=56	1.8	5.4	-	-	7.1	12.5	12.5	19.6	Sub-chapter 3.1
Import immersed <i>in situ</i> (S)*	58	13	13-15 °C n=6.5 t	0.92	-	-	-	-	-	-	20	
Import immersed <i>in situ</i> (A)	36	10-13	11-14 °C n=20	-	0.0	0.0	-	30.0	30.0	30.0	30.0	Lorenzon <i>et al.</i> (2008)
Import semi-dry <i>in situ</i> (A)	36	10-13	11-14 °C n=20	-	0.0	0.0	-	0.0	0.0	0.0	0.0	
Simulated national immersed (A)	48	12	16 °C n=8 (not acclimated)	25.0	25.0	25.0	-	25.0	37.5	37.5	-	Sub-chapter 3.3
			16 °C n=8 (not acclimated)		12.5	12.5	-	12.5	25.0	25.0	-	
Simulated national semi-dry (A)	48	4	16 °C n=8 (acclimated from 11 to 16 °C)	4	12.5	12.5	-	12.5	12.5	25.0	-	
			16 °C n=8 (acclimated from 6 to 16 °C)		0.0	0.0	-	0.0	12.5	12.5	-	

Abbreviations: (D) duration; (T) temperature; (S) spring; (A) autumn; (*) traders data report.

Vigour check was found to be fundamental tool to grade *C. pagurus* as crabs that become moribund almost inevitably die in the following hours. Therefore, the transport of weak and moribund crabs should be avoided, and also be graded immediately after transport and periodically during storage so that moribund crabs can be processed as soon as possible and the remaining fully recovered. The implementation of this simple procedure will reduce the waste of a valuable resource along the trade chain. In table 5.6 an example of a grading system and some recommendations are provided.

Table 5.6 Grading scale based on vitality condition and recommendations.

Strong

Prompt, strong, aggressive response or defensive position
 Cling to hand when held upside down
 Eyestalk response
 Mouthpart strong

Recommendation: can be live transported further in the trade chain.



Weak

Weak legs and claws, no aggressive response
 Slow reaction when touched in abdominal area
 Loosen the grip when held upside down
 Eyestalk response
 Mouthpart strong

Recommendation: process or try to recover the animals in water before further transport.



Moribund

No claw response
 No reaction when touched in the abdominal area
 No grip when held upside down
 Slow eyestalk response
 Mouthpart slack

Recommendation: remove from tanks and if possible process immediately



Dead

The same lack of reaction as in moribund crabs
 Without any movement in antennae and mouthparts when touched

Recommendation: remove from tank and do not process.



Photos by Sara Barrento ©

Comparing mortality values and haemolymph parameters (Sub-chapters 3.1 to 3.3) it can be concluded that both international and national transport in semi dry conditions promotes a similar stress response to the immersed systems as long as semi-dry transport is kept at low temperatures and preferably with proper acclimation. During recovery either from import or national transport, it can be concluded that aerial exposure in semi-dry environment improves the condition and survival of crabs (Lorenzon *et al.*, 2008; Sub-chapter 3.3). Therefore, this transport system can be used either during import transport and national distribution, which is an advantage from an economical and quality perspective.

Nevertheless, in order to implement transport in semi-dry conditions, reception tanks must be properly designed and small adaptations in the reception facilities might be needed to address crabs' physiological needs. As crabs transported in air consume much less O₂ than in seawater, and ammonia is accumulated, reception tanks must have high dissolved oxygen and efficient skimmers to accommodate crabs with high oxygen consumption rate and ammonia excretion (Uglow *et al.*, 1986; Lorenzon *et al.*, 2008). Refrigeration is also fundamental, to avoid temperature changes. As an alternative, cascade systems similarly to those already used for American lobster could be used to accommodate *C. pagurus*. This could be achieved with the development of transport boxes matching the requirements of the shipping companies in a light material with a good insulation capacity. Boxes should be designed in order to be used in the fishing vessel, so that handling would be reduced to the minimum from harvesting to international markets. Once at the importer holding facilities crabs could be flushed with good quality seawater and temperature slowly increased (e.g. 1.5 °C per hour), so that ammonia could be excreted without compromising seawater quality of the holding tanks and allowing crabs 'acclimation (Figure 5.3).



Figure 5.3 Illustration of prototype boxes to accommodate crabs from the fishing vessel to holding facilities (adapted from the EU Collective Research CRUSTASEA project).

5.2 Major outcomes and recommendations

The current work enabled the compilation of scientific data necessary to improve *C. pagurus* survival during live trade. The traditional practice of selling live crabs is a good standard to assure food safety, but limits the marketability to crabs' survival time under the extreme conditions of trade. Moreover, *C. pagurus* survival varies with season, geographic origin, sex and handling procedures, thus further limiting their marketability.

Contrary to traders beliefs survival can be improved in every step of the trade chain. Knowledge about *C. pagurus* ecological needs and tolerance to emersion and temperature changes, along with good handling practices, seems to be the key factors to success. The same underlying principle can be implemented to other species, such as *N. puber* and *C. maenas*, which also have high mortality. Semi-dry transport of *C. pagurus* seems to be efficient either during import and national distribution. This study was a first approach and the next step could be the development of *in situ* tests in industrial operating conditions. However, to test and implement this transport system the following recommendations are essential along the trade chain to assure animal survival:

1. **Capture:** non-invasive restraining of claws is highly recommended. Jackline and Lart (1995) observed that the quality of banded crabs, in terms of liveliness, lost claws and condition was superior to that of nicked crab, not to mention the observed reduced mortality.
2. **Post-capture:** holding crabs in good conditions before international import transport might be essential for crabs to recover from capture inflicted stress possibly decreasing mortality afterwards.
3. **Unloading and reception:**
 - a) special care should be taken during transfer either from boat to truck or from truck to holding premises. The mechanisation of the loading/unloading processes would be most advantageous. Less handling steps could also be achieved with a crate system designed to transport and store animals;
 - b) identification of the best time of day to load/unload and to store crabs according to the circadian rhythmicity of the animals. This can be beneficial as many crustaceans are known to have a peak of foraging activity after dusk, reflected by the circadian rhythmicity of the crustacean hyperglycaemic hormone (Kallen *et al.*, 1990). Also, it was reported by Spanoghe and Bourne (1997) that live *Panulirus cygnus* packed during evening were more likely to exhibit higher mortality rates in live shipments than those packed during the day. On the other hand, Durand and Regnault (1998) indicated that *N. puber* showed higher survival to prolonged emersion at 18 °C during the night than during the day. Danford and Uglow (1994) concluded that *C. pagurus* excretes ammonia in higher quantities during darkness and discuss that transport and storage can be improved by any means of reducing ammonia production.

