

CARACTERIZAÇÃO DO SISTEMA DE ACASALAMENTO DO BLENÍDEO *Salaria pavo* NA RIA FORMOSA

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Tese de doutoramento em Ciência Animal,
Especialidade em Morfologia e Fisiologia

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CONTENTS

AGRADECIMENTOS	5
CONTENTS	9
PUBLICAÇÕES E COMUNICAÇÕES EM REUNIÕES CIENTÍFICAS RESULTANTES DA DISSERTAÇÃO DE DOUTORAMENTO	11
RESUMO	15
ABSTRACT	21
RÉSUMÉ	25
GENERAL INTRODUCTION	31
CHAPTER I: Female mate choice and mate search tactics in a sex role reversed population of the peacock blenny <i>Salaria pavo</i> (Risso, 1810)	59
CHAPTER II: Brood size manipulation and androgen levels in nest-holder males of the peacock blenny	79
CHAPTER III: Life-history pathways associated with sequential reproductive tactics in the peacock blenny, <i>Salaria pavo</i>	107
CHAPTER IV: Social cues in the expression of sequential alternative reproductive tactics in young males of the peacock blenny, <i>Salaria pavo</i>	129
CHAPTER V: Seasonal and tidal variation of microhabitat use by the peacock blenny, an intertidal fish with alternative reproductive tactics	165
GENERAL CONCLUSIONS	191

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RESUMO

O sistema acasalamento da população do blenídeo *Salaria pavo* (Risso, 1890) do Parque Natural da Ria Formosa apresenta características peculiares que a tornam um excelente modelo para a compreensão dos mecanismos de seleção sexual: táticas alternativas de reprodução (TARs) e reversão de papéis sexuais. A presença nesta população de polimorfismos associados às TARs constitui ainda uma oportunidade para abordar os mecanismos de expressão de fenótipos reprodutivos.

A reversão dos papéis sexuais e as TARs masculinas são classicamente interpretadas como resultantes de pressões selectivas opostas. A primeira é consequência da competição intrasexual feminina e as últimas têm as suas origens na competição intrasexual masculina. Porque a competição intrasexual é teorizada como produto da escassez do sexo oposto, a competição intrasexual feminina e a masculina são tomados como mutuamente exclusivas. No entanto, elas ocorrem simultaneamente nesta população.

A população ocupa uma área onde os locais propícios ao estabelecimento de ninhos são raros. A competição entre os machos pelo acesso a estes ninhos é intensa e por consequência apenas alguns machos são capazes de estabelecer e defender um ninho. As fêmeas, por outro lado, competem pelo acesso a esses machos nidificantes (machos burgueses) que elas cortejam activamente. Tanto machos como fêmeas acasalam com diversos parceiros mas os machos são selectivos em relação às fêmeas que aceitam como parceiras. Consequentemente os papéis sexuais são revertidos nesta população. No entanto, são os machos burgueses, e não as fêmeas, que apresentam caracteres sexuais secundários. Os machos burgueses são maiores do que as fêmeas e têm como caracteres sexuais secundários uma glândula anal produtora de feromonas e de substâncias antibióticas e uma conspícua crista cefálica. Estes machos defendem um ninho e prestam cuidados parentais aos ovos que consistem em defender e limpar o ninho e em arejar os ovos ('fanning'). Os machos mais pequenos e mais jovens ('sneakers') imitam a morfologia e o comportamento das fêmeas, a fim de se aproximarem discretamente dos ninhos dos machos burgueses e fertilizarem parte dos ovos que as fêmeas depositam nos ninhos. Deste modo, os 'sneakers' parasitam o investimento dos machos burgueses em defesa do ninho, na atração das fêmeas e em cuidados parentais. A fim de integrar as perspectivas ecológica e fisiológica, foram realizados trabalho descritivos na população natural, e experimentais, em laboratório, a fim de abordar questões relativas à reversão dos papéis sexuais e às TARs, nomeadamente:

Tratando-se de um sistema com papéis sexuais revertidos, quais são os factores responsáveis pela manutenção dos caracteres sexuais secundários masculinos? Serão as fêmeas selectivas, e contribuirá essa escolha para a manutenção dos caracteres sexuais secundários masculinos?

Será que as fêmeas seleccionam os seus parceiros com base no tamanho da postura? Existirá um compromisso entre o esforço parental e os níveis de androgénios que limita o tamanho da postura?

Serão as TARs sequenciais nesta população? Que trajectórias da história vital se encontram nesta população, e estarão associadas à data de eclosão dos machos?

Que factores sociais determinam a expressão das diferentes tácticas e quais são os mecanismos endocrinológicos subjacentes à expressão das TARs?

Como se distribuem os diferentes sexos e os diferentes estágios da história vital pelos microhabitats onde ocorre a população e como muda essa distribuição em função do ciclo de maré e ao longo do ano? (Considerações de amostragem)

Apesar da reversão dos papéis sexuais, os resultados sustentam a existência de selectividade feminina na escolha de parceiros. As fêmeas dessa população não acasalam de forma aleatória, acasalam preferencialmente com machos grandes, que cortejam, machos com caracteres sexuais secundários bem desenvolvidos e com grandes posturas no seu ninho. Esta seleção contribui portanto para a manutenção dos caracteres sexuais secundários masculinos, adicionando-se ao efeito da competição intrasexual masculina identificado por Saraiva *et al.* (2009).

As fêmeas amostram os potenciais parceiros através de uma táctica de comparação com um limiar flexível de qualidade mínima ("One step decision" com "total recall"), ajustado à distribuição da qualidade e à distribuição espacial dos machos. Isto, juntamente com agregação dos ninhos, permite a redução dos custos de amostragem.

Independentemente do espaço disponível nos ninhos, o número de ovos que cada macho cuida tende a ser estável, apesar da confirmação da preferência das fêmeas por posturas maiores. Identificou-se também um trade-off entre os níveis circulantes de testosterona e tamanho da postura (como medida aproximada dos cuidados parentais). Estes dois factos sugerem que as fêmeas baseiam a sua escolha em qualidades dos machos, para além do tamanho das posturas que eles cuidam, e que a taxa de desova das fêmeas é regulada através de comportamentos masculinos dependentes de testosterona, eventualmente comportamentos de corte. Tal regulação do número de ovos dentro do

ninho revela, por seu turno, custos associados com cuidados parentais que limitam ainda mais a reprodução de machos, contribuindo assim para a selectividade masculina na escolha de parceiros, para a competição entre fêmeas e a consequente reversão de papéis sexuais.

Apesar da forte competição entre fêmeas por acesso a potenciais parceiros, a competição entre machos por acesso a recursos, nomeadamente aos locais de nidificação, impõe um regime de seleção intrasexual masculina forte, hipoteticamente, causando o aparecimento de TARs nesta população. TARs estão aqui, como classicamente descritas, relacionadas com a aquisição de recursos para a qual o tamanho do corpo e a dominância social desempenham um papel importante. Nessa população as tácticas são expressas em sequência, com o mesmo indivíduo a reproduzir-se inicialmente como 'sneaker' e posteriormente como macho burguês (nidificante). No entanto, diferentes trajectórias da história vital são observadas: a "via de sneaker" representa a expressão sequencial das duas tácticas, como acima descrito, e a "via de macho burguês" é constituída pelos machos que se reproduzem directamente como machos burgueses na primeira época de reprodução, sem nunca expressarem a táctica de 'sneaker'. Uma terceira via, em que os machos adiam a reprodução na primeira época e se reproduzem como burgueses apenas na época seguinte, é ainda possível mas não foi confirmada.

As duas trajectórias observadas estão associadas à data de eclosão dos ovos. Os machos que seguem a "via de macho burguês" eclodem no início da época de reprodução, enquanto que aqueles que seguem a "via de sneaker" tendem a eclodir mais tarde, confirmando assim o 'Birth-date effect' proposto por Taborsky (1998) como um dos mecanismos envolvidos na expressão de TARs. No entanto, as diferentes datas de eclosão não tiveram influência aparente no padrão de crescimento dos machos das duas vias. E consequentemente o efeito de data de eclosão deveu-se principalmente ao tempo decorrido entre a eclosão dos machos que seguiram uma e outra trajectória e que permitiu aos indivíduos que eclodiram mais cedo crescerem durante mais tempo até ao início da época de reprodução.

Os resultados suportam um mecanismo de expressão das TARs condicional e que pode ser descrito como consistindo num único ponto de conversão ('switching point') para o morfótipo burguês, dependente da dominância social (ou do tamanho relativo do corpo) e que, independentemente da maturação sexual, determina a expressão dos caracteres sexuais secundários, da glândula testicular e comportamento de nidificação. Os machos de ambas as vias observadas são sexualmente maduros na primeira época de

reprodução, e a expressão de uma ou outra tática depende exclusivamente do desenvolvimento ou não daquelas características, típicas do morfótipo burguês. Os machos eclodidos precocemente, que seguem a “via de macho burguês” chegam à época de reprodução com um corpo maior do que os machos nascidos mais tarde. Consequentemente, os primeiros são mais dominantes, o que determina o início da expressão dos traços típicos dos machos burgueses; enquanto os últimos, os indivíduos mais pequenos da população, são subordinados e portanto não desenvolvem tais características e podem reproduzir-se como ‘sneakers’ (“via de sneaker”). Estes ‘sneakers’ atingem o ponto de conversão apenas no final da época quando iniciam o expressão do morfótipo burguês.

A 11-cetotestosterona, mas não testosterona, mediou o efeito da dominância social no desenvolvimento da crista cefálica, característica do morfótipo burguês. No entanto, este efeito de mediação não foi detectado para os restantes traços deste morfótipo e mecanismos alternativos devem ser investigados. Não foi encontrado apoio para a hipótese de uma interação do cortisol sobre o eixo hipotálamo-hipófise-gónadas na determinação do fenótipo reprodutivo, neste caso, a tática reprodutiva. Os níveis circulantes de cortisol não estão associados à dominância social, mas sim inversamente relacionados com o tamanho relativo das gónadas.

As duas vias diferiram significativamente no seu sucesso reprodutivo reforçando o apoio à hipótese de que a expressão das táticas alternativas depende de um mecanismo condicional. O sucesso reprodutivo da “via de macho burguês” foi muito inferior ao da “via de sneaker” levantando a hipótese da existência de uma força selectiva agindo em direcção oposta, em favor dos indivíduos nascidos mais cedo. A desvantagem de um sucesso reprodutor mais baixo poderá ser compensada por uma maior taxa de sobrevivência dos ovos ou dos juvenis ou por uma maior fertilidade das fêmeas eclodido/as no início da época – hipóteses a serem exploradas.

Em conclusão, apesar da reversão de papéis sexuais, as fêmeas da população estudada são seletivas na escolha dos seus parceiros potenciais e isso pode ser facilitado pelo baixo custo de amostragem dos machos, permitindo um benefício potencial relacionado com os cuidados parentais de que os ovos são dependentes. Estes baixos custos são conseguidos através de uma tática flexível de amostragem e, devido à agregação dos machos. A estabilidade do tamanho da postura, por outro lado, sugere a existência de custos de cuidados parentais para os machos, que limitam ainda mais a sua reprodução e assim contribuem para a reversão dos papéis sexuais. Competição intrasexual feminina por parceiros coexiste com intensa competição intrasexual masculina por

recursos, permitindo assim a reversão dos papéis sexuais e as TARs que se encontram nesta população. Essas TARs são sequenciais e condicionais. Um único ponto de conversão pode determinar a expressão das táticas como uma função da dominância social (ou do tamanho relativo do corpo) e justificar a existência de duas trajetórias alternativas da história vital. A "via de sneaker" em que os machos se reproduzem primeiro como sneakers e mais tarde como burgueses, e a "via de macho burguês" em que os machos, que eclodiram no início da estação reprodutiva anterior, são grandes o suficiente para reproduzir diretamente como burgueses. A dominância social promove a mudança para o morfótipo burguês, mas não influencia a maturação sexual, permitindo a existência da tática de 'sneaker'. A 11-cetotestosterona medeia o efeito da dominância na expressão dos caracteres sexuais secundários que são típicos do morfótipo burguês mas os mecanismos que promovem o desenvolvimento da glândula testicular e da expressão do comportamento de nidificação não foram ainda identificados. O "birth-date effect" foi detectado como importante influência na expressão das TARs da população. Machos nascidos mais cedo seguiram a "via burguesa", enquanto os eclodidos mais tarde seguem a "via de sneaker". O tempo decorrido entre as datas de eclosão das duas vias confere aos machos diferentes períodos de crescimento, conduzindo a uma diferença no tamanho relativo do corpo aquando do início da primeira época de reprodução. Esta diferença de tamanho do corpo deverá determinar o grau de dominância e por sua vez a tática reprodutiva a expressar e a trajetória da história vital.

ABSTRACT

The mating system of a South Portuguese population of the peacock blenny *Salaria pavo* (Risso, 1890) presents some special characteristics that render it a challenging model for the understanding of sexual selection. These are sex role reversal and alternative reproductive tactics (ARTs), two traits that classically have been interpreted as the result of opposite selective forces. The first is a consequence of female intra-sexual competition and the second has its origins in male-male sexual competition. Because intra-sexual competition has been theorized to be driven by the scarcity of the opposite sex, female and male intra-sexual competition are taken as mutually exclusive. However, they occur simultaneously in this population of *Salaria pavo*. Additionally, the presence of polymorphisms associated ARTs in this population, strongly facilitates studying proximate mechanisms involved in the expression of reproductive phenotypes.

The population occupies an area where nesting sites are rare. Competition among males for the access to these nests is therefore intense and only few males are able to establish and defend a nest. Females, on the other hand, compete for the access to these nesting males (bourgeois males) and display courtship behaviour. Both sexes mate with multiple mates but males are selective in respect to the females they accept as mates. Therefore sex roles are reversed. However, it is the bourgeois males that present secondary sexual characters and not the females. Bourgeois males are larger than females and present a pheromone producing anal gland and a conspicuous head crest as secondary sexual characters. They defend a nest and provide uniparental parental care to the eggs which consists of nest defence, nest cleaning and egg fanning. Smaller and younger males (sneakers) parasitize the investment in nest defence, female attraction and parental care of the bourgeois males. Sneakers mimic the morphology and the behaviour of the females in order to discretely approach the nest and fertilize part of the eggs.

In order to integrate ecological and physiological perspectives both descriptive and experimental studies both in the field and in the laboratory were conducted to address issues regarding sex role reversal and ARTs, namely:

1. Why do males present secondary sexual characters in a sex role reversed system, is female choice contributing to the maintenance of these male traits?
2. Is female choice based on brood size? Is there a trade off between parental effort and androgen levels limiting brood size?
3. Are ART's sequential in this population? What life-history pathways are present and are they related to the "birth-date" of males?

4. What social factors determine the expression of the different tactics and what are the endocrinological mechanisms underlying that expression?

5. How are the different sex and life-history stages distributed through the diverse microhabitats available to the population and how do they change with tide cycle and season? (Sampling considerations)

Despite the reversed sex roles, the results support the existence of female mate choice. Females of this population did not mate randomly, rather they mated following the common female strategy in teleost fishes which is preferentially with larger courting males with more developed secondary sexual characters and larger broods. This selection contributes therefore to the maintenance of male secondary sexual characters adding to the effect of male intra-sexual competition reported by Saraiva *et al.*, 2009.

Females sampled males by means of a flexible threshold tactic (“one step decision” tactic with “complete recall”) adjusted to the distribution of male quality and to the spatial distribution of males. This, together with nest aggregation, allows for reduced costs of partner sampling.

Independently of available nest space, broods tended to a stable size which varied between individuals despite confirmation of female preference for larger broods. A trade-off was also observed between circulating testosterone levels and brood size (as a proxy for parental care). This reflected female choice for male quality, in addition to the preference for large broods, and suggested regulation of female spawning rate by means of testosterone dependent behaviour of males, possibly through courtship. Such a regulation of brood size reveals in its turn, reproductive costs associated with parental care that further limit the reproduction of males, therefore contributing to male mate choice, female-female competition and consequent sex role reversal.

In spite the strong mate competition between females, resource competition among males for access to nesting sites imposes a regime of strong intra-sexual selection, hypothetically causing the emergence of ARTs in this population. ARTs are here, as classically described, related to resource acquisition for which body size and social dominance play a major role. In this population the tactics are expressed sequentially, with the same individual expressing the sneaker, female mimicking tactic when young, and the bourgeois (nesting) tactic when older and larger. However, different life-history pathways are observed: the “sneaker pathway” represents the sequential expression of the two tactics as described above; and the “bourgeois pathway” represents males that reproduce directly as bourgeois males in their first breeding season without ever

expressing the sneaker tactic. A third pathway of postpone reproduction in which males would not reproduce in the first season but express the bourgeois tactic in the next season is possible but has not been confirmed.

The two observed pathways are associated with date of hatching. Males following the “bourgeois pathway” hatched at the start of the breeding season, while males following the “sneaker pathway” tended to hatch later in the season, thus confirming the “Birth-date effect” proposed by Taborsky (1998) as one of the mechanisms involved in the expression of ARTs. Nevertheless, different dates of hatching had no apparent influence on growth patterns of the two pathways and therefore the “Birth-date effect” was mainly due to the time elapsed between the hatching of the males following each of the two pathways, which allowed early hatched individuals more time to grow until the breeding season.

The data accords with a condition-dependent mechanism of tactic expression that can be described as consisting of a single switching point triggered by social dominance (or relative body size) and determining the expression of secondary sexual characters, testicular gland and nesting behaviour, independently of sexual maturation. Males following either pathway are mature in the first breeding season, and their expression of one or the other tactic depends exclusively on the development or not of the typical traits of the bourgeois morphotype. The early hatched males that follow the “bourgeois pathway” arrive at the breeding season with a larger body than the latter hatched males. The first supposedly due to a higher hierarchical rank, initiate the expression of the bourgeois typical traits, while the latter, the smallest of the population and therefore the most subordinate, do not develop such traits and reproduce as sneakers (“sneaker pathway”). These sneakers attain the switching point at the end of the breeding season, when they become larger and more dominant and initiate the expression of the bourgeois morphotype.

11-Ketotestosterone, but not testosterone, mediated the effect of social dominance on the development of the head crest, characteristic of the bourgeois morphotype. However, this mediating effect was not detected for the remaining bourgeois traits and alternative mechanisms mediating this effect should be investigated. No support was found for the hypothesis of an interaction of cortisol on the hypothalamus-pituitary-gonad axis in the determination of the reproductive phenotype, in this case, the reproductive tactics. Cortisol was also not associated with social dominance but was inversely related to the relative size of gonads.

The two pathways differed significantly in their reproductive success further supporting the expression of the alternative tactics through a condition-dependent mechanism. The

reproductive success of the “bourgeois pathway” was much lower than that of the “sneaker pathway” raising the hypotheses of an opposing selective force acting in favour of early hatched individuals, such as higher egg or juvenile survival or higher fertility of early hatched females.

In conclusion, despite sex role reversal, females of the studied population have been found to be selective towards potential mates and this may be facilitated by the low costs of sampling males, allowing a potential benefit related to good parental care of which the eggs are dependent. These low costs are achieved through a flexible sampling tactic and due to the aggregation of males. The stability of brood size, on the other hand, suggests costs of parental care to the male, which further limit male reproduction and contribute to the reversal of sex roles. Female-female competition for mates coexisted with intense male-male competition for resources, therefore both sex role reversal and ARTs were present in the population. These ARTs were sequential and condition dependent. A single switching point may determine the expression of the tactics as a function of social dominance (or relative body size) and justify the existence of two alternative life-history pathways. The “sneaker pathway” in which males reproduce first as sneaker and later as bourgeois; and the “bourgeois pathway” in which males, who have hatched early in the previous breeding season, are large enough to reproduce directly as bourgeois. Social dominance promoted the switching to the bourgeois morphotype, but had no effect on maturation allowing the existence of the sneaker tactic. 11-Ketotestosterone mediates the effect of dominance on the expression of secondary sexual characters that are typical of the bourgeois morphotype but the remaining mechanisms promoting the development of the testicular gland and the expression of nesting behaviour were not identified. A “birth-date effect” was detected to influence the expression of the ARTs in this population. Early hatched males followed the “bourgeois pathway”, while later hatched followed the “sneaker pathway”. The time elapsed between hatching dates confers males growing periods of different duration until the first breeding season, leading to a lag in body size (and therefore dominance rank) that determines the tactic expressed at the first breeding season, or in other words, the life-history pathway followed.

RÉSUMÉ

Le système d'accouplement de la population sud-portugaise de la blennie paon, *Salaria pavo* (Risso, 1890) présente des caractéristiques particulières qui le rend un modèle stimulant pour la compréhension de la sélection sexuelle: les tactiques de reproduction alternative (TRAs) et la réversion des rôles sexuels. En outre, la présence de polymorphismes, associés aux TRAs, dans cette population constitue une excellente opportunité d'aborder les causes proches impliquées dans l'expression des phénotypes reproducteurs. La réversion des rôles sexuels et les TRAs des mâles sont classiquement interprétés comme le résultat des forces sélectives opposées. Le premier est conséquence de la compétition intra-sexuelle féminine et le second a ses origines dans la compétition sexuelle mâle-mâle. Parce que la compétition intra-sexuelle est théorisée comme le résultat du déficit de l'autre sexe, la compétition intra-sexuelle féminine et la compétition intra-sexuelle masculine sont prises pour mutuellement exclusives. Toutefois, elles sont présentes simultanément dans cette population.

La population occupe une région où les sites de nidification sont rares. La concurrence entre mâles pour l'accès à ces nids est donc intense et seulement quelques mâles sont capables d'établir et défendre un nid. Les femelles, d'autre part, se battent pour l'accès à des mâles nidifiant (mâles bourgeois) et elles exécutent des comportements de cour vers ces mâles. Les deux sexes s'accouplent avec plusieurs partenaires, mais les mâles sont sélectifs en ce qui concerne les femelles qu'ils acceptent comme partenaires. Par conséquent, les rôles sexuels sont inversés. Toutefois, ce sont les mâles bourgeois qui présentent les caractères sexuels secondaires et pas les femelles. Les mâles bourgeois sont plus grands que les femelles et ils ont, comme des caractères sexuels secondaires, une glande anale que produit des phéromones et une crête céphalique bien évidente. Ils défendent un nid et offrent des soins parentaux aux œufs. Ces soins consistent en la défense et le nettoyage des nids et le 'fanning' des œufs (des mouvements ondulatoires du corps et des nageoires pour ventiler le nid et oxygéner les œufs). Les mâles plus jeunes et plus petits ('sneakers') parasitent l'investissement des mâles bourgeois en défense du nid, en attraction des femelles et en soins parentaux. Les sneakers imitent la morphologie et le comportement des femelles, afin d'approcher discrètement le nid et fertiliser une partie des œufs.