4. Storage: reception tanks of crabs after semi-dry transport must have refrigeration, sufficient dissolved oxygen and a proper scaled skimmer to avoid accumulation of the excreted ammonia. Alternatively a cascade system could be used, though higher quantities of good quality seawater are required to flush the crabs.

5. Whole trade chain:

- c) quality should be the keyword and grading is an essential tool to achieve high-quality products. Standardized grading should be implemented from capture to the final destination (including restaurants); animals in poorer condition should be immediately processed.
- d) seawater quality should be a priority in every step of the trade chain. Drastic temperature changes ought to be avoided. Water quality at the reception tanks must be monitored regularly, especially considering that stock densities are much variable.
- e) air exposure and handling at ambient temperature is not admissible especially in warmer months.
- f) real improvements can only be achieved if a standardized code of conduct is implemented in all countries involved in *C. pagurus* live trade chain. There is a clear need for instruction and training of all actors involved in the trade chain (fishermen, exporters, importers, wholesalers and retailers).
- g) further experiments to optimize the best transport and recovery temperatures might be needed or even to test more efficient anaesthetic concentrations that can be useful in the more stressful stages of the trade chain (e.g. capture, unloading). Traders must be aware that for the same problem different solutions can arise and different procedures might be needed throughout the year according to temperature changes. It is important that traders are willing to be flexible in their established practices.

Traceability and a narrower trade chain would also assist the technical procedures required to achieve a better transport and standardized handling and holding practices. However, presently two major drawbacks exist: crabs are still insufficiently valued, at least not as much as lobsters, and therefore, systems are less cost effective. Additionally, the implementation of a new system requires instructed and trained traders.

During the development of this work a training session was given to importers, retailers and supermarket quality managers. It was evident that there is a major unawareness of most problems and solutions. Mortality is seen as part of the business and in the end the consumers pay this extra cost. This is a major obstacle and ultimately the implementation of such technical solutions is more difficult than investment in the acquisition of scientific knowledge. Unfortunately, the solutions proposed in this work might only be put in place when unsustainable practices lead to stock scarceness and the consequent increase of *C. pagurus* economical value, which can be too late for the viability of this crab.

5.3 Commercial and nutritional value of homarids and *C. pagurus*

The enormous effort dedicated to commercialize live crustaceans has the purpose of supplying high quality fresh animals. However, in the context of live marketing, a live animal is not necessarily synonymous of a product with high quality (Uglow, 1997b). From a consumer's perspective common sense dictates that there are times of the year when crustaceans are of better quality (e.g. meat yield). From a biological perspective it is clear that during the life cycle of any crustacean the biochemical condition and quality varies due to several natural factors.

The nutritive and commercial value of seafood mainly depends on the structure of flesh and other edible parts, on the proportion of the edible tissues in the total mass of the specimen, on the chemical composition of these tissues, and on factors related to fishing and handling procedures (Sikorski, 2000). In the end, seafood commercial value is affected by traders and consumers' expectations, preferences and beliefs. Consumers' expectations in the particular case of *C. pagurus* include the amount of brown and white meat (claw muscle) content, whereas for Homarids, claw and abdominal muscle are more appreciated. It is also common knowledge among traders and consumers that *H. gammarus* taste better than *H. americanus* and thus are much more expensive (Sub-chapters 2.1; 4.1 and 4.2). A similar principle exists regarding the edible crabs from the English Channel (EC), which are known to be of higher quality comparatively to those from the Scottish Coast (SC; Sub-chapter 4.3 to 4.5). Additionally, most consumers have the perception that crustaceans are merely delicatessen starters, eaten at special festivity occasions, and associated to life style and status. Usually included in the unhealthy category of food, crustaceans are rarely associated to a nutritious full meal, and this concept is contrary to the common knowledge that seafood is healthy.

Taking into account consumers expectations and habits, several parameters were addressed in this thesis to evaluate the nutritional composition and edible tissues proportion of homarids and *C. pagurus*.

5.3.1 Meat yield of female and male specimens

Purchasing a crab or a lobster is a ritual that must fulfil consumers' expectations and, without doubt, the meat yield is one of the most important criteria when it comes to eating.

Figure 5.4 shows the percentage yields of brown meat and muscle in homarids and edible crab. While, in the latter, muscle is responsible for the biggest proportion, in the former brown meat contribution is usually higher. However, the proportion of edible meat sampled for *C. pagurus* was variable throughout the seasons due to moulting and breeding cycle. This was particularly significant in females owing to changes in the brown meat content following the development of gonads (Sub-chapter 4.6).

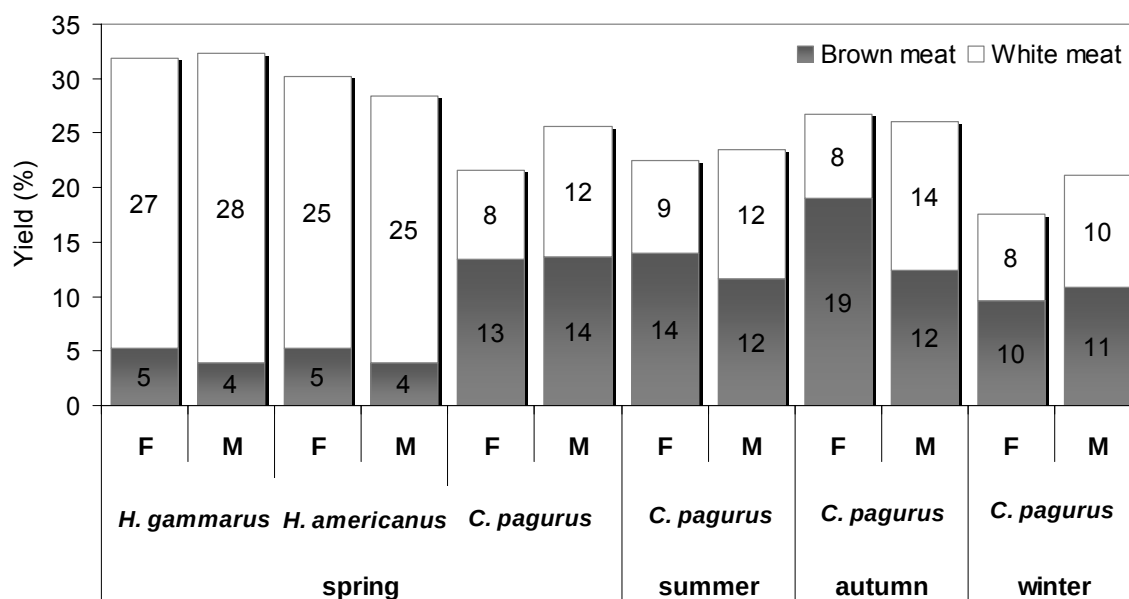


Figure 5.4 Brown and white meat yields of *H. gammarus* and *H. americanus* in spring and, of *C. pagurus* in the four seasons for both sexes. Brown meat, equivalent to hepatopancreas and gonads; white meat, claw muscle (*C. pagurus*); white meat, claw and abdomen muscle (homarids). Abbreviations: (F) females; (M) males. Data from sub-chapters 4.1 and 4.6.

The highest GSI was in autumn corresponding to the greatest proportion of ripe females. In figure 5.5 the GSI is presented with the reproductive life cycle of females. During summer months female moult and mate while they are still soft.

Ovaries start to develop in autumn, either in the same year when moulting takes place, or the following year. Ovaries mature and the eggs are spawned in late autumn or beginning of winter, depending on latitude. In the following months, the eggs are incubated and eventually larvae hatch by the end of spring and through summer (Figure 5.8; Edwards 1979; Woll 2003; Tully *et al.*, 2006). The chances of having better yields are greater in autumn than in any other season, but even so some crabs can be immature and poor in meat content.

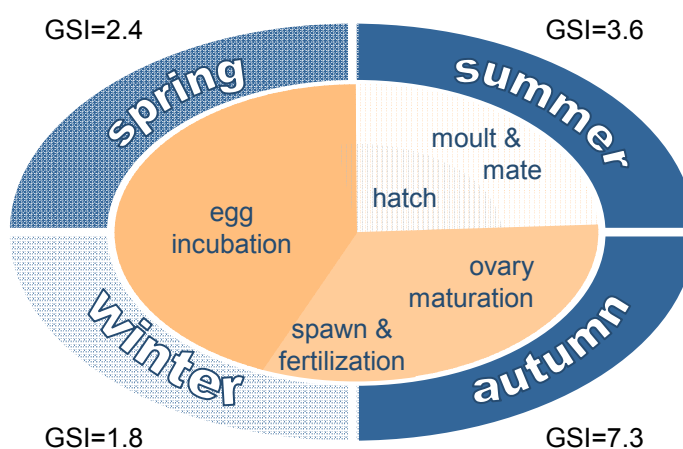


Figure 5.5 Reproductive cycle of female *C. pagurus* and gonadosomatic index (GSI).

This issue is so important that several attempts have been made to develop a practical and efficient device able to accurately grade crabs and lobsters. Recently, the European project CrustaSea carried out research to develop an image scanning device based on near-infrared spectroscopy (NIR) for determination of edible content in the edible crab shell. The NIR spectrum of a scanned crab contains information about fat, water and protein. Two prototypes were designed, one to be used at sea and another to be used at processing plants. If such a system is to be implemented a better discrimination of the quality meat yield will be obtained (EU Collective Research CRUSTASEA project).

In restaurants, the problem of having a crab with poor brown meat content is usually solved by dressing the carapace with extra ingredients that give a smoother texture and even sweeter taste. Therefore, males are preferred because even if brown meat is poor, claws are bigger than those of females. In the case of homarids, the content in brown meat is not much of a problem considering that for most consumers muscle content is far more important. Though homarids were not sampled every season it is known that after moulting, claw and abdominal muscle can be much less in proportion to the recently acquired bigger exoskeleton (Bliss, 1990).

5.3.2 Nutritional quality of homarids and *C. pagurus*

Though nutritive quality of crustaceans is not the most important criteria of consumers, nowadays developed nations are more concerned and aware about seafood benefits and risks and consequently about food labelling (Mathew *et al.*, 1999). According to data obtained in spring for homarids (Sub-chapter 4.1 and 4.2) and summer for *C. pagurus* (Sub-chapter 4.3-4.5), the muscle of all species can be categorized as low fat and protein rich food according to the classification of seafood based both on protein (15-20 %) and fat (< 5 %) content (Sikorski, 2000). Another classification only considers the fat content of muscle and according to this, homarids and *C. pagurus* have a lean flesh (< 5 % fat; Ackman, 1994). The results indicate that muscle is a structural tissue that fits the common description of seafood, *i.e.* low in fat and cholesterol, a good source of high quality protein, polyunsaturated fatty acids and essential trace elements in the range of dietary reference intakes (DRI), or even above (Table 5.7). Precisely because of its structural function, muscle had the most homogeneous composition with fewer differences detected between species and was less exposed to seasonal and sex variations than hepatopancreas and gonads.

These results are similar to other found for commercial crab and lobster marine species (Karakoltsidis *et al.*, 1995; Skonberg and Perkins, 2002; Gökoğlu and Yerlikayak, 2003; Rosa and Nunes, 2003b; Çelik *et al.*, 2004; Nacz *et al.*, 2004; Oehlenschläger, 2006; Sirot *et al.*, 2008). This balanced nutritional composition of crustaceans' muscle is not the usual idea of most consumers, as many still have the misconception that crustaceans have high cholesterol content and are, therefore, an exception among the benefits of seafood.

Table 5.7 General biochemical and inorganic composition of muscle from homarids and edible crab.