Afin d'intégrer les perspectives écologiques et physiologiques, des travaux descriptifs sur le terrain et des études expérimentales de laboratoire ont été menées pour aborder les questions relatives à la réversion des rôles sexuels et à des TRAs, à savoir:

1. Pourquoi les mâles présentent-ils des caractères sexuels secondaires dans un système où les rôles sexuels sont inversés, est-ce que le choix féminin contribue à l'entretien de ces traits masculins?
2. Est-ce que les femelles choisissent leurs partenaires en fonction de la taille de leur couvée? Y a-t-il un compromis entre l'effort parental et les taux d'androgènes limitant la taille de la couvée?
3. Est-ce que les TRAs sont séquentielles dans cette population? Quelles trajectoires de l'histoire de vie sont présentes, et sont-elles liées à la date de naissance des mâles?
4. Quels sont les facteurs sociaux qui déterminent l'expression des différentes tactiques et quels sont les mécanismes endocrinologiques sous-jacents à cette expression?
5. Comment se distribuent les individus des différents sexes et des différents stades de l'histoire de vie par les microhabitats présents dans l'aire de présence de la population, et comment changent-ils avec le cycle de marées et les saisons? (considérations pour les procédures d'échantillonnage)

Malgré les rôles sexuels inversés, les résultats confirment que les femelles sont sélectives envers les mâles qu'elles acceptent comme partenaires. Les femelles de cette population ne s'accouplent pas aléatoirement, plutôt elles se sont accouplées préférentiellement avec des mâles courtisans, qui ont des grandes couvées et des caractères sexuels secondaires plus développés. Cette sélection contribue donc à la conservation des caractères sexuels secondaires des mâles, ajoutant à l'effet de la compétition intra-sexuelle masculine rapporté par Saraiva *et al.* (2009). Les femelles examinent et comparent leurs potentiels partenaires en recourant à une tactique de seuil ("threshold") flexible - "One Step decision" avec "total recall". Cette tactique se caractérise pour un échantillonnage séquentiel des mâles et une comparaison des caractéristiques de ces mâles à un niveau de qualité minimal. Pourtant, ce niveau minimal n'est pas absolu, il est ajustable à la qualité des mâles que les femelles rencontrent et aux efforts de chercher des nouveaux mâles qui puissent surmonter ce seuil. Les femelles peuvent toujours retourner pour accepter un partenaire qu'elles avaient rejeté avant. Ceci, avec l'agrégation des nids, permet de réduire les coûts de l'échantillonnage.

Indépendamment de l'espace disponible à l'intérieur des nids, les couvées tendent à maintenir une taille stable qui varie entre les individus, en dépit de la confirmation de la préférence féminine pour les couvées plus grandes. Un compromis a également été observé entre les taux circulants de testostérone et la taille des couvées (comme un proxy pour les soins parentaux). Cela reflète le choix féminin pour des qualités individuelles des

mâles, en plus de la préférence pour les grandes couvées, et a suggéré la réglementation des taux de fraye chez les femelles à travers des comportements de reproduction des mâles dépendant des niveaux de testostérone, comme éventuellement la cour. Une telle régulation de la taille des couvées révèle, à son tour, des coûts de reproduction associés aux soins parentaux qui limitent davantage la reproduction des mâles, contribuant ainsi au choix masculin des partenaires, à la compétition entre femelles et, en conséquence, à la réversion des rôles sexuels.

Malgré la forte compétition pour des partenaires entre les femelles, la compétition pour des ressources entre les mâles - pour l'accès aux sites de nidification - impose un régime de sélection intra-sexuelle masculine très forte qui est hypothétiquement responsable pour l'émergence des TRAs dans cette population. TRAs sont ici, comme classiquement décrites, liées à l'acquisition de ressources, pour laquelle la taille du corps et la dominance sociale jouent un rôle majeur. Dans cette population les tactiques sont exprimées de façon séquentielle, le même individu exprime premièrement la tactique de 'sneaker' et plus tard, quand il est plus âgé et plus grand la tactique bourgeois (mâle nidifiant). Pourtant, des différentes trajectoires de l'histoire de vie sont observées: la "voie sneaker" représente l'expression séquentielle des deux tactiques telles que décrites ci-dessus et la "voie bourgeois" représente les mâles qui se reproduisent directement en tant que mâles bourgeois, sans qu'ils n'expriment jamais la tactique de sneaker. Une troisième trajectoire dans laquelle les mâles ne se reproduisent pas dans la première saison, mais expriment la tactique bourgeois dans la saison suivante, est possible mais n'a pas été confirmée.

Les deux trajectoires observées sont associées avec la date d'éclosion des œufs. Les œufs des mâles suivants la "voie bourgeoise" éclosent au début de la saison de reproduction, tandis que les mâles suivant la "voie sneaker" tendaient à éclore plus tard dans la saison, confirmant ainsi le 'Birth-date effect' proposé par Taborsky (1998) comme l'un des mécanismes impliqués dans l'expression des TRAs. Néanmoins, les différentes dates d'éclosion n'ont eu aucune influence apparente sur les modes de croissance des mâles des deux trajectoires. Donc le 'birth-date effect' a été principalement dû au temps écoulé entre l'éclosion des mâles qui ont suivi chacune des deux voies, et qui a permis aux individus éclos au début de la saison d'avoir plus de temps pour se développer jusqu'au moment d'initiation de la reproduction. Les résultats sont compatibles avec un mécanisme conditionnel d'expression des tactiques qui peut-être décrit comme étant composé d'un seul point de commutation ('switching point') déclenché par la dominance sociale (ou la taille relative du corps). Ce mécanisme induirait le début de l'expression des caractères sexuels secondaires, de la glande testiculaire et le comportement de

nidification, indépendamment de la maturation sexuelle. Les mâles suivants chacune des deux trajectoires sont matures durant la première saison de reproduction et l'expression de l'une ou l'autre des tactiques dépend exclusivement du développement, ou pas, des traits typiques du morphotype bourgeois. Les mâles éclos plus tôt arrivent à la saison de reproduction avec une taille plus grande que ceux qui ont éclos plus tard. Les premiers, soi-disant en raison d'un rang hiérarchique supérieur, initient l'expression des traits typiques du morphotype bourgeois et suivent la "voie bourgeois". Tandis que les seconds, les plus petits de la population et donc les plus subordonnés, ne développent pas de tels traits et suivent la "voie sneaker". À la fin de la saison reproductive les 'sneakers' atteignent le point de commutation, car ils sont à ce moment là plus dominants, et ils initient l'expression du morphotype bourgeois.

La 11-Cétotestostérone, mais pas la testostérone, a véhiculé l'effet de dominance sociale sur le développement de la crête céphalique, caractéristique du morphotype bourgeois. Cependant, cet effet de médiation n'a pas été détecté pour les autres traits du morphotype bourgeois, et des autres mécanismes de médiation doivent être investigués. L'hypothèse d'une interaction du cortisol avec l'axe hypothalamus-hypophyse-gonades dans la détermination du phénotype de reproduction n'a pas trouvé de soutien dans cette expérience. Le Cortisol n'était pas associé à la dominance sociale, mais était inversement associé à la taille relative des gonades. Les deux trajectoires de l'histoire de vie différaient sensiblement par leur succès reproducteur en supportant l'hypothèse l'expression des tactiques alternatives à travers un mécanisme dépendant de la condition. Le succès de reproduction de la "voie bourgeois" était beaucoup plus faible que celui de la "voie sneaker" en enlevant l'hypothèse d'une force sélective en faveur de l'éclosion au début de la saison, comme par exemple, des plus hauts taux de survie des œufs ou des juvéniles ou une plus haute fécondité des femelles ayant éclos plus tôt.

En conclusion, malgré la réversion des rôles sexuels, les femelles de la population étudiée sont sélectives envers leurs potentiels partenaires sexuels et cela peut être facilité par le faible coût de l'échantillonnage des mâles, ce qui permet un gain potentiel lié à des soins parentaux dont la survie des œufs est dépendant. Ces faibles coûts sont atteints grâce à une tactique souple de l'échantillonnage et à l'agrégation des mâles. La stabilité de la taille des couvées, d'autre part, suggère des coûts de soins parentaux aux mâles, qui limitent en outre la reproduction masculine et qui contribuent au renversement des rôles sexuels. La compétition intra-sexuelle féminine pour l'accès aux mâles coexiste avec la compétition intra-sexuelle masculine pour l'accès aux ressources, donc à la fois la réversion des rôles sexuels et les TRAs sont présents dans la population. Ces TRAs sont

séquentielles et conditionnelles. Un seul point de commutation peut déterminer l'expression de la tactique en fonction de la dominance sociale (ou la taille relative du corps) et peut justifier l'existence des deux trajectoires alternatives de l'histoire de vie : La "voie sneaker", dans laquelle les mâles se reproduisent d'abord comme sneakers et plus tard en tant que bourgeois, et la "voie bourgeois" dans laquelle les mâles, qui ont éclos au début de la saison de reproduction précédente, sont assez grands pour se reproduire directement en tant que bourgeois. La dominance sociale induit la commutation vers le morphotype bourgeois, mais n'a aucun effet sur la maturation sexuelle ce qui permet l'existence de deux tactiques distinctives. La 11-Cétotestostérone induit l'effet d'une position dominante sur l'expression des caractères sexuels secondaires qui sont typiques du morphotype bourgeois, mais les mécanismes qui servent à promouvoir le développement de la glande testiculaire et l'expression du comportement de nidification n'ont pas été identifiés. Le 'birth-date effect' a été influent sur l'expression des TRAs dans cette population. Les mâles éclos plus tôt ont suivi la "voie bourgeois", tandis que ceux qui ont éclos plus tard ont suivi la "voie sneaker". Le temps écoulé entre les dates d'éclosion a conféré aux mâles des périodes de différentes durées pour grandir, conduisant à un décalage dans la taille du corps (et donc du rang de dominance) qui détermine la tactique exprimée lors de la première saison de reproduction, ou en d'autres termes, la voie de l'histoire de vie suivie.



GENERAL INTRODUCTION

GENERAL INTRODUCTION

The concept of mating system

Classifications of mating systems intend to synthesize the ways in which individuals of a species reproduce (Darwin, 1871). The structuring force of mating systems is sexual selection, the mechanism through which individuals that bear traits that increase mating success (morphological, behavioural or others) will have relative higher fitness than others, therefore contributing to the maintenance of such traits in the population. Sexual selection acts through two major forces: intrasexual competition, in which individuals of the same sex interact directly or indirectly to obtain reproductive resources, including mates; and intersexual competition through which individuals differentially select potential mates. It is the strength of sexual selection that will dictate the amplitude of variation in mate numbers between the individuals of the same sex. And the direction of sexual selection (*sensu* Clutton-Brock and Vincent, 1991) will determine which of the sexes will be under stronger selective pressure, that is, the sex roles - which sex will be more strongly competing for access to mates and which sex will be more selective when choosing mates. Sexual selection will mold the forms of competition and resource defence, forms of courtship and mate choice, sperm competition and mate guarding, pair bonds and parental care, and mating skew (*i.e.* variation in the number of mates individuals may obtain) which are the central components of mating systems (Reynolds, 1996).

The evolution of the concept and the concept of evolution

Differential reproduction lies at the heart of the evolutionary theory, promoting the maintenance and invasion of those traits that increase survival and reproduction of their carriers. Phenotypes differ in their viability, which alone can lead to differential reproduction, but they also differ in their mating success with powerful consequences on differential reproduction and thus rendering sexual selection one of the fastest and strongest evolutionary forces (Shuster and Wade, 2003).

In order to explain the origin of secondary sexual characters, Darwin (1871) proposed the theory of sexual selection. According to his view, exuberant male traits that opposed survival were selected because they increased mating success. These traits would favour acquisition of mates through intra- and intersexual interactions. Sexual selection was therefore defined as the mechanism of differential reproduction through differential

number of mates, and the first attempt of mating system classification was based solely on the number of mates that individuals obtain.

Since then many authors have contributed to the development of mating system classification, emphasising different forces that contribute to the shaping of these systems, and the original concepts reflect a strong influence of bird and mammal examples (e.g. Darwin 1871).

Nevertheless, one of the first major contributions to the study of mating systems was that of Bateman with his study on *Drosophila* (1948). He proposed a process today known as “Bateman gradient” as the cause of sexual selection. He observed that females, due to their lower intrinsic reproductive rate, gain less reproductive success by multiple mating than males do and that that difference would favour male competition for access to females and female choice.

William's principle (1966) also contributed to the evolution of the concept of mating systems by introducing the notion that present reproduction has costs in terms of future reproduction. And this same idea was subjacent to Trivers (1972) theory of parental investment. The theory states that relative parental investment dictates the direction of intrasexual competition and mate choice. Individuals of the less investing sex will compete to mate with individuals of the sex that invests more; and individuals of the sex that invests more will be selective towards potential mates (of the less investing sex). This pattern is influenced by anisogamy, in that females, by definition, produce the largest gametes and therefore have a higher initial parental investment. But parental care constitutes a second layer that in most species further accentuates this difference in investment.

The concept of operational sex ratio was later introduced by Emlen & Oring (1977) and defined as the average ratio of fertilizable females to sexually active males at any given time. This ratio identifies the limiting sex (the sex in short supply) which should be selective in mate choice; and the limited sex (that in excess) which should compete but not choose. The operational sex ratio depends on the spatial and temporal distribution of the limiting sex which, in turn, determines the environmental potential for polygamy, that is, how economically defendable are multiple mates. Operational sex ratio will define the shape of the mating system to the extent to which it determines the monopolization of mates by the limited sex. When some individuals are able to monopolize diverse mates, others will be unable to access the limiting sex. This variation in mating success is the essence of sexual selection.

The concept of operational sex ratio has been criticized by difficulties in its quantification and an alternative parameter, potential reproductive rate, was proposed as an empirical measure by Clutton-Brock and co-workers (Clutton-Brock and Vincent, 1991; Clutton-Brock and Parker, 1992). The potential reproductive rate is defined as the offspring production of each sex per unit of time, if unconstrained by mate availability. This measure closely follows operational sex roles and therefore should equally identify the direction of sexual selection, namely which sex will more intensely compete for mates. However, Ahnesjö *et al.* (2001) have noted that this parameter together with the time-out model of Simmons and Parker (1996), despite excellent measures of mate competition, can neither assess resource competition nor sperm competition.

More recently, Kokko and Monaghan (2001) have argued that neither operational sex ratio nor potential reproductive rates will completely predict the direction of sexual selection, as other factors not accounted for by these parameters influence considerably the determination of sex roles. Such factors are variance in mate quality, differential mortality rates between sexes and, most importantly, the costs of reproduction, renewing attention to the parental investment concept of Trivers (1972).

Johnstone *et al.* (1996) had already modelled mutual mate choice and predicted that costs of choice should play a more important role in the determination of mate choice than variation in mate quality. The model supported the general observation that the less competitive sex is usually the more selective but predicted that in certain circumstances the more selective sex could equally be the more competitive; defying one of the most rooted ideas of the study of mating systems.

Finally, Shuster and Wade (2003) proposed a more descriptive mating system classification that recuperates the emphases of spatial and temporal distribution of resources of Emlen and Oring (1977) but is more process centred, and they also consider variation in behaviour within the same sex. Within the context of behavioural variance, the use of genetic markers has introduced the concepts of social and genetic mating systems (Avice *et al.*, 2002) through the detection and quantification of cuckoldry, and the detection of extrapair copulations involving female mate choice for indirect (genetic) benefits (Reynolds, 1996) and other alternative, and very often discrete, reproductive behaviours. Hence, more attention has been paid recently to both alternative reproductive tactics (ARTs) and female choice when considering mating systems.

Fish mating systems

The most classical and most general classifications of mating systems are based on the number of mates that reproducing individuals, males and females, have per breeding season and this may be visualized as a continuum, ranging from polygyny to polyandry, and being associated with the strength and direction of sexual selection. Birds are socially monogamous, reflecting a balanced intensity of sexual selection among the two sexes; while mammals are typically polygynous, reflecting stronger sexual selection acting on males. Teleost fish, in comparison, show a wide diversity of mating systems, commonly including polygynandrous and polyandrous species, in which males generally provide parental care and where sex role reversal can be observed particularly in polyandrous species (Awise *et al.*, 2002). But the diversity of fish mating systems goes beyond the multiple patterns of number of mates. Fish show the greatest diversity of reproduction forms: there are gonochoric species where sexes are fixed and distinct, simultaneous hermaphrodites, sex-changing species are fairly common in fish when comparing to other vertebrate taxa (see Devlin and Nagahama, 2002 for review) and ARTs are widespread in fish (see Gross, 1984; Taborsky, 1998, 2001, 2008 for reviews). Parental care varies from absent to biparental with prevalence of male parental care (see Clutton-Brock, 1991; Gross and Sargent, 1985; Smith and Wootton, 1995 for reviews). Sex roles are also very diverse, ranging from the conventional pattern to reversed sex roles (see Vincent *et al.*, 1992 for review; e.g. Syngnathidae: Jones and Awise, 2001), which in some species may even be flexible and vary in time (e.g. St. Peters fish, *Sarotherodon galilaeus*: Ros *et al.*, 2003; two-spotted goby, *Gobiusculus flavescens*: Forsgren *et al.*, 2004). Teleost fish provide therefore perfect models for studying ultimate and proximate factors of organization of mating systems.

Sex role reversal

Initial parental investment in the form of anisogamy has since ever been fundamental in the study of mating systems and in the understanding of “conventional” sex roles (Trivers, 1972). Anisogamy is probably the central driving force of sexual selection owing to the different number of gametes that the two sexes can produce and that allows for competition among male gametes. According to Bateman’s principle (Bateman, 1948), due to their lower initial parental investment in gametes, males gain more reproductive success by multiple mating than do females (Arnold and Duvall, 1994). This should select for multiple matings by males at the expense of male parental care, since in most taxa a trade-off exists between parental care and number of additional mates (Magrath and

Komdeur, 2003). In this way, males may increase their reproductive success by engaging in additional matings more than they would by tending offspring. Females, on the other hand, gain less net benefits through multiple matings and therefore will tend to defend their initial investment. If parental care has impact on offspring survival, females will tend to provide it. Parental care will thus increase the parental investment of females and reinforce the performance of conventional sex roles.

The concept of parental investment is intrinsically related to the parameters established to try to quantify the strength and direction of sexual selection: operational sex ratio (Emlen and Oring, 1977; Kvarnemo and Ahnesjö, 1996), potential reproductive rates (Clutton-Brock and Vincent, 1991; Clutton-Brock and Parker, 1992), costs of breeding (Kokko and Monaghan, 2001). Although not interchangeable, these parameters are usually correlated (Kokko and Monaghan, 2001). Parental care for instance, obviously impacts in the same direction each of these parameters.

There are however species in which, contrary to conventional roles, females compete among themselves for access to males, exhibit mate attraction behaviours, such as courtship, and may even develop secondary sexual characters, while males provide more parental care to their progeny than females and are selective in their mate choice (Andersson, 1994; Trivers, 1985). These are said to be sex role reversed species. As the research on the Syngnathidae family has demonstrated (e.g. Jones *et al.*, 2000), sex role reversed species are particularly interesting to test hypotheses on sexual selection and mating systems since they represent the opposite extreme of sexual selection forces than that found on most birds and mammals, in which the first studies on mating systems were carried out (Avise *et al.*, 2002).

In teleost species male parental care is more common than in other taxa (Gross and Sargent, 1985; Smith and Wootton, 1995). Most teleost fish present external fertilization, and not only the degree of anisogamy is usually less pronounced than, for instance, in birds, but also the male providing the care will certainly have fathered the brood (at least in the absence of ARTs). Adding to this is the fact that territorial defence, normally executed by males, may enhance offspring survival more than female parental care would. Females on the other hand will have the opportunity to desert their eggs when mating, because males are usually the last to release gametes during spawning (Trivers, 1972). And should pay them to do so, for the reason that fertility in female fish is usually associated with body size and allocating resources to parental care would compromise growth, diminishing their future reproductive success (Gross and Sargent, 1985).

Male parental care alone is not synonymous with sex role reversal but it may conduce to it if reduces the availability of “receptive” males, that is, if increases the operational sex ratio, and thus poses limits on the reproduction of females. This will lead to an excess of female gametes and competition among them for access to males. Such conditions can be brought about by limitations of space to receive eggs in nests or, in the case of Syngnathidae, in brood pouches (Berglund and Rosenqvist, 2003) or by high costs of parental care (Shuster and Wade, 2003). Female-female competition will enable male mate choice intensifying sexual selection which may lead to females exhibiting secondary sexual characters (e.g. the pipefish, *Nerophis ophidian*: Berglund *et al.*, 1996). Mate selectivity of one sex may be seen as stemming from intrasexual competition of the opposite sex (Trivers, 1972) and influenced by the weight of direct and indirect benefits and the costs of breeding (Kokko and Johnstone, 2002). In sex role reversed species, females are supposed to compete for access to males and be subject to their selection but competition and choosiness are not necessarily mutually exclusive (Kokko *et al.*, 2006), despite the rarity of mutual mate choice (Kokko and Johnstone, 2002).

Alternative reproductive tactics

Asymmetry in male mating success allows for the emergence of ARTs (Taborsky, 2001). Variation in behavioural traits should be selected in such a way that those individuals that can exploit the investment in resource acquisition of others, parasitizing the benefits of that investment but avoiding its costs, will differentially reproduce and invade the population (Shuster and Wade, 2003). ARTs are therefore intraspecific and intrasexual alternative behaviours that allow individuals to maximize reproductive success in face of strong sexual selection (Taborsky *et al.*, 2008). Typically, these behaviours allow the individuals of the class with lower mating success to exploit the reproductive investment of the other class, more successful in mating (Taborsky, 2001). In general, associated with differences in these behaviours are morphological and physiological traits that vary discontinuously within the population. Discontinuity of reproductive traits found in the ARTs is the product of disruptive selection acting on intermediate phenotypes (Gross, 1985; Brockmann, 2001; Brockmann and Taborsky, 2008). Taborsky (1994, 1997) has identified these two classes as bourgeois males (also referred to as final stage or type I males) and parasitic males (also referred to as initial stage males or type II males). Bourgeois males invest in male-male competition for reproductive resources and access to females, thus typically exhibiting secondary sexual characters and courtship behaviours. The parasitic males explore that investment in different ways like darting (e.g. pygmy swordtail, *Xiphophorus nigrensis*: Zimmerer and Kallman, 1989), female mimicry

(e.g. peacock blenny, *Salaria pavo*: Gonçalves et al, 1996), sneaking and helping (e.g. satellite males in the Azorean rockpool blenny, *Parablennius parvicornis*: Santos et al., 1996).

More than two ARTs are in fact possible and also female ARTs exist (an example of both is the side-blotched lizard, *Uta stansburiana*: Sinervo and Lively, 1996). However, ARTs are more prone to emerge in the male sex due to a stronger effect of sexual selection acting on this sex class.