	<i>H. gammarus</i> ^(a)	<i>H. americanus</i> ^(a)	<i>C. pagurus</i> ^(b)
Proteins (%)	18	17.1 - 15.6*	16.4-20.5*
Fat (%)	0.4	0.7	0.3
Cholesterol (mg 100 g ⁻¹)	34.0	43.2 -37.2*	38.73
SFA (%)	22.7	21.0	15.7-17.6**
MUFA (%)	29	30.8	30.1
PUFA (%)	45	42.0 - 44.6*	49.6
PUFA/SFA	2.0	2.0 – 2.2*	3.0
n-3/n-6	4.2	6.0	3.7
IA	0.24	0.24	0.18
IT	0.17	0.15	0.12
TAA	16.75	17.13	16.5-17.8**
EAA/NEAA	0.79	0.82	0.80
Trace elements (% DRI)	Zn (~30 %)	Zn (~30 %)	Zn (~50 %)
	Cu (~100 %)	Cu (~100 %)	Cu (~100 %)
	Se (~150 %)	Se (~100 %)	Se (100-200 %)
Toxic elements		As (< AL)	
		Cd, Hg, Pb (< ML and AL)	

Abbreviations: (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (IA) index of atherogenicity; (IT) index of thrombogenicity; (TAA) total amino acids; (EAA) essential amino acids; (NEAA) non essential amino acids; (DRI) dietary reference intake; (ML) maximum level; (AL) action level; (a) sub-chapter 4.1 and 4.2; (b) sub-chapter 4.3-4.5; (*) significant differences between sexes, data on the left represent females and males are shown on the right; (**) significant differences between populations, values on the left correspond to crabs from the English Channel, while values on the right correspond to crabs from the Scottish coast .

This misinterpretation is mainly due to the fact that crabs and lobsters are usually included in the general designation of shellfish which includes high cholesterol species such as prawns (118-163 mg 100 g⁻¹), squids (88-198 mg 100 g⁻¹) and cuttlefish (130-162 mg 100 g⁻¹; Mathew *et al.*, 1999). In fact, in the report by Mathew *et al.* (1999), 5 crab species were analysed and the cholesterol range was from 51.5 to 66.8 mg 100 g⁻¹. It is also important to state that in terms of fat quality, that is, ratio of n-3/n-6, PUFA/SFA, DHA and EPA contents, muscle fat quality is excellent but, since muscle is a lean tissue it does not provide a large amount of the important polyunsaturated fatty acids.

Generalizations about seafood nutritional quality cannot be assumed for all edible tissues. In fact, in the present study, homarids and edible crab showed great diversity in the nutritional quality of muscle, hepatopancreas and gonads, and for *C. pagurus* differences were also observed between sexes, seasons and fishing ground location. The contents of the main components in the edible tissues of homarids and *C. pagurus*, especially crude protein and lipids, were concluded to be tissue specific that is, muscle, hepatopancreas and gonads have a distinct chemical composition that is related to their physiological function in crustaceans.

Data obtained during spring for homarids and summer for *C. pagurus* reveal that hepatopancreas is a moderate to high fat (7-25 %) tissue, rich in SFA (21.2-32.1 %), with Cd concentration ($11-30 \mu\text{g g}^{-1}$) above the action level (AL), and with higher indexes of atherogenicity (0.34-0.53) and thrombogenicity (0.21-0.73) comparatively to muscle. In general, the hepatopancreas of homarids had more fat, SFA, IA and IT but lower Cd concentration than edible crab. Valuable features were also evidenced in hepatopancreas, namely the high taurine content ($0.31-0.59 \text{ g } 100 \text{ g}^{-1}$) and several macro and trace elements were in the range or above the DRI, such as Cl, Ca, Fe, Cu, Zn and Se. The PUFA/SFA ratio in hepatopancreas was also above the minimum recommended value set by the UKDH (0.4) for both sexes of *C. pagurus* (1.1-1.7) and female homarids (0.5-0.8), whereas this value was below in male homarids (0.2; Sub-chapter 4.1). These results are similar to other crustacean species, though the nutritive composition of hepatopancreas is rather scarce comparatively to that of muscle (Floreto *et al.*, 2000; Cherif *et al.*, 2008; Latyshev *et al.*, 2009).

Gonads chemical composition showed great variations between sexes, and generalizations regarding this tissue are difficult. But independently of sex, season or species, the fat content was dominated by PUFA followed by MUFA and SFA. Ovaries had more protein and amino acids as well as fat and cholesterol than testis; this was evidenced in *C. pagurus* (Sub-chapters 4.3 to 4.7). Proximate chemical analyses were not possible in testis of homarids and roe found in female *H. gammarus* due to the lack of sufficient material. The nutritional quality of gonads from wild crab species is rather scarce as most studies concern crab species from an aquaculture development perspective (Wen *et al.*, 2001; Woll and Berge, 2007; Wu *et al.*, 2007).

Cholesterol in hepatopancreas ($76-116 \text{ mg } 100 \text{ g}^{-1}$) and in female gonads ($105-170 \text{ mg } 100 \text{ g}^{-1}$) was higher than in muscle ($31-43 \text{ mg } 100 \text{ g}^{-1}$; Sub-chapters 4.1 and 4.3). Though in roe cholesterol analysis was not possible, values are also expected to be high. Previous findings of Iwasaki and Harada (1984) highlighted high cholesterol values of eggs of 25 marine species ranging from 250 to $650 \text{ mg } 100 \text{ g}^{-1}$ in *Osmerus eperlanus mordax* and *Achantopagrus schlegeli*, respectively.

While the proximate chemical composition is tissue specific and the same trends were observed between homarids and between crabs of different locations, the fatty acid and amino acid contents were different and the elemental composition was rather variable.

The fatty acids profile of muscle showed differences according to location, as crabs from the SC had higher proportion of 14:0, 18:1n-9 and 18:2n-6, and lower 18:1n-7 and 16:4n-3 than those of the EC. In hepatopancreas and gonads, the only population differences were observed in the profile of 16:1n-5 and 20:1n-9, respectively, with SC crabs having proportionally more of these fatty acids than EC ones (Sub-chapter 4.3). Also, crabs harvested in the SC were better sources of most essential elements than animals from the EC (Sub-chapter 4.4). The accumulation pattern of Br, Sr, Cd, Hg and Pb in brown crab from both locations seemed to be mostly influenced by the type of tissue and sex, whereas the accumulation of S and As was mainly influenced by the geographical location and sex (Sub-chapter 4.5).