ARTs in fish

ARTs are widespread among fish species. Teleosts show indeterminate growth and thus size differences among reproducing individuals may be extreme. Smaller males will be unable to compete for resources essential to reproduction, such as a territory, consequently creating a large asymmetry in mating success between males. However, because fertilization is generally external in fish, the smaller outcompeted males can interfere in spawning events and sire a fraction of the eggs. These two conditions promote the occurrence of ARTs in teleosts (Taborsky et al., 2008). And in species with male parental care, so common in this group, the smaller males when using ARTs parasitize not only the investment in mate attraction but also the parental care of the bourgeois males. The exploitation of the parental care provided by other males should constitute a major benefit for the parasitic tactic, particular when parental care is costly and dependent on brood size. In this way, parasites not only increase their reproductive success but also decrease that of the bourgeois males. Despite the large potential benefit of parental care exploitation, a recent phylogenetic study has demonstrated that the emergence of ARTs in ray finned fish (Actinopterygii) has multiple origins independent of parental care (Mank and Avise, 2006) therefore emphasizing the importance of mate acquisition and intrasexual competition in the emergence of ART.

Pattern and determination of ARTs

ARTs are very diverse in their pattern. They may be fixed, if each individual within a species expresses only one tactic throughout its life, or flexible, in species in which individuals reproduce in both ways along their lives. Flexible sequential tactics can in turn differ in their ontogenetic pathways presenting either reversible or fixed sequences, depending on the ability or not of moving back and forward between the tactics (Taborsky et al., 2008).

The determination of the tactics is equally variable. Some species seem to have a more heritable determination of tactics, as Shuster and Wade (2003) defend to be the most common situation, and as demonstrated in the side-blotched lizard (*Uta stansburiana*: e.g. Sinervo and Lively, 1996) and in the marine isopod (*Paracerceis sculpta*: e.g. Shuster, 1989). In other species the expression of the tactics has been shown to be conditionally dependent on environmental cues and/or on individual condition. Examples of species with conditional tactics are the Atlantic salmon (*Salmo salar* L.: Rowe and Thorpe, 1990; Aubin-Horth *et al.*, 2006) and the European earwig, *Forficula auricularia*: Tomkins, 1999; Tomkins and Brown, 2004).

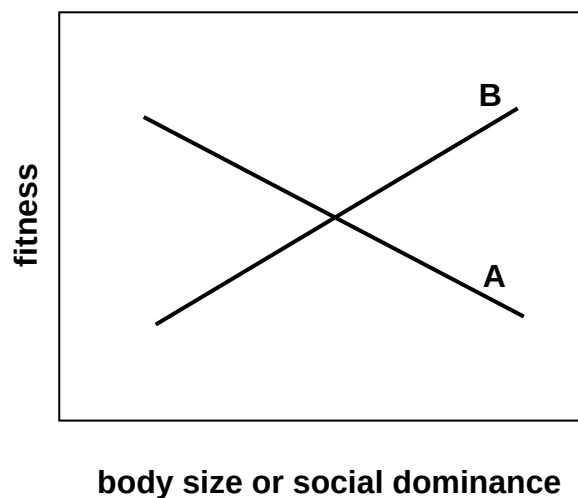


Figure 1. Crossing fitness curves. A and B represent the fitness curves of two tactics and how the fitness of individuals expressing them are dependent on individual state, such as age, body size or social dominance. Individuals with lower values of the individual state considered will have higher reproductive success if expressing the tactic A, while on the other extreme, individuals with higher values of that state will be more successful when expressing the tactic B.

Selection against intermediate phenotypes is responsible for the discontinuity of reproductive traits found between ARTs. When the relationship between a phenotypic trait and the fitness of the individuals expressing it differs between the two tactics and when these fitness curves cross (Fig. 1, concave fitness curves), intermediate phenotypes at the crossing point of the curves, the switching point, will have lower reproductive success than either of the extremes. Disruptive selection will therefore act to promote the expression of each of the tactics associated with the phenotype that maximizes their fitness. This may be achieved either through genetic polymorphisms, in which the different traits that characterize each tactic are under the control of the same genes or genes in linkage; or through condition dependent tactics in which the tactic expressed will depend on the

phenotype expressed by an individual. In the context of conditional tactics, the same mechanism may be applied to other factors determining fitness, as the relative frequency of tactics or other environmental or social cues, as well as combinations of individual and demographic effects (Gross, 1985; Taborsky, 1999; Brockmann, 2001; Brockmann and Taborsky, 2008).

Several models have been proposed for conditional expression of ARTs of which the two most prominent are the status-dependent model (Gross, 1996; Gross and Repka, 1998a, b) and the threshold model (Hazel *et al.*, 1990; Emlen, 2008). In each of these models, the reproductive phenotype is expressed according to the sensitivity to some cue. If the cue reaches a determinate critical value one tactic is expressed, if the values of the cue remain below that reference value, the other tactic will be expressed. While in the threshold model this cue may be either an environmental signal or an individual condition, in the status dependent model this cue is conceptualized as some parameter of individual condition. Both models, either since its original formulations or later through addition of new components, include in reality an interaction between environmental and genetic effects. In the two models the individual condition is assumed to have additive genetic variation and to be sensitive to environmental influence. However, the decision mechanism in the status dependent model is described to be fixed (although it may be interpreted as probabilistic) in respect to the critical value of the cue and therefore it is assumed to represent a genetic monomorphism. Contrarily, the threshold model considers genetic variation in the threshold value, *i.e.* the critical value upon which the decision to express one or the other reproductive phenotype is taken, meaning that different individuals will react differently to same value of the cue.

In specific but common circumstances in which the switching point of conditional tactics is associated with body size, the “birth-date effect” may determine the expression of one or the other ART. Taborsky (1998) has proposed this hypothesis as a possible cause for differences in body size and consequent ART expression. When the breeding season of one species is long or when reproduction is asynchronous, date of birth may vary considerably between individuals of the same species. In temperate climates, this may also coincide with different seasonal environmental conditions that influence growth patterns. For these reasons, individuals born earlier or later will differ in body size. Body size is one of the most likely determinants of conditional ARTs. The success of the bourgeois tactic is associated with resource defence and therefore body size, which is generally associated with social dominance and resource holding potential. Such circumstances allow conditional mechanisms that determine expression of tactics in function of body size to emerge. In fact, the most frequent examples of conditional tactics

involve body size as cues for tactic expression, as for example in the scarab beetle, *Onthophagus taurus* (Moczek and Emlen, 2000).

The conditional mechanisms of tactic determination can underlie different patterns of ARTs. Fixed, in which the decision mechanism presents a sensitive period, usually during early stages of development, and the tactic expressed afterwards will be constant throughout the lifetime of the individual; flexible, with fixed sequences, in which the same mechanism may determine the moment an individual will switch between tactics; or flexible and reversible sequences, where the individual switches back and forth between tactics. These latter usually involve only behavioural differences and none or minor morphological differences, but may also potentially fit the conditional models if the proximate mechanisms of tactic expression do not compromise the expression of the alternative phenotypes later on and if the changes are able to occur rapidly. Therefore proximate mechanisms are also essential in understanding the expression of ARTs.

Proximate mechanisms

In the expression of the phenotypes associated with ARTs, the neuroendocrine system plays a crucial role. The neuroendocrine system and androgens in particular are the central physiological mechanism coordinating male reproduction, from the development of primary and secondary sexual characters, to the onset of sexual maturation, to the facilitation of reproductive behaviours (Nelson, 1995; Adkins-Regan, 2005). Furthermore, hormone levels feedback with social environment in a bidirectional relationship in which hormones promote certain behaviours to be displayed in a social context but their levels also respond to the social environment in which the individual is integrated (Wingfield *et al.*, 1990; Oliveira *et al.*, 2002, 2005; Hirschenhauser *et al.*, 2003; Oliveira, 2004; Hirschenhauser and Oliveira, 2006). Sex hormones are steroids which, after binding with specific receptors in the target cells, promote gene transcription and the subsequent translation into proteins implicated in the expression of behaviour or development of certain structures. Therefore, this mechanism has not only the potential to trigger the genetic programming of a genotype but also to constitute the vehicle of an environment x genotype interaction, through the modulation of hormones by environmental factors. In this way hormones may mediate the development of the reproductive phenotype either in fixed or flexible, genetic or conditional tactics.

In fish, the two most relevant androgens are 11-ketotestosterone and testosterone. 11-ketotestosterone is the main androgen involved in the expression of secondary sexual characters and male competitive behaviours (Hoar, 1965; Borg, 1994; Oliveira *et al.*,

2005). In all teleost species with ARTs that differ in morphological traits, levels of 11-ketotestosterone of bourgeois males have been reported to be higher than those of parasitic males (Oliveira *et al.*, 2008). This is not necessarily true for species in which alternative tactics differ only in behavioural traits and do not show morphological differences (e.g. St. Peters fish, *Sarotherodon galilaeus*: Ros *et al.*, 2003). Hence, androgens alone can not fully explain the expression of the different reproductive tactics within a species (Oliveira *et al.*, 2005, 2008). Species with alternative tactics that are restricted to behavioural differences are usually flexible and switching between tactics may be very rapid suggesting the exclusive involvement of neural pathways. Neuronal mechanisms underlying alternative tactics are beyond the scope of this work but most studies have been dedicated to the effects of arginine vasotocin and gonadotropin-releasing hormone, and more recently to the effect of aromatase and neurosteroids, on reproductive behaviour and its plasticity.

An interaction between androgens and glucocorticoids as an underlying mechanism of ARTs expression has also been proposed by Knapp (2003). The enzyme complex 11 β -hydroxylase (11 β -H) and 11 β -hydroxysteroid dehydrogenase (11 β -HSD) control both the production of 11-ketotestosterone from testosterone and the activation/inactivation of glucocorticoids, more precisely cortisol. Through this mechanism a feedback between cortisol and androgens is possible, potentially mediating the effect of dominance suppression of reproduction in sex-changing (Perry and Grober, 2003) and of secondary sexual characters in ARTs species. However, diverse patterns of glucocorticoids have been reported in species with ARTs and in many, dominance is not associated with glucocorticoids circulating levels (e.g. the cichlid *Neolamprologus pulcher*, Buchner *et al.*, 2004).

The effects of hormones will also and fundamentally depend on the target tissues they will reach. Consequently, the field is now proceeding towards identifying sensibility of tissues to the different hormones, and that includes understanding the effects of steroidogenic enzymes, binding globulins, hormone receptors and the subsequent battery of genetic effects that the different tissues will be able to carry out. In these last point, understanding the activity/expression of potentially interesting genes (Gonçalves *et al.*, 2010), or random samples of genes with no known effect through microarrays (Aubin-Horth *et al.*, 2005) is a particularly promising venue for future research.

Life-history

In a more ecological perspective, a life-history theory approach to the study of ARTs is equally promising. A life-history perspective focuses on the important reproductive events in the life of individuals, like age at first reproduction, number of reproductive events in life, number and quality of offspring, age specific growth and survivorship, and the trade-offs involved between these traits (Stearns, 1976). Selection acting on individuals will not privilege one or another trait but instead promote the propagation of sets of traits that individuals carry. Successful individuals are those carrying combinations of traits that maximize their survival and reproduction. However constraints exist in the degree of expression of these traits because individuals do not possess infinity access to resources, energy or time. Trade-offs between different traits will reflect diverse solutions to maximize fitness in the presence of such constraints. This is actually what is seen in ARTs and consequently life-history theory is central to the understanding of ARTs and its evolution. A life history perspective will not only allow a description of the patterns of reproductive tactics and the details and timing of its expression but it will also allow the acquisition of reliable information on lifetime reproductive success of individuals, although typically in ARTs parental genetic assessment will be necessary in addition. Reproductive success, as a proxy to fitness, will be essential in understanding the function, i.e., the adaptative benefits of ARTs and the stability and dynamics of the co-occurrence of these alternative tactics, shedding light into the evolutionary dynamics of ARTs.

Model species

The peacock blenny, *Salaria pavo* (Risso, 1810) is a benthic fish included in the family Blenniidae. It inhabits the littoral shores of the East Atlantic Ocean, from Morocco to the North of France and the Mediterranean Basin, typically occupying rocky intertidal areas (Zander, 1986).

However some populations have colonized sandy shores, of which the two most studied are situated in barrier islands in the Mauguio Lagoon, Gulf of Lion, France (Ruchon *et al.*, 1995) and in the South of Portugal, in Ria Formosa (Almada *et al.*, 1994). As typical of blenniid species, males of the peacock blenny defend a territory around the nest, which is usually established in a crevice of a rock, and provide parental care to eggs until hatching (Patzner *et al.*, 1986). The species presents marked sexual dimorphism. Males are larger than females and have secondary sexual characters, namely a head crest, composed of adipose tissue, and an anal gland which consists of a modification of the two first rays of the anal fin (Fishelson, 1963; Papaconstantinou, 1979; Patzner and Seiwald, 1986)

transformed into a pheromone (Laumen *et al.*, 1974; Zeeck *et al.*, 1996; Barata *et al.*, 2008) and antibiotic (Giacommelo *et al.*, 2006) producing organ. Females present a somewhat dull coloration and no secondary sexual characters. While males are territorial and therefore sedentary at least during the breeding season, females defend no territory and rove around, searching for food and mates. Females typically spawn in the nests of diverse males. Similarly, males court (see Fishelson, 1963; Patzner *et al.*, 1986 for mating behaviour ethogram) and receive eggs from diverse females.

The habitat of the Ria Formosa is different from that normally occupied by the species. As a barrier island system, its substrate is mobile and mostly composed of sand and silt, leaving males with few or no natural rocky substrate to establish their nests (Almada *et al.*, 1994). However, this mudflat area is used for clam culture and artificial structures such as bricks and other objects are used to delimit these clam fields and to help retain sediment. Such structures are used as nests and shelters by males and are scarce and agglomerated, creating areas of reproduction where the nests are concentrated. Males have to compete intensely to access these potential nests and once nests are established males rarely leave their nests (Almada *et al.*, 1994).

The limited number of nests and its saturation with eggs at the peak of the season (Almada *et al.*, 1995) reduces male potential reproductive rates and consequently biases operational sex ratio towards females (Saraiva *et al.*, 2009). Females compete for the access to males while males are selective towards females, rejecting part of the courting females (Almada *et al.*, 1995). In this way the species has been described as presenting sex role reversal (Almada *et al.*, 1995). However, nests are not saturated with eggs throughout the whole breeding season and the size of broods is associated with the body size of males (Oliveira *et al.*, 1999; Gonçalves *et al.*, 2002; Ros *et al.*, 2009) so the reproductive rates of males may be limited by factors other than nest space. Additionally, the fact that males, and not females, show secondary sexual characters, and that males vary in the size of their broods suggests that female mate choice may also occur.

In this population, the intense male-male competition for access to nests has lead to the emergence of ARTs in which smaller males mimic female morphology and courtship behaviour (parasitic tactic) to mislead the nesting males (bourgeois tactic) in accepting their presence. In this way, the parasitic males attain proximity to the nest, maximizing their chances of participating in the fertilization of the eggs that the females lay in the nests of the bourgeois males (Gonçalves *et al.*, 1996). Bourgeois males in this species, also referred to as nest-holders, differ from the parasitic males, referred to as sneakers, in their behaviour, morphology, gonad traits and physiology. The intense male-male

competition is proposed to cause the reduction of the territory to the nest itself and the shortening of the male courtship repertoire, very often performed without the need to leave the nest (Almada *et al.*, 1994). In this way males defending a nest minimize the chance on nest takeovers by other males. Sneakers approach the nests and court bourgeois males similarly to females (Gonçalves *et al.*, 1996; Gonçalves *et al.*, 2005). They will try to fertilize the eggs that females lay in the nest from either inside or from the entrance of the nest. Morphological differences involve the expression of secondary sexual characters by the bourgeois males, as mentioned above (Fishelson 1963; Papaconstantinou 1979; Patzner and Seiwald 1986). Sneakers mimic the morphology of females and are therefore smaller, but also younger (Gonçalves *et al.* 1996), than bourgeois males and do not present secondary sexual characters. The age difference between parasitic and bourgeois males suggests that the expression of the ARTs in this population may be sequential.

In terms of gonad morphology, sneakers present a higher gonadosomatic index (Gonçalves *et al.*, 1996) as it is common in the parasitic phenotype of most species with ARTs. The larger relative size of their gonads is considered to optimize their sperm competition abilities to cope with the disadvantage of fertilizing the eggs from a larger distance than bourgeois males do (Taborsky, 1998). Additionally, the gonads of bourgeois males present a testicular gland that is absent in sneakers. This accessory gland participates in the process of maturation of sperm (Lahnsteiner *et al.*, 1990; Lahnsteiner and Patzner, 1990) suggesting potential differences in the development of the spermatozoa of bourgeois and sneaker males, like in other species of blennies (e.g. black goby, *Gobius niger*: Rasotto and Mazzoldi, 2002; Locatello *et al.*, 2007). The testicular gland is also the major source of 11-ketotestosterone in this species (Reinboth and Becker, 1986; Oliveira *et al.*, 2001a). At the endocrinological level, like it happens in most teleost species with ARTs, circulating levels of 11-ketotestosterone are higher in bourgeois males than sneakers (Oliveira *et al.*, 2001a; 2001b, 2001c). In this population testosterone is equally higher in bourgeois than in sneaker males (Gonçalves *et al.*, 2008; Saraiva *et al.*, 2010).

Goals of the study

The goal of the thesis is the description of the mating system of the population of the blenny *Salaria pavo* at Ria Formosa, with the main focus on the description of the ARTs from a life-history perspective, and on the investigation of the mechanisms underlying the expression of those tactics. Sex role reversal is also approached from the perspective of reproductive costs and female choice.

Chapter I: Female mate choice and mate search tactics in a sex role reversed population of the peacock blenny, *Salaria pavo* (Risso, 1810)

In sex role reversed species, females compete for access to males allowing males to be selective in their mate choice. Both intra and intersexual selection will then contribute to strengthen sexual selection on females and drive the development of secondary sexual characters in females. However, the peacock blenny and other sex role reversed species do not show the reverted pattern of secondary sexual characters and males are in fact the ones who continue to exhibit these characters. The reasons for males carrying secondary sexual characters, despite the strong competition between females, are discussed.

Chapter II: Adjustment of brood size and androgen levels in a teleost species with exclusive male parental care

In the studied population of the peacock blenny the proposed sex role reversal is predominantly behavioural whereas secondary sexual characters are expressed by males, as is the norm for most vertebrates. Is female choice contributing to the maintenance of male's sexual characters? Are females choosing mates on the basis of their morphological quality, nest features or brood size? Furthermore, since reproducing individuals face a trade-off between parental care for their current offspring and investment in androgen-dependent sexual traits to produce further offspring, a trade-off between parental effort and androgen levels has been proposed. Do changes in male parental effort due to brood size manipulation induce changes in androgen levels? These questions are approached by analysing long term effects of brood size manipulation on female spawning rate and hormone levels of parenting males.

Chapter III: Life-history pathways associated with sequential reproductive tactics in the peacock blenny, *Salaria pavo*

In this population larger and smaller males present different ARTs that correspond to different ages (Gonçalves *et al.*, 1996), raising the hypothesis that the pattern of expression of ARTs is sequential. The description of the life-history pathways followed by males of this population allows to verify this hypothesis and to describe the patterns of tactic expression. Additionally, the "birth-date effect" is also tested to explore the possibility that early or later hatched males follow different growth trajectories that are associated with conditional expression of ARTs.

Chapter IV: Social cues in the expression of sequential ARTs in young males of the peacock blenny, *Salaria pavo*

In conditional tactics, different types of cues may determine the expression of ARTs including certain aspects of the social environment. Social dominance, for instance, reflects both the condition of the individual and the environment in which it is integrated, and it is classically proposed as a likely determinant of ARTs. This stems from the fact that access to resources, which is central to the reproductive success of the bourgeois tactic, is highly influenced by dominance. Density, on the other hand, may reflect competition. In this population the number of nesting sites is limited and density could possibly direct tactic expression, as a higher density will mean a higher proportion of individuals that will not be able to establish nest and therefore will compromise their reproduce success as bourgeois males. The effects of these two factors on the life-history trajectory of males were evaluated at the onset of maturation. The endocrinological mechanisms underlying these effect were also explored, namely, the associations with 11-ketotestosterone and cortisol. Later in the life-history trajectory the effect of the removal of the bourgeois male, *i.e.*, the removal of male-male competition, and the access to a nest on the development of sneaker males was also tested.

Chapter V: Seasonal and tidal variation of microhabitat use by the peacock blenny, an intertidal fish with alternative reproductive tactics.

In conditional ARTs, environmental or individual conditions are determinant for the expression of one or the other tactic. Therefore knowing the conditions under which individuals develop in nature may provide some cues to the mechanisms of decision of life-history pathways. Is the distribution of individuals uniform through the different microhabitats present in the mudflat where the population is established? Do different sex classes and different life-history stages within a sex class distribute uniformly? Are these distributions constant through tidal cycle and season and how do they affect sampling and social environment in which individuals are integrated? Would distribution through different microhabitat within the same area be associated with the different male life-histories pathways? These are the questions approached in this chapter.

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CHAPTER I

Female mate choice and mate search tactics in a sex role reversed population of the peacock blenny *Salaria pavo* (Risso, 1810)

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Fagundes, T., Gonçalves, D.M. & Oliveira, R.F., 2007. Female mate choice and mate search tactics in a sex role reversed population of the peacock blenny, *Salaria pavo* (Risso, 1810). J. Fish Biol. 71: 77-89.

ABSTRACT

Behavioural observations were carried out on a sex role reversed population of the blenny *Salaria pavo* to investigate possible mate choice behaviour of the females. Females mated with relatively larger males, with a larger head crest, anal gland and genital papilla, which had more eggs in their nests and that courted the female. Thus, these male traits were potentially assessed by females when choosing their mates. In order to mate, females visited one to four males (eight females spawned with the first male they had visited and eight visited more than one male). The majority of the females spawned with the last male visited but three of them returned to mate with males visited previously (two with the penultimate visited male). Additionally, the outcome of the first visit of each female depended on the quality of the males: males that were accepted on this first visit had larger head crest than rejected males. Therefore, the mate searching model that best fitted the data was a threshold tactic that allowed the searching individual to return to any potential mate visited before, *i.e.* that allowed for complete recall. Moreover, three females returned to mate with males rejected earlier during the search, which indicates that they lowered their thresholds. These results suggest that females use a 'one step decision' tactic to search for mates. In this tactic, females mate with males that satisfy an adjustable threshold criterion, balancing the quality of the mates expected to find in the next step of the search and the effort of finding them.

INTRODUCTION

Mate choice is favoured when the benefits of choosing mates exceed the costs of being choosy. The benefits of mate choice have always been central to sexual selection theory (Darwin, 1871; Fisher, 1915; Trivers, 1972; Williams, 1975; Zahavi, 1975; Hamilton & Zuk, 1982; Heywood, 1989; Hoelzer, 1989). Contrary to selectivity benefits, choosiness costs have been largely overlooked. These costs can be related to mate sampling tactics and involve time and energy costs, increased risk of predation, risk of being attacked when entering the male's territories, or risk of losing a potential mate to a competitor while sampling mates (Daly, 1978; Parker, 1983; Real, 1990). The extent of such costs, amongst others, depends importantly on environmental characteristics, distribution of potential mates and time constraints. Empirical studies on mate searching (Trail & Adams, 1989; Choudhury & Black, 1993; Rintamäki *et al.*, 1995; Dale & Slagsvold, 1996; Gibson, 1996; Uy *et al.*, 2001) were the logical consequence of several theoretical models proposed in the 1980s, but very few of them were carried out on fishes (Gronell, 1989; Milinski & Bakker, 1992; Forsgren, 1997).

Several theoretical models of mate search tactics have been proposed. In the most simple model, the 'random mating' model, there is no active choice of mates and thus individuals mate randomly. In the 'fixed threshold' model (Janetos, 1980; Wittenberger, 1983) individuals mate with the first potential mate they visit that exceeds a minimum threshold of some quality. Therefore, the search ends when that individual is found and the chosen mate is always the last of the sampling sequence while the number of individuals sampled may vary. The 'one step decision' model (Janetos, 1980; called 'sequential mate choice' by Real, 1990) is similar to the 'fixed threshold' model but the threshold is not fixed. In this model, the individual balances the quality of the mates expected to be found in the next step of the search and the effort of finding them. As in the former model the number of individuals sampled is variable. According to the definition of Janetos (1980), the chosen mate is the last visited individual, but Real (1990) considers the possibility of recall, or in other words the possibility that the searching individual is able to find a potential mate again after having visited others. The 'sequential comparison' model (Wittenberger, 1983) is a sequential sampling tactic that stops when the quality of the potential mates visited starts to decrease. At this point the searcher 'decides' whether to mate with the individual last visited or the one before it, balancing the difference in quality between the two and the costs of returning to the second last. A variable number of potential mates is visited and the chosen mate is either the last or the second last individual sampled. Finally, in the 'best of n' model (Janetos, 1980; Wittenberger, 1983; Real, 1990), also named the 'pool comparison' tactic, complete recall is assumed. In this model, a fixed number of potential

mates are sampled, or the search has a fixed duration or searching distance before the best of the potential mates sampled is chosen. The choice of the mate in this model is independent of the visiting order of the females' sampling sequence.

Real (1990) stressed that different degrees of selectivity are to be expected in different searching individuals, depending on the searchers' perception of the distribution of the quality of potential mates in the population, and on the costs associated with the search. This variation in the selectivity of the searchers leads to variation in the threshold level for mate acceptance, or in the number of potential mates sampled (Real, 1990). As a consequence, the quality of mates accepted and the number of males visited may not be sufficient to distinguish which tactic the searchers use. Moreover, when assuming the occurrence of complete recall, information about the order in the sampling sequence in which the mate was visited before being accepted may also not suffice to identify the actual mate searching tactic. Having realized that, Real (1990) proposed that an additional difference between the 'best of n' and the 'one step decision' tactic would be the quality of potential mates rejected or accepted in the first visits the searcher makes. This is so because the optimal behaviour in the 'best of n' model is to sample a certain number of potential mates and only then decide which one to choose; whereas in the 'one step decision' model the searcher mates as soon as it finds a potential mate that exceeds an adjustable threshold. Therefore, the rejection or acceptance of the first individuals visited depends on their quality, when the latter model is followed, while, it is independent of the potential mate qualities (*i.e.* it depends only on the selectivity of the searcher, reflected in the number of individuals sampled before mating) when the 'best of n' model is followed.

In this study, female mate choice criteria and searching behaviour were analysed in a sex role reversed population of the peacock blenny *Salaria pavo* (Risso, 1810). This species lives in intertidal areas in the Mediterranean Sea and adjacent North Atlantic coasts (Zander, 1986). The mating system is polygynandric and males, which are territorial and larger than females, establish their nests in rock crevices where they provide parental care for the eggs without the help of the female (Patzner *et al.*, 1986). The population studied is situated in Ilha da Culatra, a sand barrier island located off a coastal lagoon in Ria Formosa (southern Portugal). There, due to the lack of rocky substratum, males use holes in debris, mainly bricks, as nests and since these are scarce and agglomerated there is a strong competition among males for the access to nesting sites (Almada *et al.*, 1994, 1995; Oliveira *et al.*, 1999). The probability of nest take-overs by other males is thus high and males seldom leave their nests. Consequently, in this population the breeding territories are restricted to the nest itself (Almada *et al.*, 1994) and male courtship behaviour is rare (Almada *et al.*, 1995). Another consequence of nest limitation is a

shortage of the number of males that can reproduce, probably resulting in a female biased operational sex ratio. The limited space inside the nests, which at the peak of the breeding season may be saturated with eggs (Almada *et al.*, 1995; Oliveira *et al.*, 1999), intensifies this bias and renders the males the limiting reproductive factor. These conditions seem to be the cause of the observed sex role reversal, where males are very selective, rejecting a high proportion of the females and females are the sex that courts more actively and that initiates most of the courtship episodes (Almada *et al.*, 1995).

In spite of the reversed sex roles in courtship behaviour, males exhibit the most developed secondary sex traits during the breeding season: an anal gland in the first two rays of the anal fin and a conspicuous head crest (Fishelson, 1963; Patzner *et al.*, 1986; Patzner & Seiwald, 1987). Although Oliveira *et al.* (1999) and Gonçalves *et al.* (2002a) found no correlation between head crest size and reproductive success, other studies show a positive correlation between reproductive success and body length, anal gland size (Oliveira *et al.*, 1999) or head crest size (E. Barata, unpubl. data), which suggests that these traits might be under sexual selection. This study focused on inter-sexual selection and its goals were to determine: (1) whether females select mates and, if so, which characteristics are used to assess male quality and (2) how do females search for males and which tactics are they using to sample them.

MATERIALS AND METHODS

This study was conducted during the peak of the breeding season of this population, from the third week of May until the end of July 2000 at Ilha da Culatra (Ria Formosa, southern Portugal). A 35 m long transect, composed of 40 bricks was monitored throughout the study. Every 2 weeks, all exposed bricks were inspected during low tide, and the individuals' location and biometrical variables were recorded. Standard length (LS) was registered to the nearest mm and an ordinal scale ranging from 0 (not developed) to 9 (fully developed) was used to score the height of the head crest and the size of the anal gland and of the genital papilla. The percentage of the nest that was covered with eggs was registered frequently for all the nests (checked on average 2.1 days before the visits of the females, and never more than 8 days before) and the number of eggs estimated (Gonçalves *et al.*, 2002a). Fish were captured individually by gently shaking them out of the bricks. Fish were tagged with a combination of coloured beads attached to a fishing line (diameter = 0.3 mm) inserted at the base of the dorsal fin for individual identification, following the procedure described by Patzner & Seiwald (1984). To minimize stress, fish were lightly anaesthetized with MS-222 (tricaine methanesulphonate) before manipulation.

After measurements fish were recovered in a container with abundant water and aeration, and returned to the brick from which they were captured. No mortality due to manipulation or anaesthesia occurred.

Females used in field observations were captured during low tide from exposed bricks or other debris and transported to a field station located at the island. To ensure that females had no prior knowledge of the males to which they were being presented, females were caught on different parts of the island, some distance from the transect where the field observations were to be carried out. Females were anaesthetized, measured and tagged as described above. Additionally, to standardize their motivation to spawn, they were injected, in the peritoneal cavity, with 150 µl of a solution of a gonadotropin analogue (des-Gly¹⁰, [d-Ala⁶]-luteinizing hormone releasing hormone ethylamide, Sigma), at a concentration of 2.5 mg per 100 µl. Females were maintained for 48 h, the time expected for the hormone to be most effective (A.V.M. Canário & R.F. Oliveira, unpubl. data) in a 115 l aquarium without any males. The aquarium reproduced the natural conditions of the population habitat (sandy substratum with shelters, temperature = 20° C, range $\pm$ 2° C and 16L:8D cycle). Females were fed daily with chopped common cockles. To confirm their breeding motivation, they were presented to a nesting male, prior to their release. Only females that courted the 'stimulus' male in the pre-test were subsequently used in the field observations. A total of 39 females were released in the transect, one at a time. No more than four females were released during each day and most abandoned the transect soon after their release (87% in <5 days; recapture or sighting rate = 16%). Hence no inflation of the number of females on the transect was observed throughout the study. Before being released, females were placed in a glass box (16 l) in a random location of the transect for 5 min before the start of the observations in order to acclimate to the new conditions. Continuous focal observations of 60 min (Martin & Bateson, 1993), with a time resolution of 30 s, were conducted by following females while snorkelling. The number and duration of courtship displays of the focal female and of the males they approached were registered (durations registered with a time resolution of 1 s) on an underwater slate. The location of the female was also recorded to calculate the total swimming distance.

Binomial and χ^2 tests (Statistix, version 1.0, from Analytical Software) were used to analyse the frequencies of behaviours. The two-tailed significance level used to reject the null hypotheses was 0.05.

Female preference for male traits

To test which male traits could potentially be assessed by females, males that were visited by females and that defended a nest were divided in two groups, 'accepted males' and 'rejected males'. A visited male was defined as a male that received courtship displays and spawns from females, since these were the only unambiguous criteria to define a visit. Spawns are not possible to observe as they take place inside nests, thus a uniform criterion was used to define spawning. Using only the criterion of a female entering a nest was not sufficient for defining spawning because some females left the nests immediately after they had entered. Therefore, 300 s was set to be the necessary minimum time for a female to spawn after entering a nest [as defined by Neat *et al.* (2003) for the sister species *Salaria fluviatilis* (Asso, 1801)]. 'Accepted males' were defined as those that received at least one spawn from a female and 'rejected males' those that never mated. Floater males were excluded from the analyses. These males usually swim around the transect area and have no established nests, for this reason, they may not have the necessary conditions to mate, due to intra-sexual rather than inter-sexual selection. The behavioural and morphological variables measured in these two groups of males (*i.e.* 'accepted' v. 'rejected') were compared. The number of eggs present in the nests before the focal observations was also compared. Since many males were visited by more than one female, the behavioural and morphological variables presented by each male during the different observations were averaged. In this way there were no repeated male subjects in the statistical analysis. Moreover, some 'accepted males', although accepted by some, were rejected by other females. In these cases, the averages calculated included only the observations where these 'accepted males' were in fact accepted, *i.e.* where they did mate. Thus, data about the same 'accepted' males obtained during the observations where they did not mate were excluded from the analysis. In this study, only one female spawned more than once during the focal observations. Data obtained from both the searching sequences of this female, that spawned twice, were included in the analysis and this was the only source of non-independent data. As data did not conform to the assumptions of parametric procedures Mann–Whitney U-tests (SPSS 14.0 for Windows) were applied throughout. Tests were two-tailed and null hypotheses were rejected at probabilities of type I error <0.05.

Besides the fact that males included in the analysis had an established nest, they should have had space in their nests to receive spawning from females. Only if these two conditions were met were males considered to be available for mating. The available nest space of the males observed was checked to verify this latter condition. The males had a mean $\pm$ S.E. $36.6 \pm 3.6\%$ of the internal area of the nest covered with eggs. Ninety five

per cent of the males in the analysis had <80% of eggs, so they were able to receive new eggs from visiting females. Two males had 90 and 98% of the nests covered with eggs but 63 and 59% of the nests area had eggs ready to hatch (within 3 days). Since these measurements were taken 3–4 days, respectively, before the visit of the focal females, it was assumed that by time the female visited these males, their nests would have available space to receive more eggs.

Mate searching tactic

The visiting sequences of the females were analysed and compared to the distinctive features of each of the mate search models described in the introduction, namely the number of males visited and the order of the accepted males in the sequence of the females' visits. The traits of the males that were accepted during the first visits of the females were compared to traits of rejected males in those first visits, to evaluate if the probability of a male being accepted at first was independent or not of its quality. Because some females visited the same males first in their sampling sequence, data from these males were repeated. The data from each of the repeated male inside each of the groups compared (accepted and rejected males in the first visits of the females) was averaged and the three males that were still included in both groups were randomly excluded from one of them. One-tailed, Mann–Whitney U-tests (SPSS 14.0 for Windows) were applied to these data with a significant level 0.05. The use of one-tailed tests is justified by the theoretical hypothesis tested: H0: no differences between the quality of males accepted and rejected in the firsts visit of each of the females (which would support the 'best of n' tactic) against H1: accepted males are higher quality males than the rejected ones (which would support a threshold tactic and oppose the 'best of n' tactic).

RESULTS

General results

Of the 39 focal females released in the transect, 15 spawned. Four females did not spawn although they courted males, and 20 were not active or presented some other kind of non-sexual activity, usually foraging. The latter were excluded from further analyses. Of the females that spawned, only one did so twice during the same observation. Out of the 54 courtship interactions observed, 14 were initiated by male courtship displays and 39 by female sexual displays [binomial test (14, 53, 0.5), $P < 0.001$]. In one case, mating occurred without any of the individuals involved courting the other one. The occurrence of

spawning was independent of whether females, males or both displayed courtship behaviour (exclusive male courtship: two with spawn, 18 without spawn; exclusive female courtship: four with spawn, 15 without spawn; mutual courtship: nine with spawn, 13 without spawn; χ^2 , d.f. = 2, $P > 0.05$). From the 42 courtship episodes in which males courted the females, 31 (74%) were not followed by spawning and six of the 10 females that sampled more than one male, received courtship displays from males that they did not mate with.

Female preference for male traits

'Accepted males' were larger [Fig. 1(a)], and had more developed sexual characters: larger head crests [Fig. 1(b)], larger anal glands [Fig. 1(c)] and larger genital papillae [Fig. 1(d)] than 'rejected males'. Accepted males had more eggs in their nests [Fig. 1(e)] relatively to 'rejected males' and displayed courtship behaviour [Fig. 1(f)] while most of the 'rejected males' did not.

Female mate searching tactic

The duration and extent of the searching sequences were variable (mean $\pm$ S.E. duration: 1283 ± 252 s, $n = 16$; path length: 2.15 ± 0.48 m, $n = 16$). Females visited one to four males before they spawned (mean $\pm$ S.E. = 1.75 ± 0.25 , $n = 16$). Eight females mated with the first visited male, six visited two males before mating (one of these was actually the second searching sequence of the female that spawned twice) and two visited four males until they spawned. In 13 of the 16 sampling sequences (15 females), the females mated with the last male visited. In eight out of these 13 sequences, females mated with the first, and only, male visited. Three females returned to previously courted males, having meanwhile courted others. Two of these females, returned to the second last male visited and in both these cases the second last and the last males visited had adjacent nests in the same face of the same brick. The other female returned to spawn in the nest of a male visited in the beginning of the searching sequence, having to swim back from nearby bricks.

Considering only the first males that each female visited during the focal observations, the males that did not mate in these first visits had smaller head crests than the ones that mated [Fig. 2(b)]. The other male traits measured, with the exception of LS, showed the same tendency as the head crest, although differences were not significant (Fig. 2). Two focal observations, in particular, deserve more attention. The females (A and B) in these observations visited first a large male ($\sigma^1A1 = 10.9$ cm LS; $\sigma^1B1 = 10.9$ cm LS) with a with

a medium head crest ($\delta A1 = 5$; $\delta B1 = 5$) that had plenty of eggs in the nest ($\delta A1 = 21600$ eggs; $\delta B1 = 21600$ eggs) and that courted the females once. The second visited males were smaller ($\delta A2 = 9.0$ cm LS; $\delta B2 = 9.6$ cm LS), with less developed head crests ($\delta A2 = 2$; $\delta B2 = 1$), were not guarding eggs and they did not court the females. Both females returned to spawn with the first male they visited and this time males did not court females.

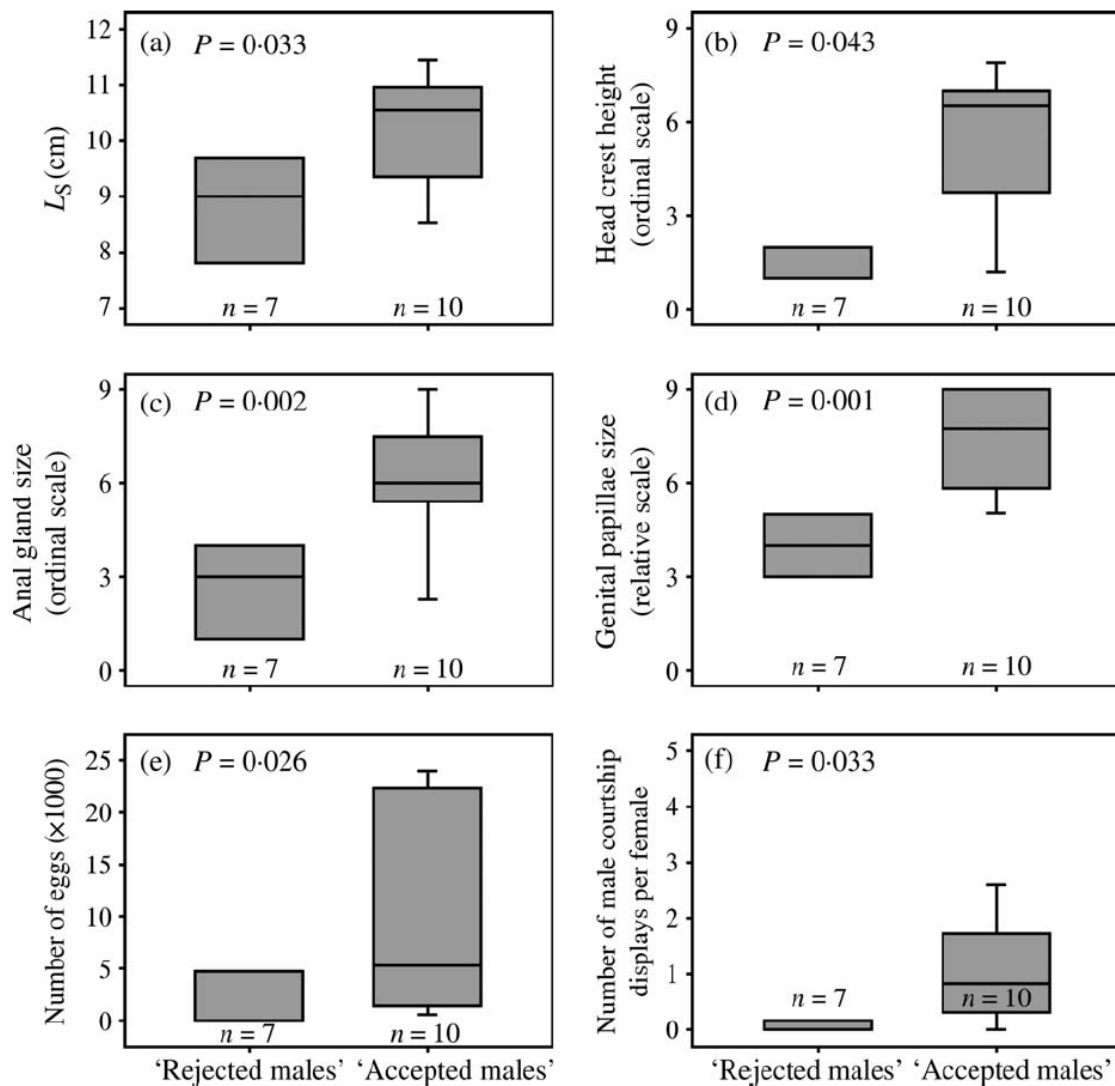


Figure 1. Boxplots (box: median with quartiles; whiskers: 5 and 95% boundaries; P-values are results of two-tailed Mann–Whitney U-tests) of different male traits of *Salaria pavo* (a) standard length, (b) head crest height, (c) anal gland size, (d) genital papillae, (e) number of eggs and (f) number of male courtship displays per female. 'Rejected males' are the males that did not mate with the focal females, 'accepted males' are those that mated, at least once, with the focal females.

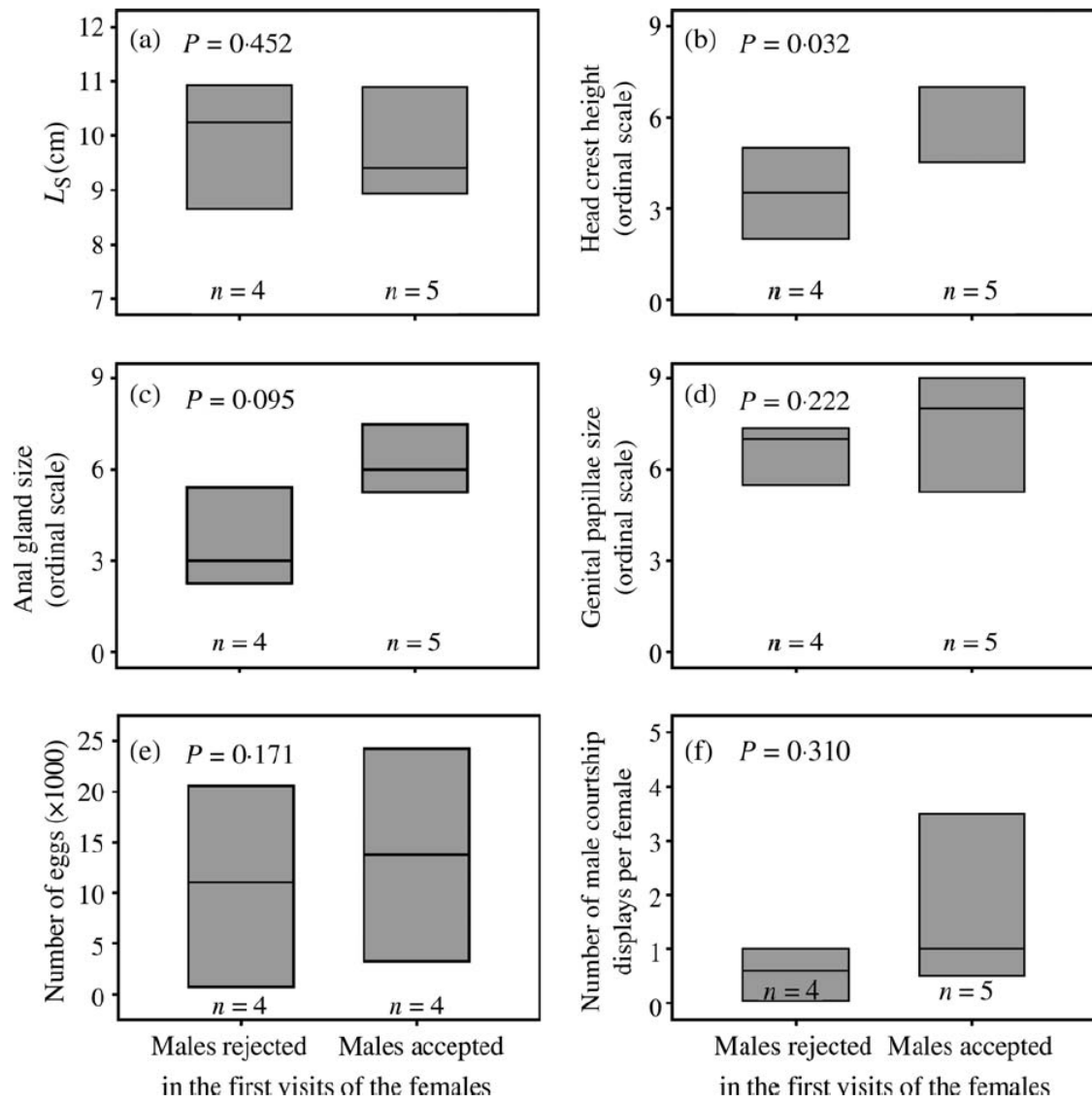


Figure 2. Boxplots (box: median with quartiles; whiskers: 5 and 95% boundaries; P-value is the one-tailed result of Mann–Whitney U-test) of male traits of *Salaria pavo* (a) standard length, (b) head crest height, (c) anal gland size, (d) genital papillae, (e) number of eggs and (f) number of male courtship displays per female. Accepted males are males that received spawnings from females during the first visit these females paid to males. Rejected males did not mate with the females that visited them first.