Among homarids, differences in elemental composition were also found; *H. americanus* had more Na (muscle) and, Na, Mg, Fe and Se (hepatopancreas) than *H. gammarus* but less Cu and Br

(hepatopancreas), S and, Zn (gonads). Generally, *H. americanus* had proportionally more MUFA, particularly eicosenoic acid, than *H. gammarus*, but less n-6 fatty acids in all tissues. In hepatopancreas, *H. gammarus* had more tyrosine and taurine than *H. americanus*, but less histidine. In general, gonads of European lobster had higher concentration of most amino acids than the American counterpart.

In general, homarids and *C. pagurus* were excellent sources of most macro and trace elements and the only limiting elements were Mg, K and Mn. In particular, Se content was above the (DRI) in all tissues, but ovaries, testis and roe analysed were the best sources of this antioxidant.

Of all the contaminants determined only cadmium concentration was above the action level (AL = 0.3 mg 100 g⁻¹) in the hepatopancreas of both homarid species and of *C. pagurus*. A number of studies have reviewed cadmium content in different foods, its concentrations ranged from non-detected in many foods to a high mean of 1.20 mg kg⁻¹ for cephalopods (EC, 2004a). Edible free-living food organisms such as shellfish, crustaceans and fungi are natural accumulators of cadmium (EC, 2004a). According to EFSA, (2004) the food categories that show more Cd are “edible offal and offal products” (0.2057 mg kg⁻¹); followed by meat and meat products, offal (0.0974 mg kg⁻¹) and fish and seafood (0.0923 mg kg⁻¹).

5.3.3 Influence of season on nutritional quality of *C. pagurus*

In *C. pagurus*, Cd concentration and most other biochemical and inorganic components showed marked seasonality mainly in gonads and hepatopancreas in opposition to muscle that showed less variation. It was concluded that the only tissue that can pose risks in every season was hepatopancreas due to the high Cd content. However, Cd concentration data must be interpreted with care because the importance given to the positive or negative characteristics of any given food must be rationalized and contextualized.

Without a well defined consumption purpose, characterization of a given food item is merely a catalogue of measurements as the nutritional quality of food is intimately related to the nutritional needs and habits of its consumer. These habits are important because, in the case of the edible crab, the abundant brown meat content is a consumer criterion, whereas in homarids this is not the case. Therefore, the negative contribution of brown meat is much less in homarids than in the edible crab and, among crab gonads' contribution of testis is lower than that of ovaries.

To truly reflect the potential hazards of hepatopancreas consumption several variables must be taken into consideration such as the annual average consumption and its seasonality, seasonal elemental variations and, interaction between cadmium and other minerals affecting absorption rates, just to name a few. Another key issue is the relative Cd contribution to the diet as some of the food items that contain high Cd levels are rarely consumed by the general European population (e.g. oilseeds and edible offal) and therefore might not be important for overall intake. While, other food items with high consumption in the total population or in some sub-populations can be major contributors to the overall cadmium intake even if they contain only low cadmium levels (EFSA, 2009).

Though this evaluation is out of the scope of the present thesis in sub-chapter 4.7 an attempt was made to calculate the Cd intake per week and compare it to the provisional tolerable weekly intake (PTWI). Considering that presently no data is available concerning the average consumption of edible crab in the Portuguese population, table 5.8 shows the maximum hepatopancreas portion that an adult can eat weekly which was calculated considering the PTWI set for Cd. This calculation did not consider the consumption pattern, or the interactions of Cd with other elements such as Se, which is known to have a protective action on many toxicological effects of Cd and Hg (Skowerski *et al.*, 1997ab). The only variable considered was the minimum and maximum Cd concentration obtained in each season for females and males. Cadmium PTWI is 7 µg/body weight, that is 0.42 mg of Cd tolerable on a weekly basis by a 60 kg adult. Cadmium concentration was much variable even for crabs sampled in the same season. According to these rough calculations an adult can eat a maximum of 37.3 to 111.4 g per week of male hepatopancreas in spring, but only 10.2 to 27.3 g of male hepatopancreas in summer and 7.9 to 35.4 g of female hepatopancreas in winter.

Table 5.8 Minimum and maximum Cd concentration in hepatopancreas of female and male crabs seasonally and calculated maximum portion per week according to the PTWI set value of 7µg/body weight.

Season	Sex	Hepatopancreas weight (g)	Cd (mg 100 g ⁻¹)	Weekly portion (g)
Spring	F	85.3	0.31-1.83	136.8 - 23.0
	M	104.0	0.38-1.13	111.4 - 37.3
Summer	F	77.5	0.82-4.11	51.0 - 10.2
	M	76.8	1.54-4.11	27.3 - 10.2
Autumn	F	91.5	0.39-1.14	107.4 - 36.7
	M	88.3	0.48-5.02	88.1 - 8.4
Winter	F	78.1	1.19-5.29	35.4 - 7.9
	M	85.9	0.54-1.15	77.8 - 36.6

Abbreviations: (PTWI) provisional tolerable weekly intake. Maximum portion calculated considering a 60 kg adult. Hepatopancreas average weight.

Therefore, consumption moderation is advised in both high seasons at least consumption of males in summer, and of females in winter.

The wide seasonal variation in the Cd concentration was also observed in the biochemical and other inorganic elements composition. Therefore, in sub-chapters 4.6 and 4.7 it was concluded that adding to the intrinsic physiological differences between tissues, biotic and abiotic factors also affects the nutritional status of the animals and consequently the yield and biochemical and inorganic composition of tissues. The biotic endogenous factors include the life cycle of each species, age, sex, and condition, which are governed by abiotic factors such as season, food availability, geographical location, substrate, depth, salinity, temperature and anthropogenic influence (Karakoltsidis, 1995; Rainbow and Black, 2001). This was not observed in homarids because sampling only took place in spring. In *C. pagurus*, the meat yield and chemical contents of hepatopancreas and gonads showed the widest variations mainly due to sex and seasonal differences: hepatopancreas was mostly influenced by season, while in gonads both season and

sex contributed to the differences observed (Sub-chapter 4.6 and 4.7). These differences are important for consumers and nutritionists when it comes to make wise decisions about what to eat and when.