DISCUSSION

Male traits preferred by females

Females of *S. pavo* mated with the relatively larger males of the transect, and that had relatively larger head crests, anal glands and genital papillae. They spawned with males that courted them and that had relatively high numbers of eggs in their nests. The preference for males found with these traits suggests that these traits are potentially assessed during mate choice.

Males are very selective in the studied population (Almada *et al.*, 1995) which complicates the analysis of female choice, even more so, because courtship displayed, either by males or by females, is not strictly associated with the acceptance of the potential mate. Yet, the pattern of preferences found attests the existence of female choice. In the present study, all the males analysed were able to reproduce, they were all defending nests and all except two had been parental males during the same breeding season. Moreover, they were in conditions to receive more eggs in their nests. Therefore, the clear differences found between 'accepted' and 'rejected' males cannot be explained by the lack of reproductive abilities of some males. Additionally, some of the earlier studies have shown that larger males and males with larger secondary sexual characters (Oliveira *et al.*, 1999; E. Barata, unpubl. data) have higher reproductive success. Being so, it would be expected that these males would have higher potential for being selective towards females. In such a situation, male selectivity alone would not have explained the results obtained. The 'rejected males' of this study, those that never mated would be interpreted as the most selective, having rejected all focal females that visited them, and would be expected to have larger secondary sexual characters and body size. Yet, the results of these study show the 'rejected males' (*i.e.* males that would be interpreted as more selective) to be smaller males and have less developed secondary sexual characters than the males classified as 'accepted males' (*i.e.* males that would be interpreted as the less selective since they have accepted some of the focal females). Therefore, the differences between 'accepted' and 'rejected' males can also not be explained by male selectivity.

Finally, 73% of the courtship episodes in which males courted the females were not followed by spawning, and 60% of the females that sampled more than one male did not mate with some males that courted them, which indicates that these females were not rejected by the males. Therefore it can be concluded that, despite the selectivity of the males, the observed patterns strongly suggest the existence of female choice in this population.

The results presented here are in agreement with previously reported higher reproductive success of larger males in this population (Oliveira *et al.*, 1999; Gonçalves *et al.*, 2002a). Although intra-sexual competition is expected to be the strongest driving force of this higher success due to male–male competition for the access to nests. The present study also is consistent with laboratory controlled studies that have shown that males with larger head crests receive more eggs (E. Barata, unpubl. data) and more courtship displays from females (Gonçalves *et al.*, 2002b) and that females spend more time close to males with larger crests (Gonçalves & Oliveira, 2003). In spite of all these studies showing the same trend, choice tests that control for the correlations between male traits are needed in order to disentangle which male traits are effectively assessed.

Female mate searching tactic

In this study, the head crest of the males accepted in the first visits of the females was larger than the head crest of those rejected. This result supports a threshold tactic while opposing the comparative tactics: the ‘best of n’ and the ‘sequential comparison’ of Wittenberger (1983). The mate searching models that are based on thresholds, as the ‘fixed threshold’ and the ‘one step decision’ models predict the comparison of the males’ characteristics to a threshold of quality that can be fixed or adjustable. Different females are expected to use different levels of threshold, depending on their perception of the distribution of the quality of males in the population and of the cost of the search (Real, 1990). Furthermore, whenever females are also subjected to male mate choice, as it is the case in this population, the qualities of the searching females that will influence their probabilities of being accepted by the males, should also influence the definition of the threshold. Hence, a definite level of threshold of mate acceptance shared by all the females is not likely to be found (Real, 1990). The comparative methods, on the other hand, assume the sampling of a group of males and the comparison of the qualities they possess. For the ‘best of n’ tactic, a female should sample an optimal number of males independently of the quality of the males she finds as the search goes on. This optimal number of males is also expected to vary between females, again depending on the perception of the distribution of the quality of males, of the costs of the search and other factors. Consequently, the number of males sampled is not enough to identify the mate-searching tactic (Real, 1990). Moreover, when considering Real’s (1990) perspective of the ‘one step decision’ tactic which does not preclude complete recall, the order in which the mated males were visited in the sampling sequence is an insufficient criterion to distinguish between tactics as well. Real (1990) predicts, though, that females that use the ‘best of n’ tactic should sample the optimal number of males irrespectively of the quality of

the males they find. In this case, as in the 'sequential comparison' of Wittenberger (1983), the probability that a male is accepted at the first visit a female makes, depends on the selectivity of the female and not on the quality of the male encountered. This implies that males accepted and males rejected in the first visit should not differ. The threshold tactics on the other hand are expected to reveal a difference between males accepted and rejected in the first female visits, as the choice of a male is dependent on its quality and not on the comparison with other males. Females of *S. pavo* did accept males with larger head crests in their first visits and rejected those with smaller crests, which supports the use of a threshold tactic by these females.

Additionally, in concordance with the hypothesis of a threshold tactic, is also the fact that the number of males sampled, the time and the duration of the search varied between females and that most of the females spawned with the last male visited, although these are not distinctive features of the different tactics. And, when considering the possibility of recall, as Real (1990) did, the fact that two females spawned with the penultimate visited males and one of them returned to a male visited in the beginning of the search, is not incompatible with the assumptions of this class of tactics.

Finally, assuming that all females use the same searching tactic in the population, the analysis of the searching sequences of the two females that returned to the penultimate visited male, described in the results, excludes the hypothesis of a 'fixed threshold' tactic, since in order to accept the male rejected in the beginning of the search, the females had to lower their threshold of acceptance.

The mate tactic that is closest to this description is the 'one step decision' tactic in case complete recall is admitted. Because females belong to population and they were caught in the wild, they are presumed to have a perception of the distribution of the males' quality. For this reason, they may have already established some threshold that would allow them to accept a good male even if that was the first male encountered. On the other hand, because the 'one step decision' tactic is a tactic of adjustable threshold, females can adjust the more precise notion of the distribution of males quality and of the costs of search they might achieve during the search and, for instance, lower their threshold if male quality was overestimated or costs of the search underestimated. This decrease in the threshold level would justify the fact that females sometimes return back to mate with a male initially rejected.

According to Real (1990), the 'one step decision' tactic is more effective than the 'best of n' tactic when the costs of search are considered and the difference in performance between this two tactics is maximal when the intensity of the costs is not extremely high or

extremely low. Peacock blenny females do have time constraints, because they have a limited breeding season and also because spawning can only be carried out during high tide, when nests are submerged. Searching for a mate also has costs for these females since predations by eels *Anguilla anguilla* (L., 1758) and marine birds such as the little egret *Egretta garzetta* were observed (unpubl. obs.). Costs related to female–female competition over mates are expected to be high in this population due to the promiscuous mating system and to the hypothesized limiting reproductive rate of males.

In summary, these results provide evidence for female mate choice in this sex role reversed population and suggest some male traits that might be used by females to assess male quality, namely: male's body size, development of the anal gland, genital papillae and, in particular, head crest, male courtship frequency and the number of eggs brooded by the male. The results indicate that females use the 'one step decision' tactic, theoretically considered the optimal mate searching tactic when medium searching costs are expected.

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CHAPTER II

Brood size manipulation and androgen levels in nest-holder males of the peacock blenny

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ABSTRACT

In maximizing reproductive success, individuals face a trade-off between parental care for their current offspring and investing in androgen-dependent sexual traits to produce further offspring. It has thus been proposed that parental effort would suppress androgen levels. Here, we studied parental effort by manipulating brood size in the peacock blenny, *Salaria pavo*, a littoral fish species in which males show long periods of paternal care. We focused on the effects of brood size on female spawning rate (measured as number of eggs received in the nest by focal males) and androgen levels. We found a positive linear relationship between brood size and the number of eggs received subsequently. Accordingly, spawning rate increased for males with experimentally enlarged broods while it decreased for males in which brood size was reduced. However, over a longer time interval, brood sizes of both treatments returned to the nonmanipulated brood size, suggesting an effect of additional factors such as male quality. Brood size did not show the expected negative relationship with levels of testosterone and 11-ketotestosterone. On the contrary, testosterone levels were positively correlated with brood size. However, in agreement with the prediction, changes in the level of parental care by manipulation of brood size showed an inverse relationship with testosterone levels. As with brood size, given time, testosterone levels tended to return to nonmanipulated levels. Such changes suggest that males adjust their brood size to an individual value through androgen modulation of courtship or other traits influencing female spawning rate.

INTRODUCTION

Animals are expected to invest in their brood so as to maximize their remaining lifetime reproductive success (Williams 1966; Trivers 1972; Sargent & Gross 1986). Because parental effort in the form of providing care and protection for offspring is both time and energy consuming, it will reduce resources available for growth and survival and thereby result in a trade-off between current and future reproduction (Clutton-Brock 1991; Stearns 1992; Deerenberg & Overkamp 1999). Therefore, to maximize lifetime reproductive success, selection should favour mechanisms that adjust parental effort to the reproductive value of the current brood, for example via parental assessment of the number or age of the offspring and the chance of brood loss from predation (Carlisle 1982; Lessells 1993; Magnhagen & Vestergaard 1993). Such adjustment of parental effort to the reproductive value of the offspring has been well documented in vertebrates (e.g. Lessells 1993; Vélez *et al.* 2002). Brood size may also be adjusted to parental quality via investment in sexual behaviour or via brood reduction (Clutton-Brock 1991). There is still little knowledge about the proximate mechanisms of brood size regulation (Ziegler 2000). Here we investigate such mechanisms in a teleost fish with exclusive male parental care, the peacock blenny, *Salaria pavo*.

Because of their wide range of effects on reproductive physiology and behaviour, androgens have been commonly studied to analyse the proximate mechanisms underlying life history trade-offs (Wingfield *et al.* 1990; Ketterson *et al.* 1996). In many vertebrates including teleost fishes, androgens facilitate competitive behaviours that are typically displayed in sexual competition, and androgen levels have been found to correlate negatively with parental effort (Wingfield *et al.* 1990; Ziegler 2000; Oliveira 2005). This interaction between androgens and parental effort has been studied experimentally by manipulating androgen levels (*Trichogaster trichopterus*: Kramer 1972; *Lepomis macrochirus*: Kindler *et al.* 1991; *Neogobius melanostomus*: Murphy & Stacey 2002; *Gasterosteus aculeatus*: Páll *et al.* 2002b; *Parablennius parvicornis*: Ros *et al.* 2004), or by manipulating parental effort (*L. macrochirus*: Magee & Neff 2006). Contrary to expectation, experimentally elevated androgen levels did not reduce parental care in these studies. However, an experimental reduction in parental effort via brood size reduction (see Coleman *et al.* 1985 for the relationship between brood size manipulation and parental effort) did result in a significant increase in androgen levels in comparison with control males (Magee & Neff 2006). Thus, parental effort might modulate androgen levels and thereby the extent to which animals are active in sexual competition (reviewed in Hirschenhauser & Oliveira 2006).

In blennies, males defend nests in a hard substrate to which they attract females to spawn. Nest-holding males provide uniparental care to the eggs until they hatch, and this care consists of agonistic nest defence behaviours and some specialized care behaviours such as fanning, cleaning the nest and removal of infected eggs (Almada & Santos 1995; Kraak 1996). Blenniid females prefer to spawn in nests with males that care for many eggs (Kraak 1996; Fagundes *et al.* 2007). Thus it is beneficial for males to have large broods. However, nest-holders in the peacock blenny are often seen to reject courting females while most nests are only partially filled with eggs (Almada *et al.* 1995). This suggests that caring for a large brood is costly. Large successful broods require more parental effort such as fanning behaviour (see Oliveira *et al.* 2000; Karino & Arai 2006). Successfully breeding males also attract small males that try to 'steal' fertilizations or eggs, and this would increase the need to defend the nest with territorial behaviour (Oliveira *et al.* 2002; Gonçalves *et al.* 2003). Furthermore, males of the peacock blenny continuously receive eggs from many females and thus their broods are not sequential but rather composed of overlapping batches of eggs. Therefore, during the reproductive period nest-holders are found almost exclusively in their nest, which curtails their opportunity to forage (Almada *et al.* 1994; Gonçalves & Almada 1998), and results in a decrease in their body condition (Gonçalves & Almada 1997). Consequently, defence and care for larger broods is costly and thus the brood size that a male may sire successfully is expected to depend on his quality.

The aim of this study was to investigate mechanisms of brood size regulation in the peacock blenny. We used the following approaches.

(1) A database from individually tagged territorial males sampled along an intertidal transect was examined for correlations between morphological characteristics and changes in brood size of nest-holders. Here we expected to find a positive relationship between male morphological characteristics and brood size, and between brood size and the number of eggs the nest-holder receives subsequently (Magnhagen & Vestergaard 1993; Fagundes *et al.* 2007).

(2) Brood size was manipulated experimentally to test its effect on subsequent egg number received by the nest-holder. If females base their choice to spawn only on the number of eggs present in the nest, enlarged broods should remain large, while reduced broods should remain small in comparison to nonmanipulated broods. If females spawn independently of the number of eggs in the nest, for example by mate choice for characteristics of the nestholder or the nest site, or when males regulate the number of

eggs in their nest by changing the level of mating behaviour, the number of eggs in the nest should return to the premanipulated brood size.

(3) Blood samples were drawn from the manipulated males to check for a relationship between androgen levels (11-ketotestosterone and testosterone) and brood size. High androgen levels were expected to be incompatible with providing elevated levels of parental care, and thus males with a large or enlarged brood were expected to have lower androgen levels than males with a small or reduced brood.

METHODS

The study was carried out on an intertidal sand flat in the Ria Formosa Natural Park (Ramsar RS212) on an island, Culatra (36°59' N, 7°51' W), Algarve, Portugal (for a detailed description of the area see Almada *et al.* 1994). The local fishermen cultivate clams on the intertidal area of the island and border their clam fields with artificial materials such as bricks, tiles, sand sacks and stone debris. This has created artificial reefs providing shelter and nesting sites for marine life. Peacock blenny males, which normally breed in rocky coasts, have populated these reefs where their nests can be found highly aggregated in cavities in the bricks (Almada *et al.* 1994). The breeding season of the peacock blenny lasts from the end of April to the beginning of September.

Correlates of brood size

As part of a long-term longitudinal study on the life histories and alternative reproductive tactics in the peacock blenny, a transect consisting of an isolated row of 80 bricks (55 m) on the intertidal flat was sampled from 2002 to 2006 at monthly intervals outside the breeding season and every other week during the breeding season. During low tide, blennies were extracted from brick holes by shaking the bricks. All individuals captured in the transect that were larger than 5.2 cm total length were individually marked with passive transponders (passive integrated transponder tag; ID100A, Trovan, Aalten, The Netherlands; 11 × 2.1 mm). Passive transponders were inserted through a small incision in the abdominal cavity in animals that were anaesthetized with MS-222 (tricaine methanesulphonate; Pharmaq Ltd, Oslo, Norway, dilution 1:10 000 sea water), and wounds were closed using medical grade adhesive glue. After recovery from anaesthesia the marked fish were released at the site of capture. The identity of marked fish could be obtained in a noninvasive manner by holding the fish close to a portable reader (LID-500, Trovan).

For the current analysis, we used only data of nest-holders. Males were classified as nest-holders if they expressed secondary sexual characters (*i.e.* a conspicuous head crest and an anal gland) and were found with eggs in their nests. During the breeding season we visited the population every other week during low tide. As part of these regular observations, we collected the following data: (1) standard length (± 1 mm); (2) the relative size of the head crest, as head height/body size (± 1 mm); (3) body weight, measured using a Pesola spring scale (± 0.5 g); (4) the number of eggs in each nest, calculated by multiplying the area covered with eggs in each nest by the average number of eggs per area, estimated a priori from a subset of nests (80 eggs cm², Gonçalves *et al.* 2002); and (5) the egg development stage, classified according to a scale of three categories based on colour and visual signs of embryo development: 1 $\frac{1}{4}$ orange or reddish eggs with no embryo visible (1–3 days old); 2 $\frac{1}{4}$ brownish eggs with embryo in early stages of development (3–6 days old); 3 $\frac{1}{4}$ greyish eggs with embryo clearly visible (6–10 days old).

To characterize the changes in reproductive activity over the breeding season, the number of eggs of all nests in the transect were summed over 2-week intervals and the interval values were averaged over the years. To detect which parameters might influence the brood size of males, we calculated Pearson correlations on data collected during June, the peak of the breeding season (Oliveira *et al.* 1999; Fagundes *et al.* 2007).

Brood size manipulation

During the breeding season of 2006 we carried out an experiment to study the effects of a brood size enlargement or reduction on the reproductive fitness of the nest-holder. The result of this brood size manipulation was measured by following the change in egg number in the nest over a 2-week interval and comparing this result with the change in egg numbers during a sham treatment period of equal duration. At the start of a sham treatment period ('sham treatment'), males and nests were handled as during a manipulated period. At the average temperatures observed on Culatra (between 20.7 and 24.7 °C, see Ros & Oliveira, 2009), the time estimated from spawning until egg hatching is about 10 days (Fishelson 1963; von Westernhagen 1983), and thus all the eggs that were found in the nests at the beginning of the 2-week period had hatched before the next sampling point. Therefore, these brood size measurements were reliable correlates of the recent spawning activity of females and of the number of eggs received by the males. However, the measurements might somewhat underestimate spawning rate as a consequence of nest-holder males eating some of the eggs in the nest ('filial cannibalism', Manica 2002), which was not quantified in our experiment. A large proportion of nestholders in the peacock blenny have been shown to have some blennioid eggs in their

gut (Gonçalves & Almada 1997). Nest-holders might eat some of the eggs to compensate in part for the loss in condition they suffer from the reduction in foraging opportunity during breeding (Gonçalves & Almada 1997). Alternatively, they might selectively eat eggs that are diseased and thus nest-holder egg cannibalism could be part of parental care (Almada & Santos 1995; Kraak 1996). Nevertheless, in the blennioid *Aidablennius sphynx*, the effect of cannibalism on brood size is negligible for broods larger than about 1000 eggs (Kraak 1996). In the population we studied brood sizes varied between 2000 and 20 000 eggs. Even taking into account that *A. sphynx* is a smaller species than the peacock blenny, differences in egg cannibalism between nestholders must have had little effect on the total number of eggs and thus on the outcome of our experiments.

Brood size manipulation

There is a high level of male–male competition for nest sites on Culatra (Almada *et al.* 1995). Therefore, extra nesting space is readily accepted. We provided artificial nests (110 transparent PVC tubes (Cristalflex, Heliflex, Gafanha da Encarnação, Portugal) sealed on one side and placed in brick holes) 3 months before the breeding season. Two sizes of tubes were used depending on the available space in the brick (length 15 cm with a diameter of 31 mm or 28 cm with a diameter of 38 mm). Tubes were inspected at low tide in the morning hours every other week during the breeding season (average inspection interval = 14.5 days). Over the whole breeding season a male was found at least once in 85 of these tubes.

At the start of the breeding season we selected 18 males that were site attached and had received clutches of several females in their nest (seen as different egg patches in the tube). From May to the end of July 2006 brood size was estimated from the area of the tube covered with eggs. Additionally, the total length of the occupying nest-holder was measured (± 0.1 mm) at each sampling point. During the first four sampling points in this period, we manipulated brood size by swapping tubes between nest-holders, resulting in three groups: reduced broods (to $35 \pm 8\%$ of premanipulated brood size, $N = 9$), sham-treated broods (to $100 \pm 2\%$ of premanipulated brood size, $N = 14$) and enlarged broods (to $607 \pm 5\%$ of premanipulated brood size, $N = 11$).

To test whether brood size plays a role in female choice, we estimated the effect of brood size manipulation by comparing the brood size after manipulation with the brood size these males had after a sham manipulation. For statistical analysis, this resulted in nine reduced-control pairs and 11 enlarged-control pairs. The order in which animals were treated first, that is, sham, reduced or enlarged, was randomized.

To estimate brood size within the transparent PVC tubes we used the method described by Kraak & Videler (1991): a sheet of transparent plastic was folded around the tube and the outlines of the areas covered with eggs (deposited in one layer against the wall) were drawn. Sheets were scanned using an Epson flatbed scanner and areas were measured using Adobe Photoshop (accuracy 1 mm²). Exact egg counts were made of five broods and by linear regression (without an intercept) the number of eggs per cm² was calculated. The average number of eggs per cm² was then used to estimate the number of eggs present in each tube at each sampling point.

Based on previous work (Fagundes *et al.* 2007) we predicted that female spawning rate in the nest would correlate positively with the size of the nest-holder and with the number of eggs in the nest (see *A. sphynx*, Kraak & Videler 1991). From this, we had the following predictions for the manipulation experiment: (1) if brood size is the major determinant of female choice, the enlargement or reduction of brood size through experimental manipulation should correlate positively with spawning rate and thus subsequent brood size (Fig. 1, scenario A); (2) if characteristics of the nest-holder or of the nest are more important determinants for female choice, brood size should return to the control brood size following manipulation (Fig. 1, scenario B).

An alternative explanation for possible effects of brood size manipulation is that males value nests differentially depending on the age of the eggs in the brood (see Magnhagen & Vestergaard 1993). A post hoc analysis showed that nests with more than about 3500 eggs did not differ significantly in age structure of the eggs, but smaller broods held younger eggs (T. Fagundes, D. Gonçalves & R.F. Oliveira, unpublished data). Using the relationship between brood size and age resulting from this post hoc analysis, we found that estimated ages of the broods were not significantly different after manipulation between groups (estimated developmental stage (scale 1–3): reduced: 2.01 ± 0.10; control: 2.03 ± 0.06; enlarged: 2.11 ± 0.07; paired t tests: $t < 1.84$, $P > 0.20$, Bonferroni corrected). We therefore assumed that differences between broods in the age of the eggs had no systematic effect on our results.

Measurement of androgen levels

To measure the relationship between brood size and androgen levels, at the end of the last manipulation period (fourth) and at the end of the subsequent sampling period in which no manipulations of brood size were carried out, all males were captured and a blood sample was drawn. Before blood sampling, fish were anaesthetized using MS-222. Blood was sampled from the caudal vein with a 1 ml heparinized syringe fitted with a 25-gauge needle and placed in 1 ml Eppendorf tubes.

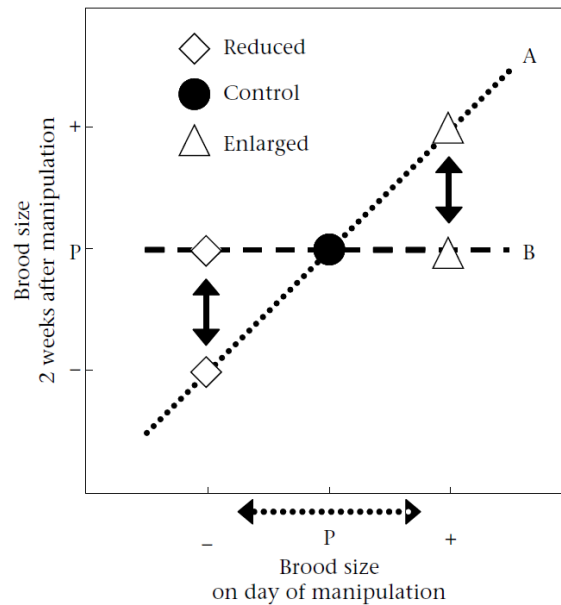


Figure 1. Hypothetical effects of brood size manipulation on subsequent spawning rate. The reduced group received a smaller brood (-) and the enlarged group received a larger brood (+), while the control group remained at premanipulation levels (P). 'Brood size' scenario (A, dotted line): spawning rate is a function of the number of eggs in the nest and brood size will remain at the manipulation level (-, P, + depending on the group); 'male quality' scenario (B, dashed line): spawning rate is independent of the number of eggs in the nest and after manipulation the brood size will return to the premanipulation level (P).

Blood was transported on ice to the laboratory, where it was centrifuged at 3000 rpm for 5 min. Plasma was collected and stored at -20 °C until radioimmunoassays were carried out. To assess circulating levels of testosterone (T) and 11- ketotestosterone (11-KT), the free steroid fraction was extracted from plasma using the methodology described in Scott & Vermeirssen (1994). Steroid residues were resuspended in 1 ml of assay buffer and stored again at -20 °C until assayed for 11-KT and T. For the quantification of 11-KT, an antibody donated by D.E. Kime was used and its cross-reactions are described in Kime & Manning (1982). The antibody for T was purchased from Research Diagnostics (RDI-TRK2T2, Concord, MA, U.S.A.); the cross-reactivities provided by the supplier are 16% for 5 α -dihydrotestosterone, 1% for androstenediol, 0.4% for androstenione, <0.1% for androsterone, <0.1% for dehydroepiandrosterone, <0.1% for progesterone, 0.001% for oestradiol and 0.001% for cortisol/pregnenolone. Intra-assay coefficients of variation for both hormones were 5% and all samples were analysed within a single assay.

Ethical note

In the longitudinal research project, in total 1192 individuals were tagged using a transponder (T. Fagundes, J. Saraiva, D. Gonçalves & R.F. Oliveira, unpublished data). Only animals above 5.2 cm total body length were tagged. Surgery was performed only after fish attained a deep state of anaesthesia, as characterized by a total loss of equilibrium and reflexes to stimulation (Summerfelt & Smith 1990). Fish were allowed to recover from anaesthesia in a small isolation aquarium before they were returned to the site of capture. Research on perch, *Perca fluviatilis*, has shown that animals of this size do not show severe negative effects of surgical pit tagging (Baras *et al.* 2000). Of the 1192 marked only eight did not survive this procedure. We could not follow the healing process of the fish after release in the field. However, more than 50% of the fish were recaptured which is a high recapture percentage for freelifving animals.

For the brood manipulation experiment, males were recognized on external cues and animal handling time was kept to a minimum. All animals survived the experimental procedures. Blood sampling was carried out by a certified researcher (Direcção-Geral de Veterinária) under anaesthesia (see above). In other experiments (*e.g.* Ros *et al.* 2004) we found no evident side-effect of blood sampling, and all animals were found in their nests in subsequent visits. The experimental procedures were reviewed by a veterinarian and a licence was granted by the Direcção Geral de Veterinária, Ministério da Agricultura do Desenvolvimento Rural e Das Pescas. The project was approved by the Fundação Para a Ciência e a Tecnologia.

RESULTS

Correlates of brood size

The reproductive period started at the end of April and lasted until the end of August, with very few nests in the transect still with eggs at the beginning of September (Fig. 2). During this period on average 13 ± 1 nests were found to contain eggs. Estimated total numbers of eggs in the transect (sum of 80 bricks) increased steeply (rate ca. 5000 eggs/day) until reaching stable values in June. After June the estimated total number of eggs slowly declined (rate ca. 2000 eggs/day).

To test the stability of brood size within individual nest-holders, we selected nest-holders that were observed to have eggs in their nest for at least three consecutive periods (Fig. 3). The number of eggs gradually built up during the first three observations after the start of the breeding season (paired t test: 0→1 and 1→2: $t_{79} > 4.7$, $P < 0.0001$; P values

corrected for multiple [5] comparisons with the Bonferroni correction). From the third observation onwards brood size remained stable (paired t tests: 2→3: $t_{47} = 0.94$, $P = 0.35$; 3→4: $t_{24} = 1.5$, $P = 0.47$; 4→5: $t_{14} = 1.4$, $P = 0.40$; P values corrected for multiple [5] comparisons with the Bonferroni correction; Fig. 3).

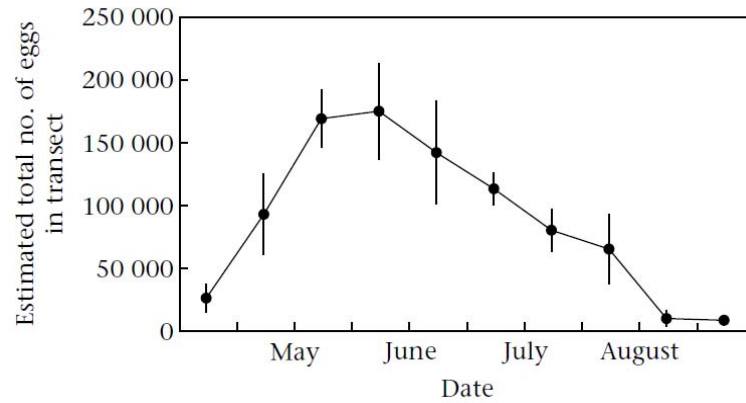


Figure 2. Seasonal variation in total egg number in the studied transect (total number of nests with eggs per season was 23 ± 2). Means $\pm$ SEM are shown for data collected between 2000 and 2006.

The average interval between two observations was 18 days and thus all eggs present at the beginning of the interval had hatched before the following measurement of brood size. This effect of previous brood size thus suggests that the number of eggs a nest-holder received was stable over a relatively long period. This stability was not a result of physical limits of the nest because the internal nest area covered by eggs varied widely (range 2-100%, mean 55%).

We tested the predictive value of the following characters on brood size in a multiple regression analysis: total length, relative head height (head height/body size), condition factor (weight/body size³) and previous brood size. Data were selected from the peak of the reproductive period (*i.e.* the beginning of June). This analysis showed a highly significant effect of both previous brood size and body size ($r^2 = 0.57$, $N = 62$, $P < 0.001$; in the regression model: previous brood size: $P < 0.001$; body size: $P = 0.005$; removed from the model: relative head height, condition, year: $P > 0.13$; Fig. 4).

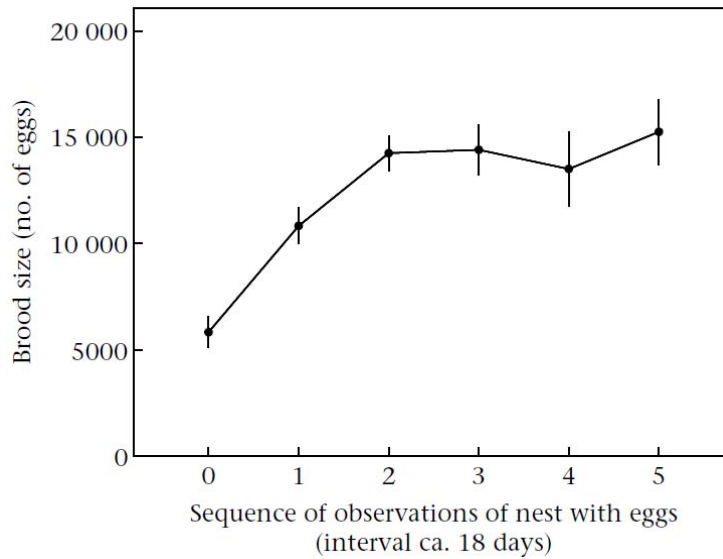


Figure 3. Change in brood size over consecutive sampling intervals (average for all the nests in the transect). Broods were aligned on the first event at which a nest-holder was found to have eggs. Means $\pm$ SEM of individual data are shown.

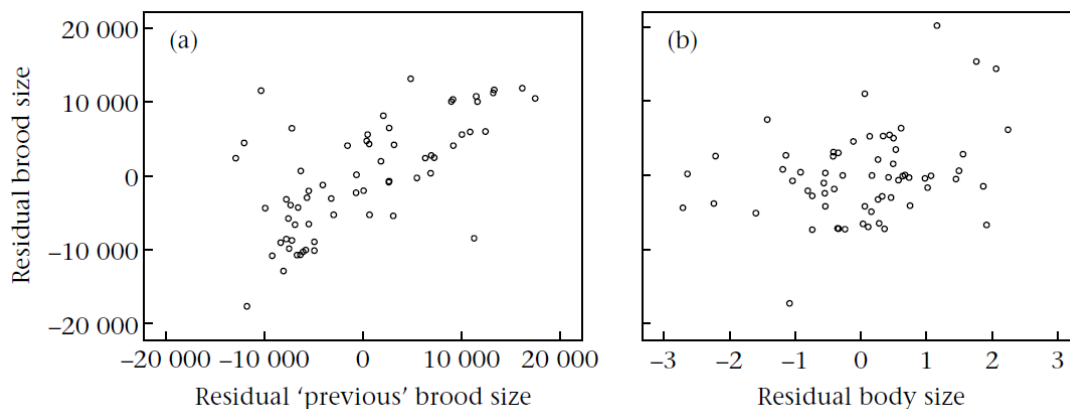


Figure 4. Plot of residuals obtained from a multiple regression analysis of (a) previous brood size and (b) total length, on brood size of nest-holders in the transect. Residuals in (a) were obtained by partialling out the effect of total length, while residuals in (b) were obtained by partialling out the effect of brood size.

Brood size manipulation

Effects on brood size

During control periods, brood size of sham-manipulated males correlated positively with body size but not with relative head height (total length: $r_{27} = 0.58$, $P < 0.001$; relative head height: $r_{27} = 0.16$, NS). Additionally, a strong positive correlation was found for brood size in the first two consecutive sampling points (approximately 2 weeks apart) for the sham-manipulated brood size treatment ($r_{16} = 0.96$, $P < 0.001$; Fig. 5).

Two weeks after manipulation males with experimentally enlarged broods had larger broods, while males with experimentally reduced broods had smaller broods, than after a sham treatment period (paired t tests: enlarged versus control: $t_{10} = 3.67$, $P = 0.008$; reduced versus control: $t_8 = -3.10$, $P = 0.015$; P values corrected for multiple [2] comparisons with the Bonferroni correction; Fig. 6a). To test whether brood size varied as a function of factors independent of brood size (see Fig. 1, scenario B), we computed residual values of the correlation between manipulated brood size and number of eggs received after the manipulation. To do this, we subtracted brood size values as a consequence of manipulation from the predicted value based on the regression of subsequent brood size on current brood size found in sham-treated nest-holders (Fig. 5).

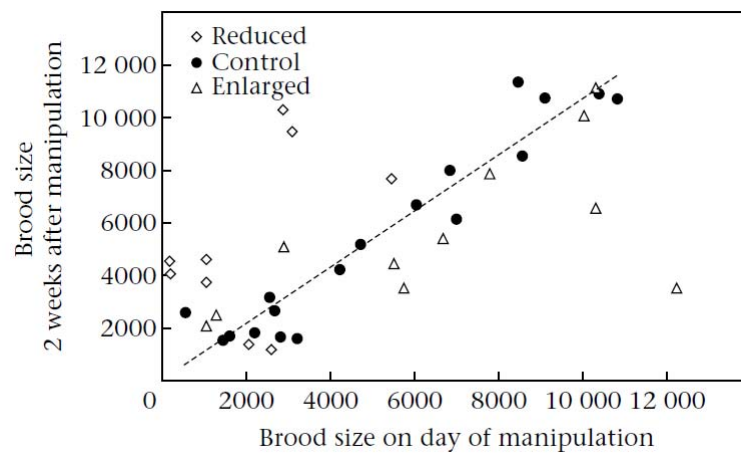


Figure 5. Effect of the brood size manipulation on subsequent spawning rate. Brood sizes on the day of manipulation and 2 weeks later of individual nest-holder males are shown. In a 2-week period all eggs hatch and thus the value on the Y axis can be used as an estimate of the spawning rate after manipulation.

The averages of these residual values were then plotted according to treatment (Fig. 6b). This residual analysis showed that males with enlarged broods had brood sizes as large as expected from their manipulated brood size, while males with reduced broods had significantly larger broods than expected (paired t test: enlarged broods: $t_{10} = -1.02$, $P = 0.3$; reduced broods: $t_8 = 3.37$, $P = 0.020$; P values corrected for multiple [2] comparisons with the Benferroni correction). Thus, although nestholders with reduced broods received fewer eggs (*i.e.* smaller subsequent broods) than during a control period, they still received significantly more eggs (*i.e.* larger subsequent broods) than expected from their brood size at manipulation.

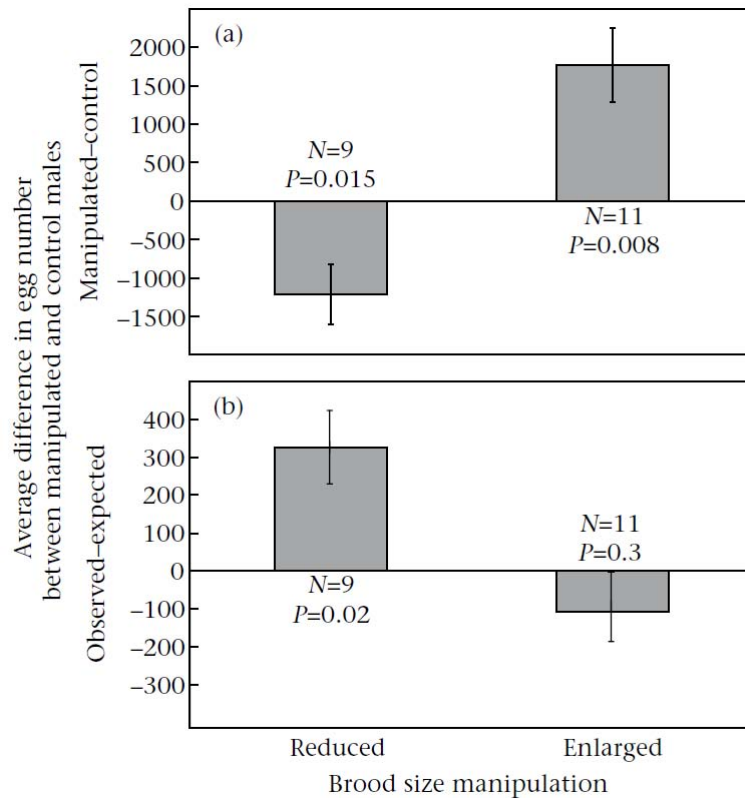


Figure 6. Results of the brood size manipulation experiment. (a) Difference between the number of eggs that a male received after a brood size manipulation and after a control treatment (reduced-control brood size and enlarged-control brood size, respectively). The values reject the hypothesis that spawning rate is associated with 'male quality' only (Fig. 1, scenario B) because this would predict that brood size after manipulation returns to premanipulation (control) levels and thus that values would not differ significantly from zero. (b) Difference between the number of eggs received after manipulation and the number of eggs expected to be received as a function of the brood size attributed by the experimental treatment. The expected values are determined by the linear relationship of Fig. 5. The values reject the hypothesis that spawning rate is associated with 'brood size' only (Fig. 1, scenario A) because this would predict that values would not differ significantly from zero. Means $\pm$ SEM are shown.

Effects on androgen levels

At the first sampling point, about 2 weeks after manipulation, males had brood sizes that correlated positively with their brood sizes at manipulation (Fig. 7a). However after 1 month broods tended to return to the premanipulation levels (Fig. 7a). These results thus confirm the conclusion of the longitudinal study that spawning is a function of both the number of eggs present in the nest and some character of the nest-holder independent of brood size.

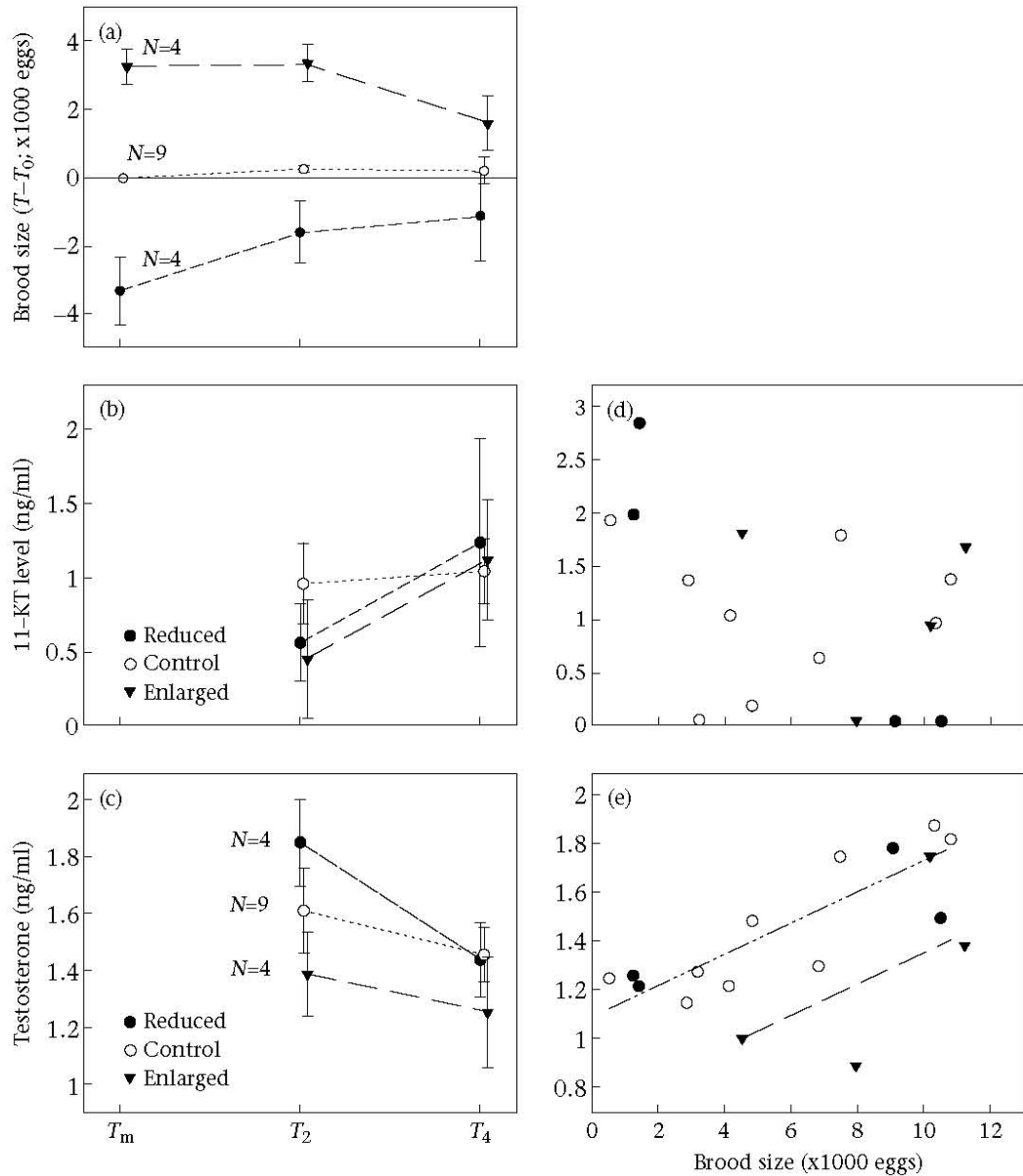


Figure 7. (a, b, c) Changes in (a) brood size and (b) 11-ketotestosterone levels, and (c) testosterone levels after manipulation of brood size. T_m = time of manipulation; T_2 : 2 weeks after manipulation; T_4 : 4 weeks after manipulation. (d, e) Regression of brood size on (d) 11-ketotestosterone and (e) testosterone levels for control broods (open dots), enlarged broods (closed triangles) and reduced broods (closed dots).

The effect of brood size manipulation on the androgen levels was measured in a repeated measures ANCOVA with androgen level at each of the two sampling points as repeated measure and average brood size at these sampling points as covariate. 11-KT levels did not show a significant correlation with brood size ($F_{1,13} = 0.95$, $P = 0.3$; controls only: $r_6 = -0.004$, $P = 0.99$; Fig. 7d); neither was there a significant effect of brood size manipulation ($F_{1,13} = 0.06$, $P = 0.8$; Fig. 7b). In contrast, T levels showed a significantly positive correlation with brood size ($F_{1,13} = 6.6$, $P = 0.023$; controls only: $r_6 = 0.86$, $P = 0.007$; Fig.