To clarify the main differences observed in season and sex, a diagram is shown in figure 5.6 and 5.7, summarizing some chemical parameters that varied in hepatopancreas and gonads of both sexes and that are important for human nutrition. For instance, hepatopancreas was the best source of inorganic elements in all seasons, particularly in autumn and winter (Cu, Se and Ca in males; Figure 5.6). Ovaries were the best source of Zn (summer and autumn; Figure 5.7).

Considering *C. pagurus* life cycle, the fat and cholesterol contents suggest an increase in energy demand during summer, possibly for the production of gametes, and during autumn and winter, for incubation and egg laying. Similarly, the high concentration of all macro elements and Fe during winter might be a consequence of the intense feeding during the post moult and mating period (autumn).

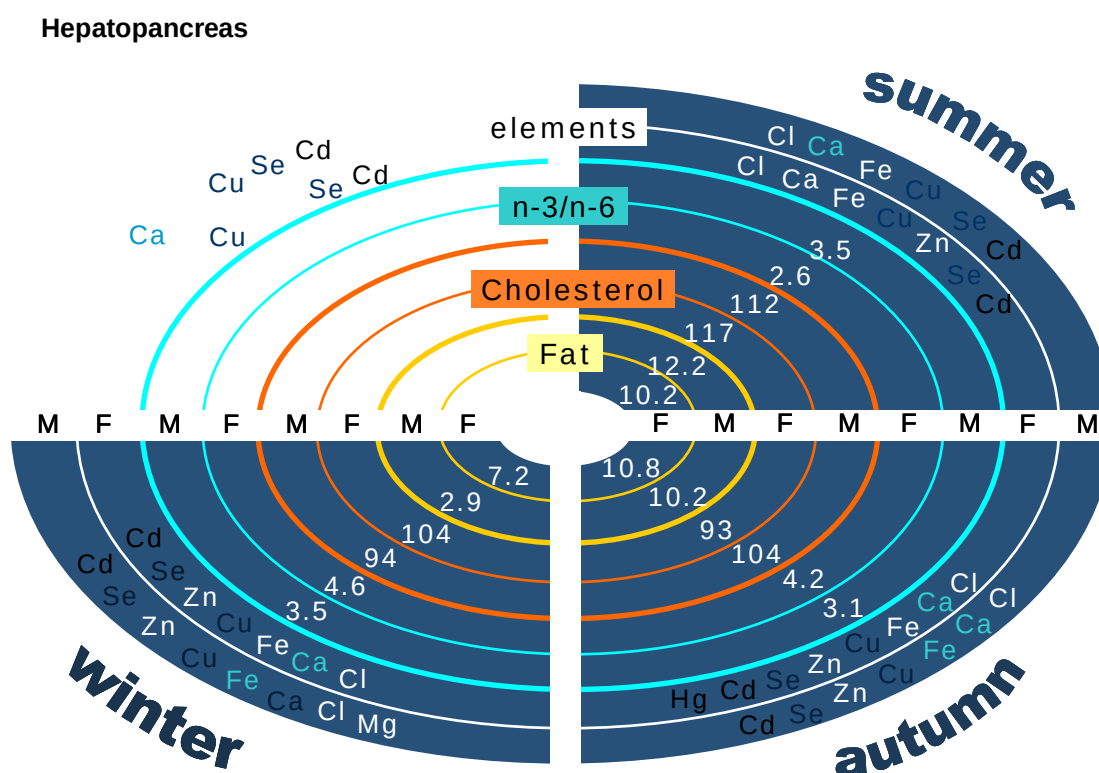


Figure 5.6 Representation of average fat (%), cholesterol ($\text{mg } 100 \text{ g}^{-1} \text{ ww}$), n-3/n-6 and inorganic elements (% of dietary reference intakes, DRI) in hepatopancreas of female and male *C. pagurus* through the four seasons. Each parameter is represented by a different colour ring (e.g. fat is represented as yellow) the inner and outer rings represent females and males, respectively. Elements in white represent (DRI) above 20 % and below 50 %; those in black are contaminants above maximum level (ML) or action level (AL), those in light blue are elements above 50 % and below 100 % DRI; elements in dark blue are above 100 % DRI.