7e) and a significant effect of brood size manipulation ($F_{1,13} = 5.03$, $P = 0.043$; Fig. 7c). T levels were increased in the reduced group and decreased in the enlarged group (Fig. 7c). They tended to decrease with time ($F_{1,13} = 3.98$, $P = 0.067$), and similarly to what was found for brood size, 1 month after the manipulation androgen levels did not differ between groups ($F_{1,13} = 1.71$, $P = 0.21$; Fig. 7c).

DISCUSSION

In a species with exclusive male parental care, the peacock blenny, a large individual variation was found in brood sizes, which remained relatively stable during the peak of the reproductive period. This reflects consistent variation in female spawning rate, as sampling intervals were longer than hatching time and because, in our sample, brood size was not limited by space for eggs in the nests. Here we discuss possible causal mechanisms by which brood size might be regulated to individual characteristics of nest-holders (for mechanisms by which they might be established see Kraak & van den Berghe 1992).

Effect of brood size manipulation on spawning rate

We expected that if females base their mate choice only on the number of eggs present in the nests, brood size should remain at manipulation levels (Fig.1, scenario A). Alternatively, if females spawn independently of the number of eggs in the nest, brood sizes should return to the nonmanipulated brood size (Fig. 1, scenario B). The results showed that, after the first 2 weeks after manipulation, the number of eggs received by the males was clearly predicted by the manipulated brood size. Thus, spawning rate was influenced by brood size. An effect of eggs in the nest on female spawning rate has been shown for several species of teleost fish (*Cottus bairdi*: Downhower & Brown 1980; *G. aculeatus*: Ridley & Rechten 1981; *Cottus gobio*: Marconato & Bisazza 1986; *Ethiostoma flabellare*: Knapp & Sargent 1989; *Hypsypops rubicundus*: Sikkel 1989; *Abudefduf luridus*: Afonso & Santos 2005) including a blenny species (*A. sphynx*, Kraak & Videler 1991). However, 1 month after manipulation brood size returned to premanipulation levels. This return could not be explained by a limited carrying capacity for eggs in a nest, because the effect was found for both reduced and enlarged nests. Thus, both brood size dependent and brood size-independent effects appeared to play a role in the reproductive success of individual males.

Two mechanisms that are not mutually exclusive may explain the return of brood size to premanipulation levels. (1) Females may choose characteristics of the male that are independent of brood size variation. Such characteristics might be secondary sexual characters or male body size (Fagundes *et al.* 2007; this study). (2) Males may adjust brood size to some individually set value. Males may do so through modulation of female spawning rate via sexual and agonistic behaviours and through filial cannibalism (Manica 2002). Functionally, this may optimize their current and future reproductive effort, because care and defence behaviours are costly activities (van den Berghe 1992; Almada *et al.* 1994; Gonçalves & Almada 1997, 1998; Cooke *et al.* 2006) and parental effort has been found to be proportional to brood size in several fish species (e.g. Magnhagen & Vestergaard 1993; Karino & Arai 2006). Brood size might also drop to a level at which the expected fitness benefit of raising that brood becomes lower than the expected loss of future reproduction as a consequence of the parental effort necessary to do so. For example, parental males of several fish species that received a small brood have been observed to cannibalize or abandon this brood and start to attract new females (Taborsky 1985; Smith & Wootton 1995; Balshine-Earn & Earn 1998).

In accordance with our study, two other studies on blennies have reported that female spawning is positively correlated with brood size (peacock blenny: Fagundes *et al.* 2007; *A. sphynx*: Kraak & Videler 1991). Kraak & Groothuis (1994) tested in *A. sphynx* whether female choice for large brood size was a direct stimulatory effect of the number of eggs in the nest, or a consequence of choosiness for some feature in male courtship. In a series of choice tests they did not detect obvious differences in the behaviour of males of *A. sphynx* that were holding a nest with many or few eggs, a result that has also been found in an experimental study in the sand goby, *Pomatoschistus minutus* (Nyman *et al.* 2006). Moreover, based on visual cues from the males alone, females of *A. sphynx* choose randomly in respect to the number of eggs in the nest for their first approach. When subsequently females were allowed to inspect both nests they showed the expected preference for spawning in nests with many eggs. Thus, female inspection of the number of eggs in the nest plays an important role in female spawning and affects the resulting brood size. Still, Kraak & Groothuis (1994) did not rule out the possibility that male courtship display and characteristics (Fig. 1, scenario B) might play a role in a different context, for example to attract females to the nest.

In accordance with the data on *A. sphynx*, field observations in the peacock blenny have shown that fecund females inspect several nests before they spawn (Fagundes *et al.* 2007). These females sometimes return to a nest they have already inspected suggesting that females do compare some feature of quality between males or their nests. Indeed,

females spawned with relatively larger males, with a larger head crest, anal gland and genital papilla, that had more eggs in their nests and that courted the female (Fagundes *et al.* 2007). Here we have confirmed that brood size plays a role in this choice process, since males with experimentally enlarged broods received more eggs than in a control situation while males with experimentally reduced broods received relatively fewer eggs.

Androgen levels and variation in brood size

Based on comparative data for a wide range of vertebrate species a trade-off has been proposed between parental behaviour and androgen levels (reviewed in Ziegler 2000; Hirschenhauser & Oliveira 2006). For example in birds, T facilitates behaviours that play a role in sexual competition. The energetic and time requirements of such androgen-dependent mating and fighting behaviours are mostly incompatible with providing protection and food for young (Wingfield *et al.* 1990). Therefore we expected to find a negative relationship between the number of eggs in the nest (cared for by the male) and androgen levels. This effect should be found especially in the androgen 11-KT because this is the major biologically active androgen in male teleosts (Kime 1993; Borg 1994). Contrary to this expectation, we did not find evidence for such a negative relationship between 11-KT levels and brood size, either correlative or as a result of experimental manipulation of brood size. Recently, several experimental studies have suggested that 11-KT does not 'trade-off' with parental care in fish (*e.g.* *Trichogaster trichopterus*: Kramer 1972; *Parablennius parvicornis*: Ros *et al.* 2004; *Lythrypnus dalli*: Rodgers *et al.* 2006). The lack of a negative relationship between 11-KT and parental care might be explained by the fact that male competitive behaviours and parental care are not mutually exclusive in fish. In many species of fish males care for several overlapping clutches and mating and parental care behaviours are often carried out in the nest territory (Smith & Wootton 1995).

In contrast to 11-KT, T showed a positive curvilinear relationship with brood size. Since males that have more eggs in their nests are expected to participate in more spawning episodes with females, this might explain the positive relationship between plasma levels of T and brood size. In adult males that compete within the conventional male reproductive context, that is, are exposed to male–male competition and female choice, exposure to fecund females appears to induce the production of gonadotrophins and the growth of the testes (Kobayashi *et al.* 2002). Social experience is also known to affect the activity of steroidogenic enzymes involved in androgen metabolism in fish, namely aromatase, which converts T into oestradiol, and 11-beta-HSD which metabolizes T into 11-KT (Leitz 1987; Frisch 2004). Therefore, it is possible that these biochemical switches

(i.e. aromatase and 11-beta-HSD) are differentially activated by mating experience, resulting in a decoupling of the social effects on the two measured androgens (T and 11-KT). For example, in *Sarotherodon galilaeus*, a tilapia with both male and female mouthbrooding, T levels increased and 11-KT levels decreased in reproductively active males that were in a female biased operational sex ratio group in comparison to males in a male-biased group (Ros *et al.* 2003). Males in such female-biased groups had higher spawning rates than those in male-biased groups and also showed higher gonadosomatic indexes suggesting again a positive relationship between testis size and T production.

The temporary reduction in T following brood size enlargement and its rise following a reduction in brood size, support a 'trade-off' between T and parental investment since larger broods require more parental care. The positive relationship between T and brood size discussed above does not necessarily contradict the existence of this trade-off since good-quality males may support the costs of higher levels of T while performing parental care. The changes in T levels might explain the consistency of individual male brood size found in this species, since spawning rate after manipulation mirrored changes in T levels. Although not studied so far for the peacock blenny, T might facilitate oestradiol-dependent sexual displays and thereby spawning rate. In guppies, aromatization of T to oestradiol affects two out of three of the investigated male sexual displays (Hallgren *et al.* 2006). Therefore, the changes in T levels as a result of brood size manipulation found in the peacock blenny make it worth considering that nest-holders actively maintain the number of eggs in their nest.

Two other studies on teleosts have measured the effect of brood size manipulation on androgen levels. In *L. macrochirus* control broods were compared with broods from which half of the eggs were removed experimentally. In males with these reduced broods both T and 11-KT levels increased (Magee & Neff 2006). In *G. aculeatus* brood size was manipulated by allowing three, one or no females to spawn in a nest. Males with three females showed a stronger decline in 11-KT levels than males with only one female, while 11-KT levels in males that had no brood in their nest remained elevated. T levels were not measured in this study (Páll *et al.* 2002a). Thus both studies show an effect of brood size manipulation on androgen levels as we have shown in the peacock blenny. However, in contrast to the peacock blenny, *L. macrochirus* and *G. aculeatus* 11-KT responded to the manipulation. There is too little knowledge on the effect of the different androgens on the breeding behaviour of the different species to explain this partial difference in results.

In conclusion, the available evidence suggest that brood sizes in the peacock blenny are associated with quality characteristics of individual nest-holders. Experimental

manipulation of brood size resulted in a correlated change in spawning rate but this effect was only temporary suggesting that brood size is regulated to an individually set value. Both female choice and male sexual and aggressive behaviour might play a role in maintaining this value. Changes in androgen levels (T in this study) in response to changes in brood size might assist in individual regulation of brood size because androgens facilitate sexual competitive behaviours. The function of such a mechanism might be to optimize brood size to the quality of the male given its social environment.

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CHAPTER III

Life-history pathways associated with sequential reproductive tactics in the peacock blenny, *Salaria pavo*

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ABSTRACT

Life-history pathways were followed on a population of the peacock blenny that presents male alternative reproductive tactics with smaller parasitic, sneaker males and larger bourgeois (nest-holder) males. Two main pathways were identified: 1. early hatched immature males become bourgeois males at age 0+/1 and reproduce as such in their first breeding season and subsequent ones ("bourgeois pathway"); 2. later hatched males reproduce as sneakers in their first breeding season switching to the bourgeois tactic after the end of that season, at the start of age 1, and they reproduce as bourgeois males in the subsequent seasons ("sneaker pathway"). These results confirm that the pattern of alternative tactics is flexible with fixed sequences as suggested previously by Gonçalves *et al.* (1996) and supports the "birth-date effect" proposed by Taborsky (1998) as a factor influencing tactic expression. The estimated reproductive success of the "bourgeois pathway" was lower than that of the "sneaker pathway". Therefore early hatching may constitute a constraint that we hypothesise to be maintained by an opposing selective force that favours early hatching of females.

INTRODUCTION

Within the same species, individuals of the same sex may use different behaviours in order to reproduce and morphological differences are usually coupled with these distinct behaviours. These contrasting reproductive phenotypes are referred to as alternative reproductive tactics and they show great diversity in their forms. A wide variation in behaviour and morphology associated with alternative reproductive tactics can be found throughout different species of different taxa, from exuberant secondary sexual characters like the horn of the horned beetles, genus *Onthophagus* (Emlen, 1997) to the diverse colours of the three morphs of the side-blotched lizard, *Uta stansburiana* (Sinervo and Lively, 1996), to cooperatively breeding in the *Neolamprologus brichardi* (Taborsky, 1984) and the female mimicry in the peacock blenny, *Salaria pavo* (Gonçalves *et al.*, 1996). This diversity is also reflected in the basic pattern of expression of the tactics which may be fixed or flexible. While in some species each individual reproduces in a single fashion during the whole duration of its life, in others species individuals switch from one tactic to another either irreversibly or opportunistically, switching freely between tactics (Brockman 2001; Taborsky *et al.*, 2008). Additionally, environmental cues may be determinant in the expression of the tactics by each individual, in an ontogenetic perspective. In such cases the tactics are said to be conditional (Hazel *et al.*, 1990; Gross, 1996; Emlen, 2008), that is, the tactic adopted is dependent on environmental or social cues to which the individual is exposed. In some species body size is one of the factors that has been shown to influence the tactic that is expressed (e.g. european earwigs, *Forficula auricularia*: Tomkins, 1999). Taborsky (1998) has proposed one possible cause for size-dependent expression of alternative reproductive tactics: the “birth-date effect”. In species with either a relatively long reproductive period or with hatching asynchrony, individuals may present a great disparity in birth dates and associated environmental characteristics. Birth date difference alone could determine differences in body size of individuals at the onset of reproduction. The “birth-date effect” may also influence growth patterns with life long consequences for the developing individual, particularly if environmental conditions will be very different for early offspring than for the later offspring. Since alternative tactics may have very different reproductive success depending on body size (Gross, 1985; Taborsky, 1999), the birth date may determine how early individuals will be able to successfully reproduce and which tactic will be the most successful for them (Pen *et al.*, 1999). The “birth-date effect” may consequently select for alternative life history pathways related to such alternative tactics.

Studying populations with alternative reproductive tactics in their natural environments is essential not only to understand the patterns of tactic expression and to explore potential

underlying proximate mechanisms, but also to understand the functional processes and selective forces that have shaped them. Monitoring populations with individually marked subjects allows to follow the development of those individuals and to reveal the possible sequences of alternative tactics they express. But it also permits gathering information on life-history events that will be useful in understanding the ultimate benefits of the alternative combinations of reproductive traits that characterise each tactic, in other words, how do the organisms manage their constraints, and by which trade-offs do they maximise their reproductive potential (Stearns, 1992).

In order to study factors influencing life-history pathways and to test the “birth-date effect” hypothesis, we have monitored a population of the peacock blenny *Salaria pavo* (Risso, 1810). This population presents alternative reproductive tactics in which smaller males may reproduce as sneakers, while larger males reproduce as nest-holders (bourgeois tactic) defending a nest and providing parental care to eggs (Gonçalves *et al.*, 1996). Such tactics are thought to be sequential and sneaker males younger than bourgeois males (Gonçalves *et al.*, 1996). Sneakers have no secondary sexual characters and mimic females to try to fertilize eggs when females spawn inside the nests of nest-holders (Gonçalves *et al.*, 1996). Contrarily, bourgeois males present developed secondary sexual characters, namely a head crest and an anal gland (Fishelson, 1963; Patzner *et al.*, 1986). Immature males, who are morphologically similar to sneakers but not sexually mature, constitute a third class of males. These may represent a class that only reproduces with the bourgeois tactic later in life.

METHODS

Field methods

The population studied inhabits the intertidal area of a coastal lagoon at Culatra island (36°59′ N, 7°51′ W), Parque Natural da Ria Formosa (Ramsar RS212), Algarve, Portugal (for a detailed description of the area see Almada *et al.*, 1994). The local fishermen cultivate clams on the intertidal area of the island and border their clam fields with artificial materials such as bricks, tiles, sand sacks and stone debris. The males of the peacock blenny, which normally breed in rocky coasts, use the cavities in these structures as nests which can be highly aggregated (Almada *et al.*, 1994). One breeding area of the population was monitored during 7 years, from May 2000 to August 2006. The area was composed of 80 brick structures linearly spread over 55m in the intertidal area in sandy substrate or over *Zostera* sp. These bricks contain diverse hollow cavities that have only

one constricted opening on one extremity and can be used as shelters or nests. Ninety nine regular *censi* were conducted in this area every month outside the breeding season, and every two weeks during the breeding season (from the beginning of May to middle September). The *censi* were conducted during low tide, when the area is emerged and fish can easily be forced out of the bricks. Once captured the individuals were anaesthetised with MS-222 (tricaine methanesulphonate; Pharmaq Ltd, Oslo, Norway, dilution 1:10 000 sea water) and marked with a passive transponder (passive integrated transponder tag; ID100A, Trovan, Aalten, The Netherlands; 11 × 2.1 mm) when captured for the first time. Because the minimum body size that can accommodate the tagging device is 5.2cm of total length, individuals below this size could not be tagged. This device was inserted through a small incision in the abdominal cavity. The incision was closed with medical grade adhesive glue. In each census the sex and morphotype of each individual, its position, morphological characteristics and the number of eggs in its nest were registered, as described below. After recovery from anaesthesia in an oxygenated container the marked fish were released at the site of capture. Thereafter, the identity of marked fish could be obtained in a non-invasive manner by holding the fish close to a portable reader (LID-500, Trovan).

The morphological measures taken included: total and standard length, body height (measured at the level of the insertion of the pelvic fins) and maximum head height measured to the nearest mm, and relative abdominal volume, measured in a scale ranging from 0 (minimum) to 9 (maximal). The weight was measured with a dynamometer (Pesola, Switzerland) to the nearest g. The relative development of male secondary sexual characters (head crest, anal gland) and of genital papilla were also classified using an ordinal scale from 0 to 9 (from absent to maximum development).

Males and females were discriminated according to the genital papilla: the genital papilla of females presents an atrium that precedes the opening of the papilla, while the male papilla is tubular and protuberant. Male morphotypes were further classified based on the development of secondary sexual characters and presence or absence of milt during the breeding season. This allows the identification of bourgeois, sneaker and immature males. Bourgeois males present developed secondary sexual characters (Fishelson, 1963; Papaconstantinou, 1979; Patzner & Seiwald, 1986) whereas both immature and sneaker males lack secondary sexual characters. During the breeding season, immature males are distinct from sneakers in the developmental state of their testes. While immature males have vestigial and immature gonads, sneakers have large sperm producing gonads. In practice, sneakers are identified by the release of milt when their abdomen is pressed, whereas immature males do not release milt (Oliveira *et al.*, 2001).

The number of eggs inside the nests was also monitored. The nest surface area covered with eggs was estimated and the number of eggs was calculated based on the Gonçalves *et al.* (2002), that reports that in this population eggs are attached to the surface of the nest in a single layer with an average density of 80 eggs per cm².

Analytical procedures

A database (Microsoft ® Office Access 2003) was constructed to compile the information collected in the censi and to dynamically manage it in order to easily assess information relative to each individual, nest or census. In this study we report exclusively information of male individuals gathered in this database. By reviewing individual data of males, we identified the date of the onset of secondary sexual characters expression, that is, the switching event between males without secondary sexual characters, either sneakers or immature males, and bourgeois males. We identified distinct periods of time in which individuals switched tactic, and assigned them, according to these peaks of switching events, to different classes which were analysed as independent units. We have tested the association of these classes with the initial ethotype expressed by the males using simulation statistics (ACTUS - analyses of contingency tables using simulations; Estabrook and Estabrook, 1989).

The individual growth trajectories, that is the evolution of observed total length (TL) in time, were analysed on FISATII (FAO-ICLARM stock assessment tools, Gayanilo *et al.*, 1996) as pairs of dates and respective TLs of all individuals in each class, and introduced in a growth increment data file. The data was analysed by the Appeldoorn's method to identify the best fit of the Von Bertalanffy growth curve: $LT = L^{\infty} (1 - e^{-K(t-t_0)})$, where L^{∞} is asymptotic length, the maximum length the fish would reach would it live indefinitely; K is the growth constant, the rate at which TL approaches L^{∞} ; and t_0 is the age a fish would have had at length zero. Constraints were, however, taken into account, namely the timing of the breeding season and consequently the time interval in the year in which hatching is possible, and a TL at hatching of 0.5 cm (Westernhagen, 1983; Patzner and Brandstätter, 1988). The best fit of the growth curves varied from $r^2 = 0.51$ to 0.63, which may reflect the fact that the species presents two "winter points" in which growth slowed down and that the software can not account for. These "winter points" occurred in winter and in summer, during the breeding season. The best fitting curves were adjusted to the average date and body size of switching, therefore deducing the average hatching moment. In order to compare growth rates of different curves, growth performance indices, ϕ' , were calculated for each class: $\phi' = \log K + 2 \log L^{\infty}$ (Pauly and Munro, 1984).

Reproductive success was measured as the sum of the number eggs found in the nests of each male in all the censi. This is actually a measure of relative reproductive success because eggs were counted every two weeks but, in fact, in this population eggs hatch in about 10 days (Ros *et al.*, 2009). Therefore, each male had most likely more eggs than those counted but because the censi are regular and systematic each males was sampled with the same effort. Additionally, the fact that some of the eggs in the nests of the bourgeois males were fertilized by the sneakers was neglected, despite not contributing to the reproductive success of the bourgeois male. Nonetheless, this measure should be a reliable approximation of the relative reproductive success of the males while reproducing as bourgeois, if the proportion of eggs fertilized by the sneakers is fairly constant throughout the nests. For the calculation of the average reproductive success all bourgeois males were included, even those who did not have eggs.

Longevity was calculated as the time between the estimated date of hatching and the last re-capture of the male. This is in fact a local parameter but should reliably measure longevity of reproducing males because bourgeois males occupy preferentially breeding areas, like the one monitored during this study, and maintain that preference throughout the year (Fagundes and Oliveira, unpublished).

Survival rate per class until each of the breeding seasons was calculated dividing the number of individuals that had reached that season by the total number of individuals included in that class. Average reproductive success of surviving males in each of the breeding seasons was calculated similarly to reproductive success as bourgeois males but accounting only for the number of eggs in the nests during that season and only for the males that had survived until then. To obtain the average reproductive success of a class in a breeding season, average reproductive success of surviving males should be multiplied by survival rate in the respective season.

Analyses of variance were conducted on SPSS 14.0 (statistical package for the social sciences) for Windows (Chicago: SPSS Inc.). All tests were two sided and significance level was set to 0.05.

RESULTS

The analysis of individual ontogenetic trajectories indicates that the alternative tactics expressed in this population are sequential but not reversible. The switching from immature or from sneaker (both without secondary sexual characters) to larger bourgeois males with secondary sexual characters was unidirectional. None of the males that were

identified as holding the bourgeois tactic (N = 165 marked bourgeois males) switched back to being a sneaker and therefore the morphotype transformation is irreversible. On the other hand, small individuals that reproduce as sneakers were never recaptured as sneakers in subsequent breeding seasons instead were always recaptured as bourgeois males (sneakers: N = 203 marked; of which N = 130 recaptured; of which N = 23 recaptured in the next breeding season: as sneakers N = 0 or as bourgeois males N = 23), indicating a sequential and irreversible expression of the two tactics in the same individual. Males classified as immature were rare but equally not recaptured as sneakers nor immatures in the next breeding season (immature males marked: N = 38 marked; of which N = 9 recaptured; of which N = 1 recaptured in the next breeding season as bourgeois males). The size of sneakers, mature individuals with no secondary sexual characters, ranged from an absolute minimum of TL = 4.4cm to TL = 10cm, but the size at first maturity was calculated as TL = 4.8, which corresponds to the smallest size class in which 50% of the males are sexually mature.