Gonads

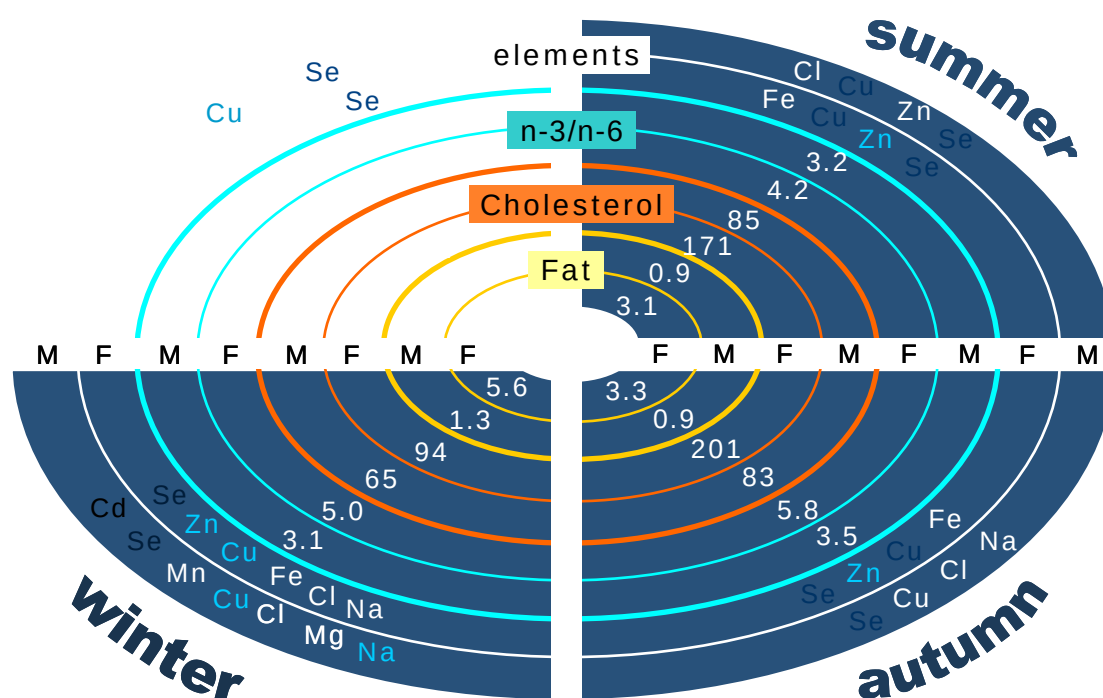


Figure 5.7 Representation of average fat (%), cholesterol ($\text{mg } 100 \text{ g}^{-1} \text{ ww}$), n-3/n-6 and inorganic elements (% of dietary reference intakes, DRI) in gonads of female and male *C. pagurus* through the four seasons. Each parameter is represented by a different colour ring (e.g. fat is represented as yellow) the inner and outer rings represent females and males, respectively. Elements in white represent (DRI) above 20 % and below 50 %; those in black are contaminants above maximum level (ML) or action level (AL), those in light blue are elements above 50 % and below 100 % DRI; elements in dark blue are above 100 % DRI.

In general, seasonal fat changes occur as reserve lipids are utilized in various parts of the body whenever needed, for example, during starvation, overwintering, fast movements, reproduction and growth. During ovarian maturation, lipids are transported from the hepatopancreas and muscle to gonads. During the winter spawning period, a rapid decrease in lipid content is observed. After spawning, animals undertake intensive feeding and the lipid content increases in the muscle and hepatopancreas, whereas it decreases in gonads (Sikorski *et al.*, 2000).

From a consumer perspective, autumn is the best season to eat edible crab, particularly females considering the brown meat yield and the maximum calculated hepatopancreas portion allowing tolerable Cd concentration (Table 5.8). Other beneficial characteristics were also observed in this season, such as the high taurine content in both hepatopancreas and gonads, Fe and Ca above 50 % DRI in hepatopancreas and Zn above 50 % DRI in ovaries. However, autumn was also the season with more fat and less n-3 fatty acids in hepatopancreas adding to the fact that in both hepatopancreas and ovaries high values of AI, TI and cholesterol concentration but low PUFA/SFA were also registered.

Moreover, in this season, Hg concentration was above the ML in muscle and in the hepatopancreas of females. Even though autumn is not the peak consumption season of edible crab, the industry usually processes female crabs (boiled and frozen) and brown meat spread

during this period precisely because females have higher meat yield. Male crabs are usually not processed because they are more valued alive. Therefore, future studies considering risk assessment should also analyse the potential risks associated to Cd intake of boiled and frozen female crabs and brown meat spread.

In order to have a global perception of the nutritional quality of *C. pagurus*, *H. gammarus* and *H. americanus* edible tissues as far as fat and essential elements are concerned, data was compared to other food items (Table 5.9).

Comparing the cholesterol content of muscle in homarids and edible crab, lower values were found compared to most food items common in the Mediterranean diet, with the exception of hake (Table 5.9). Therefore, the muscle of these crustaceans has nutritious value and, therefore, should be included in the healthy category of seafood. Even in hepatopancreas and gonads, cholesterol values were lower than in liver of cow and pork. The same is true for IA values.

Table 5.9 Fat and elemental quality of several daily food items.

	Species (common name)	Cholesterol (mg 100 g ⁻¹)	PUFA/SFA	IA	Elements >20 % DRI
Muscle					
Crustaceans	Homarids ^(a)	37	2.1	0.24	Cu, Zn, Se
	Edible crab ^(b)	37-47	2.6-3.5	0.14-0.20	Cu, Zn, Se
Fish	Sole ^(c)	85	1.3	-	-
	Hake ^(c)	19	3.0	-	-
Mammals	Lamb ^(c)	68	0.09	1.00*	Fe, Zn
	Rabbit ^(c)	48	0.54	0.82*	Fe, Zn
	Beef ^(c)	61	0.11	0.72*	Fe, Zn
	Pork ^(c)	74	0.48	0.69*	-
Poultry	Chicken ^(c)	99	0.88	0.50*	-
Viscera					
Hepato	Homarids ^(a)	43-104	0.22-0.80	0.48	Fe, Cu, Zn, Se
	Edible crab ^(b)	104	0.8-2.2	0.24-0.57	Cl, Ca, Fe, Cu, Zn, Se
Liver	Pork ^(c)	237	0.47	-	Ca, Fe, Zn
	Cow ^(c)	283	0.12	-	Fe, Zn
Ovaries	Homarids ^(a)	110	2.1-2.8	0.24	Fe, Cu, Zn, Se
	Edible crab ^(b)	94-201	1.7-2.9	0.2-0.4	Na, Fe, Cu, Zn, Se
Eggs					
Roe	European lobster ^(a)	-	-	-	Cu, Zn, Se
	Japanese crab ^(d)	529	-	-	-
Eggs	Chicken ^(c)	408	0.78	-	Fe

Abbreviations: (hepato.) hepatopancreas; (IA) index of atherogenicity; (PUFA) polyunsaturated fatty acids; (SFA) saturated fatty acids; (DRI) dietary reference intake; (a) sub-chapter 4.1 and 4.2; (b) sub-chapter 4.3 to 4.7; (c) Porto and Oliveira (2006); (d) Iwasaki and Harada (1985); (*) Rosa and Nunes (2005).

In conclusion, muscle of both female and males of edible crab and homarids is a typical seafood product with a fairly stable healthy composition and thus can be eaten with almost no special restrictions in every season. The same however, is not equally valid for brown meat, mostly due to Cd concentration in hepatopancreas. Therefore, its consumption should be moderate particularly in summer (for male specimens) and winter (for female specimens). For those consumers with heart problems or restricted cholesterol diets, brown meat of female crabs should be avoided in autumn. These considerations regarding hepatopancreas and gonads consumption are typical given the type of food and tissue that they represent. Even though both tissues are from seafood products the generalization about the flesh quality are not applied to all organs, species, sex or season.

5.4 Future research related to nutritional quality of *C. pagurus* and homarids

In this study, a general approach to the biochemical and inorganic composition of homarids and *C. pagurus* was made. It was concluded that the composition of various edible tissues of these wild crustaceans is widely variable due to season, life cycle, sex, geographical location and other factors affecting each species, in opposition to farmed food animals. Therefore generalizations about chemical composition mainly of hepatopancreas and gonads must be careful. Nevertheless, this investigation made available important information that fills a gap in the knowledge of these species nutritional composition. This data is essential for the industry, health services, research, regulation agencies, international organizations and the general public. In fact, industry can use this information to be included in nutritional labels as they become compulsory; nutritionists are especially interested in the nutrient composition of seafood in order to recommend or not its consumption; regulation agencies and organizations can use these data to improve recommendations and research areas; the public in general can be better informed and therefore be able to make choices and more reliable decisions.

These results are also a stand point that can be used by academic research to trigger other areas of interest.

From a risk assessment perspective, several studies still need to be developed:

1. consumption surveys are needed to quantitatively characterize consumption seasonal patterns. These surveys must also consider different sub-populations, as coastal populations usually consume more seafood and have different eating habits than inland communities. This information is essential to elaborate different risk scenarios.
2. determination of the organic polychlorinated dibenzo-p-dioxins (PCDD), dibenzofurans (PCDF), and polychlorinated biphenyls (PCB), considering that they are ubiquitous environmental contaminants, which persist and bioaccumulate through the food chain (Domingo and Bocio, 2007; Karouna-Renier, 2007).
3. monitorization of biotoxins, such as diarrhetic shellfish poisoning (DSP), as several reports highlight intoxication from *C. pagurus* consumption in Norway (Torgersen *et al.*, 2005) and *C. maenas* consumption in Portugal (Vale and Sampayo, 2002).

4. determination of cooking procedure effects in the availability of organic and inorganic toxic elements in foodstuff and subsequent bioavailability of these contaminants both *in vivo* and *in vitro* models. Presently the European project Riskfoodcont “BioAvailability and risk assessment of polycyclic aromatic hydrocarbons (PAH) and toxic elements (As, Cd, Hg and Pb) in processed meat and seafood products” is focusing these issues. *C. pagurus* is one of the chosen seafood species due to its high Cd content.

Other issues that were not considered in this thesis and require further research:

1. determination of the influence of starvation periods on the nutritional quality of the crustaceans’ edible tissues with respect to season and sex variability. This issue can be most important considering that crabs are stored for weeks without feeding in deposit facilities, and starvation can influence not only crab survival but also the meat content and quality.
2. conjugate data from the crustaceans’ biochemical composition to those of physiology in order to have valuable information regarding both fitness and nutritional quality. Several parameters could be seasonally measured in haemolymph (e.g. glucose, total lipids, tryglicerides, cholesterol, inorganic elements and proteins) and linked with the chemical composition of edible tissues including glycogen and also the meat yield, life cycle and sex. Similar data has been compiled for other crustaceans but only from an ecological and metabolic perspective (Dutra *et al.*, 2007; Oliveira *et al.*, 2007; Buckup *et al.*, 2008)

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Appendix

Common Portuguese and English names of commercial crustaceans

Species latin name	Species common name (English)	Species common name (Portuguese)
Clawed lobsters		
<i>Homarus gammarus</i>	European clawed lobster	lavagante Europeu
<i>Homarus americanus</i>	American clawed lobster	lavagante americano
<i>Nephrops norvegicus</i>	Norway lobster	lagostim
Spiny lobsters		
<i>Panulirus regius</i>	royal spiny lobster	lagosta-verde
<i>Palinurus elephas</i>	common lobster	lagosta-castanha ou nacional
<i>Palinurus mauritanicus</i>	pink spiny lobster	lagosta-rósea ou da Mauritânia
Crabs		
<i>Cancer pagurus</i>	edible crab or brown crab	sapateira
<i>Maja squinado</i>	spider crab	santola
<i>Carcinus maenas</i>	green crab	caranguejo
<i>Necora puber</i>	velvet crab	navalheira

Annex

Eurostat codes

Description as in Eurostat	Eurostat code
Live lobsters " <i>Homarus</i> spp."	03062210
Norway Lobsters " <i>Nephrops norvegicus</i> ", whether in shell or not, live, dried, salted or in brine, including Norway lobsters in shell, cooked by steaming or by boiling in water.	03062930
Rock lobster and other sea crawfish " <i>Palinurus</i> spp.", " <i>Panulirus</i> spp." and " <i>Jasus</i> spp.", whether in shell or not, live, dried, salted or in brine, including lobsters in shell, cooked by steaming or by boiling in water.	03062100
Crabs " <i>Cancer pagurus</i> ", whether in shell or not, live, dried, salted or in brine, including crabs in shell, cooked by steaming or by boiling in water.	03062430
Crabs, " <i>Paralithodes camchaticus</i> ", " <i>Chionecetes</i> spp.", and " <i>Callinectes sapidus</i> " whether in shell or not, live, dried, salted or in brine, including crabs in shell, cooked by steaming or by boiling in water.	03062410
Crabs, whether in shell or not, live, dried, salted or in brine, including crabs in shell, cooked by steaming or by boiling in water (excluding " <i>Cancer pagurus</i> ").	03062480
Crabs, whether in shell or not, live, dried, salted or in brine, including crabs in shell, cooked by steaming or by boiling in water (excluding " <i>Paralithodes camchaticus</i> ", " <i>Chionecetes</i> spp.", " <i>Callinectes sapidus</i> " and " <i>Cancer pagurus</i> ").	03062490
Shrimps and prawns of the Pandalidae family whether in shell or not, live, dried, salted or in brine, including shrimps and prawns in shell, cooked by steaming or by boiling in water.	03062310
Shrimps of the genus <i>Crangron</i> whether in shell or not, live, dried, salted or in brine, including shrimps in shell, cooked by steaming or by boiling in water	03062339
Shrimps and prawns, whether in shell or not, live, dried, salted or in brine, including shrimps in shell, cooked by steaming or by boiling in water.	03062390