Based on the timing of tactic switching (*i.e.* beginning of the expression of secondary sexual characters) two different classes were identified (Fig. 1): individuals that switched to the bourgeois morphotype between March and June and those who switched between October and January. These two classes were significantly associated with the initial morphotype expressed by the individuals that composed them (Actus chi-square_{1, 130} = 46.826, $P < 0.001$). So that from those individuals that switched to bourgeois morphotype between March and June, only a minority were sneakers before switching (N = 10), the majority were individuals never captured as sneakers, that is, they were either immature males or captured before the breeding season, and therefore could not be classified as sneakers or immatures. Contrary, those males that switched to bourgeois morphotype between October and January were mainly sneakers. The two classes of males that switch tactics at different times of the year were further subdivided into the following groups for further analysis:

“Immatures”- a large group of males without secondary sexual characters that were never captured as sneakers presumably immature males, (N = 40) that switched to bourgeois males between March and June;

“Large sneakers”- a small group of sneakers (N = 10) that switched to the bourgeois tactic between March and June;

“Sneakers” – a large group of sneakers (N = 25) that switched to the bourgeois tactic between October and January;

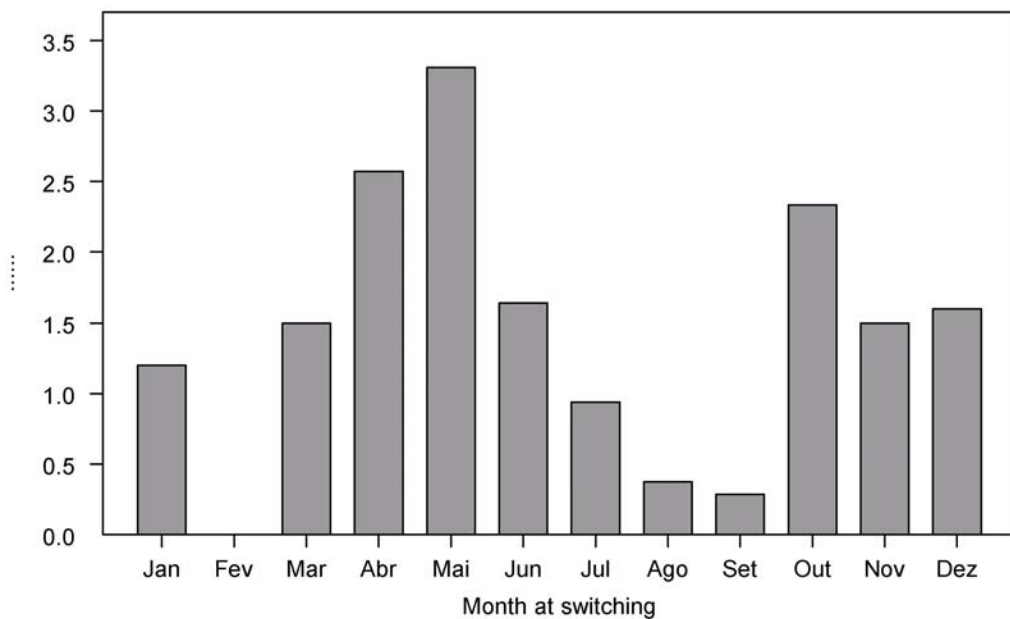


Figure 1. Frequency of switching to the bourgeois morphotype, corrected for the number of sampling points (censi) conducted on each month, i.e., number of observed switching events / number of sampling points within each month.

“Non-sneakers” – relatively few individuals never captured as sneakers (N = 16), presumably immature males, that switched to the bourgeois tactic between October and January.

Growth trajectories were estimated for each of these four classes (Fig.2) with the resulting von Bertalanffy growth functions and growth performance indices (ϕ'): “immatures”- $LT = 13.80(1-e(-1.2(t+0.031)))$; $\phi' = 2.359$; “Large sneakers”- $LT = 13.2(1-e(-0.93(t+0.042)))$; $\phi' = 2.210$; “Sneakers” – $LT = 13.20(1-e(-1.00(t+0.039)))$; $\phi' = 2.241$; “Non-sneakers” – $LT = 14.5(1-e(-0.65(t+0.054)))$; $\phi' = 2.136$. Hence, immature males tend to grow faster than others but all classes showed fairly similar growth trajectories. When adjusted to the moment time and size of switching, the growth trajectories of the main classes, “immatures” (N = 40) and “sneakers” (N = 25), revealed different hatching moments. Two distinct life-history pathways were consequently identified: males of the “immature” class switched to bourgeois males before or at the beginning of their first breeding season (age 0+) and most likely never reproduced as sneakers since their body size during the season in which they have hatched was below the size at first maturation. Therefore, their

classification as “immatures” was not an artefact of sampling procedure. “Sneakers” reproduced as such in their first breeding season and switched to bourgeois males directly afterwards (age 1). The less representative classes included the “large sneakers” (N = 10) and the “non-sneakers” (N = 16). The “large sneakers” were males that, similarly to the “immature” group, switched to the bourgeois tactic at the beginning of the breeding season. However, they presented slightly lower growth rates and reached the breeding season with a smaller body size that allowed them to still reproduce as sneaker for a short period of time at the beginning of the season. The remaining class, “non-sneakers”, was composed of males that were never captured as sneakers, similarly to “immatures”. However, they were identified for the first time at the end, or after the end, of the breeding season when it is not possible to distinguish sneakers from immature males.

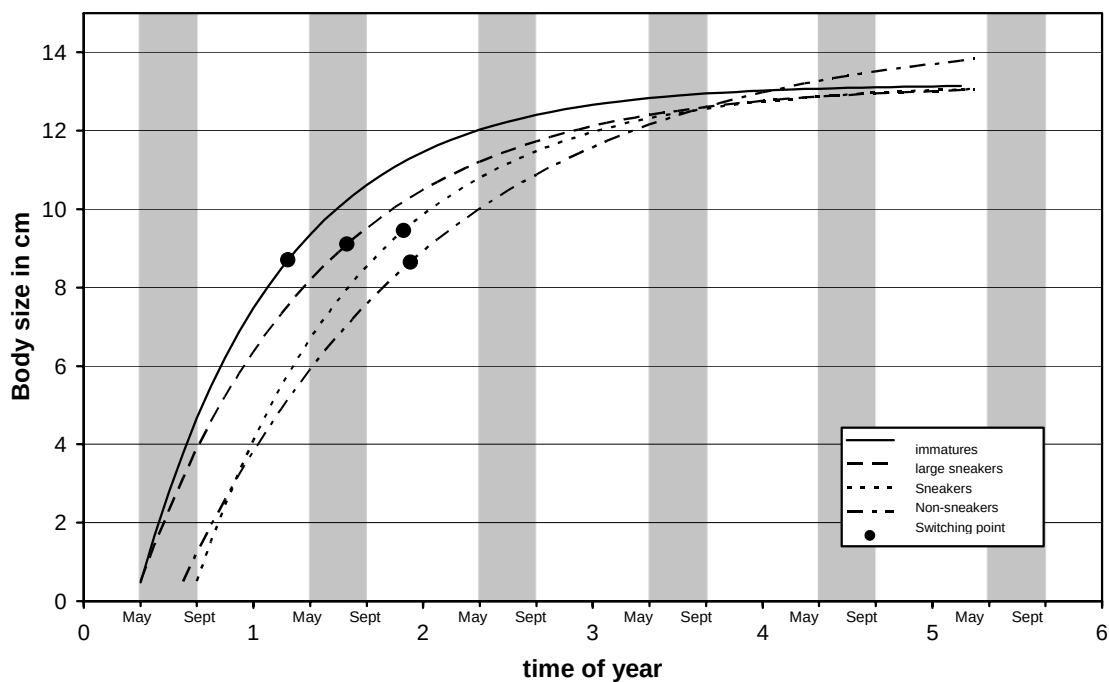


Figure 2. Best fit of the von Bertalanffy growth curve (total length) of the different classes identified on the bases of timing of switch to bourgeois tactic and initial reproductive tactic. Grey bars represent breeding season and black dots the average switching time to bourgeois morphotype.

Moreover, according to the estimated growth curve, their body size during the breeding season was higher than the size of first maturation (TL = 4.8cm) and therefore compatible with a previous reproduction as sneakers. These distinct trajectories were associated with the hatching period of individuals: “Immatures” tend to have hatched at the start of the

breeding season as well as “large sneakers” but “sneakers” and “non-sneakers” were late hatched, with “non sneakers” hatching probably slightly earlier than “sneakers” and entering the reproductive area after their first breeding season.

The body size of “sneakers” at the moment of switching to bourgeois males was larger than that of males of “immature” and “non-sneaker” classes, but it did not differ significantly from that of “large sneakers” (Fig. 2, ANOVA $F_{3,81} = 3.945$; $P = 0.011$, TL: “immatures” = 8.75 ± 0.14 ; “sneakers” = 9.45 ± 0.18 ; “large sneakers” = 9.12 ± 0.29 ; “non-sneakers” = 8.66 ± 0.21 ; Tukey test “immatures” vs. “sneakers” $P = 0.015$, “sneakers” vs. “non-sneakers” $P = 0.032$, for all remaining pair wise comparisons $P > 0.100$). Age at switching also differed between classes. “Sneakers” and “large sneakers” switched to bourgeois males at a later age than the “immature” class did but younger than “non-sneakers” (Fig. 3, ANOVA $F_{3,81} = 62.674$; $P < 0.001$, Tukey test “large sneaker” vs. “sneakers” $P = 0.841$, for all remaining pair wise comparisons $P < 0.001$). Namely, “immatures” and “large sneakers” switched to bourgeois morphotype at the beginning of their first breeding season while “sneakers” did it after their first breeding season. “Non-sneaker” males also switch after the first breeding season.

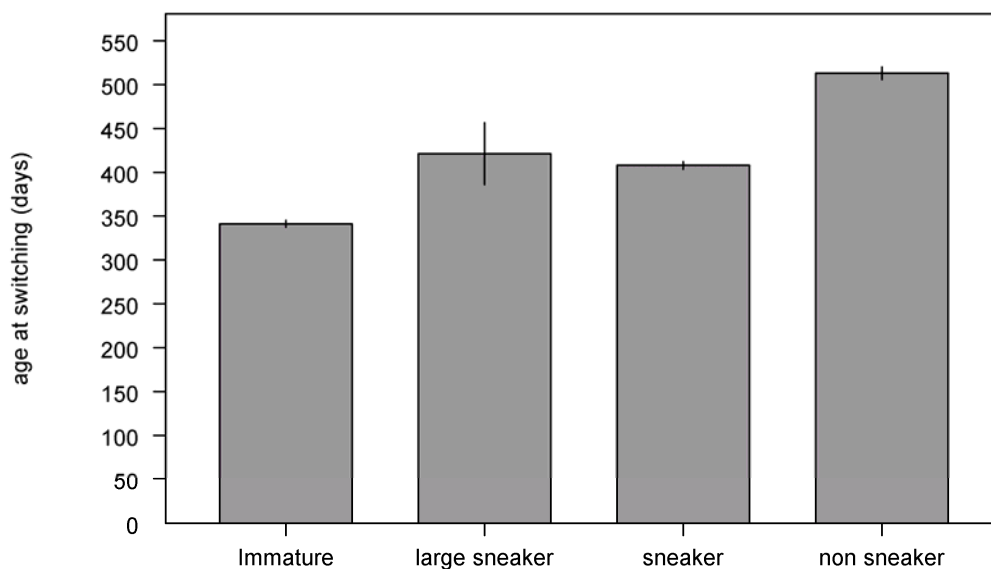


Figure 3. Age of individuals (average±S.E) at the moment of switching to bourgeois males. Identification of classes is detailed in the results section.

The longevity of each of these classes as well as their reproductive success once they became bourgeois males, were also assessed. Reproductive success was associated with time in the area monitored that was assumed to represent longevity, since most

individuals remained in the reproductive areas even outside the breeding season. The reproductive success of the “sneaker” class was higher than that of the “immature” class (Fig.4, ANOVA $F_{3,84} = 2.716$; $P = 0.050$, Tukey test “immatures” vs. “sneakers” $P = 0.045$, for all other pair wise comparisons $P > 0.100$).

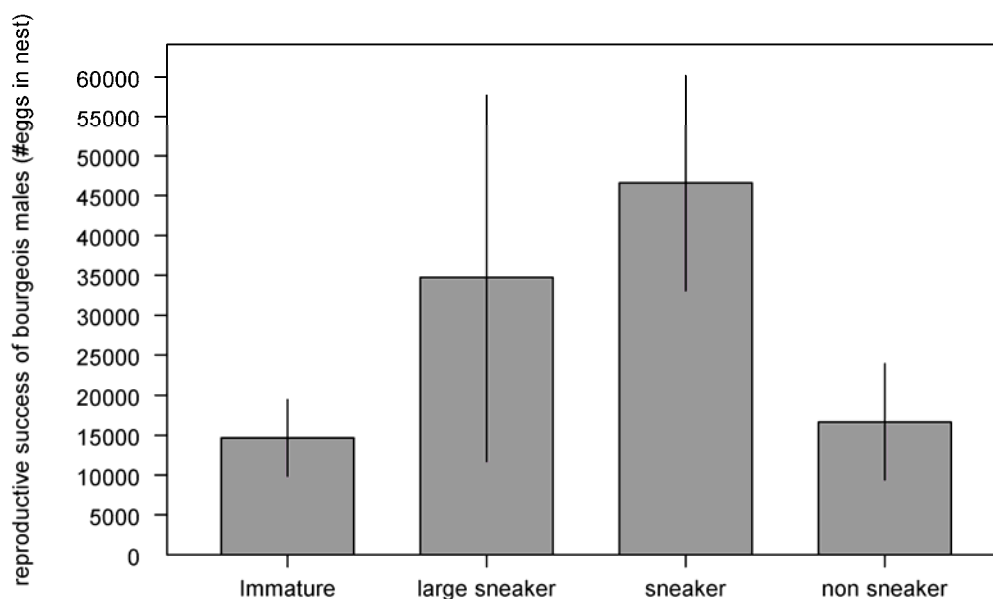


Figure 4. Estimated total number of eggs (average $\pm$ SE) inside a bourgeois male's nest during its lifetime. Identification of classes is detailed in the results section.

Indicative values of average reproductive success of surviving males per season are presented in Table 1 as well as survival rates. The longevity of “sneakers” and “non-sneakers” was higher than that of “immature” males (Fig. 5, ANOVA $F_{3,84} = 10.322$; $P < 0.001$, Tukey test “immatures” vs.: “sneakers” $P < 0.001$, “non-sneakers” $P < 0.001$, for all other pair wise comparisons $P > 0.100$). The maximal longevity observed was 1378 days (*i.e.*, approximately 3 years and 10 months).

Table 1. Characteristics of the bourgeois and of the sneaker pathways, and of the “non-sneaker” group: age represents average age in days and TL the average total length in cm, both at the moment of switch to bourgeois morphotype. For each of the breeding season, RS represents the reproductive success in number of eggs of the males that survived until that season and SR represents the survivorship rate since tagging until that season, measured in days (for the first breeding season survivorship rate is 1).

	Hatch	Switch		1 st breeding season		Switch		2 nd breeding season		3 rd breeding season		4 th breeding season	
Pathway		age	TL	RS	age	TL	SR	RS	SR	RS	SR	RS	
Bourgeois	Early	341	8.7	10356	-	-	0.07	25046	0.03	96760		-	
Sneaker	Late	-	-	?	407	9.5	0.68	30068	0.23	44400	0.09	31919	
Non-sneaker	Late	-	-	?	512	8.7	0.94	9854	0.13	59192	0.06	0	

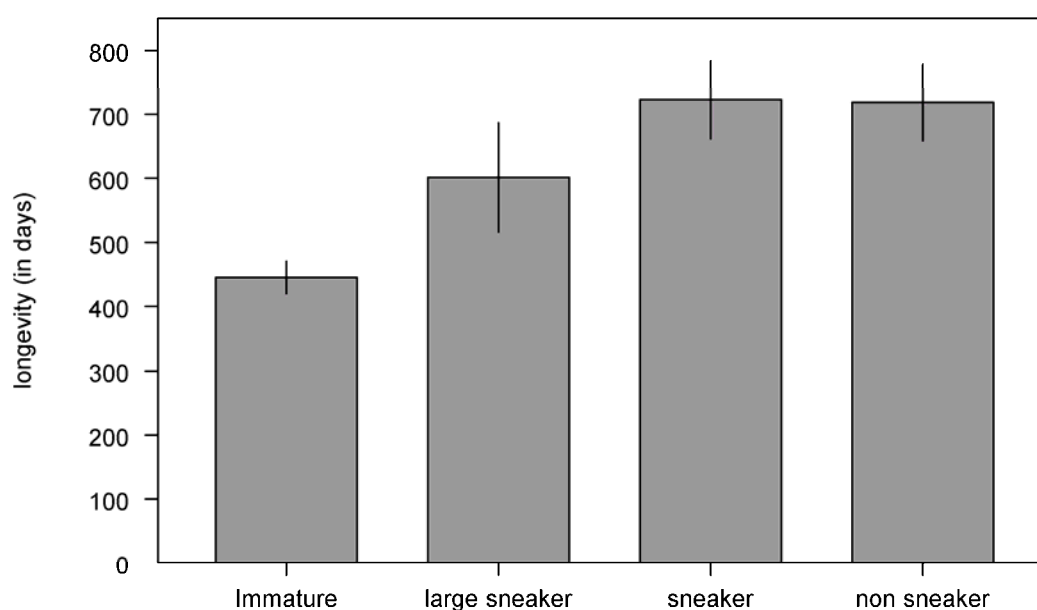


Figure 5. Longevity (average $\pm$ SE) of the males of each class measured in days. Identification of classes is detailed in the result section.

DISCUSSION

The results confirm the initial expectation that male alternative reproductive tactics in this species are sequential. Young males may initially reproduce as sneakers and later as bourgeois males, defending a nest and attracting females. However, once the secondary sexual characters are expressed, males do not return to reproduce as sneakers. Therefore, the tactics are flexible with fixed sequences (sensus Taborsky *et al.*, 2008). Moreover, not all individuals express the sneaker tactic and consequently at least two different life-histories trajectories are present in this population. The two main life-history trajectories are:

1. Individuals that reproduce directly as bourgeois males in their first breeding season. We may call it “bourgeois pathway” and it corresponds to the “immature” class analysed.
2. Individuals that reproduce as sneakers in their first breeding season switching to the bourgeois tactic after the end of that season. We may call it “sneaker pathway” and it corresponds to the “sneaker” class.

Males classified as “non-sneakers” could not be attributed to any of the two pathways. These males have been captured after their first breeding season, and their size was estimated to have been above the size at first maturation during the breeding season. Therefore these males could have been reproducing already as sneakers before being marked and followed. Nevertheless, from a previous work Fagundes and Oliveira (unpublished) report that immature males show a preferential occupation of non reproductive areas. In this way, their absence from the area monitored until after the breeding season could be due to postponed maturation in the first breeding season and associated preference for other microhabitats. This preference could also justify the very low number of immature males captured on the area monitored. According to the classical trade-off between reproduction and somatic growth (Stearns, 1992), postponing reproduction would imply losing one breeding season but would increase the reproductive success in the subsequent season through a size advantage. Such size advantage is not verified by the estimated growth curves. Nevertheless, the data does not allow either to confirm or to exclude the existence of a third pathway of postpone maturation. .

The growth trajectories of these life-history pathways coincide with the results of Gonçalves *et al.* (1996) that report an age of 0+/1 year for both sneakers and immatures.

Reproducing directly as a bourgeois might seem advantageous since the fertilization success of sneakers is usually low in comparison to that of bourgeois males (e.g. 5% - 10% in the sand goby, *Pomatoschistus minutus*: Malavasi *et al.*, 2001; 40% in the

fifteenspine stickleback, *Spinachia spinachia*: Jones *et al.*, 1998; 19% in the Bluegill sunfish, *Lepomis macrochirus*: Fu *et al.*, 2001). If one accepts the assumption that the fertilization success is higher for bourgeois males in this population, greater reproductive success would be expected for the “bourgeois pathway” since males of this pathway initiate reproduction as bourgeois earlier than those of the “sneaker pathway”. This would allow them to reproduce as bourgeois during more breeding seasons than the alternative pathway therefore achieving higher reproductive success. However, lower reproductive success of the “bourgeois pathway” was estimated from our data. This pathway shows shorter longevity than the “sneaker pathway”. Individuals in both pathways reproduce as bourgeois males on three seasons at most. Additionally, when comparing the first, second and third seasons in which the males have reproduced as bourgeois males, the “sneaker pathway” tends to show higher reproductive success (survival rate * reproductive success of surviving males) than the “bourgeois pathway”, although values are only indicative and cannot be analysed statistically due to low sample size. These two pathways also differ in body size when initiating expression of the bourgeois tactic, which may constitute an important factor determining their subsequent reproductive success as bourgeois male.

In this species, the fitness of each tactic may be a consequence of body size. Sneakers mimic females which are smaller than bourgeois males and therefore it will be more difficult for larger sneakers to deceive the bourgeois males when trying to approach the nests. This is supported by the fact that larger sneakers receive more aggression from bourgeois males than smaller ones (Gonçalves *et al.*, 2005). For bourgeois males, on the other hand, the larger they are, the easier it should be to establish and defend a nest, especially in an area with shortage of nest sites as is the case of this population. This is supported by field data showing that nest-holder males are significantly larger than nestless bourgeois males (Oliveira *et al.*, 1999).

When reproductive success is associated with body size but this relationship is positive for one tactic and negative for the other, fitness curves of the two tactics will cross (Gross, 1985; Taborsky, 1999; Brockmann, 2001). The crossing point of the two curves should determine the ideal body size at which tactic switching should occur. However, constraints may lead to tactic switching at non optimal body size. This may be the case in the “bourgeois pathway”; the large size with which these males enter the breeding season, prevents them to reproduce as sneakers, but it is also not ideal to reproduce as a bourgeois males either. When switching, at the start of the breeding season these males are the smallest nest-holders in the population, even smaller than the 2 year old males from the “sneaker” pathway. Therefore, males from the “bourgeois pathway” will be in clear disadvantage regarding their potential lifetime reproductive success.

If we consider that the early hatched males arrive at the start of the breeding season with a body size close to the switching point it is expected that some of these individuals will switch but others not. And in fact, a few of the early hatched males, the smallest, reproduce initially as sneakers for a short period early in their first breeding season and switch to bourgeois males later, during that season still. Three reasons could account for this fact:

First, small variations in growth rates or even hatching date may cause variation between the body sizes of individuals at the onset of the breeding season. Because growth rates are fairly similar for all individuals, a switching point at a fixed body size in accordance with the status-dependent model (Gross, 1996) would create a gradient of ratio of pathways along hatching time. In other words, males hatching at the beginning of the breeding season would mostly reproduce as bourgeois in the next season but as hatching progresses through the season the proportion of individuals that will reproduce as bourgeois will decrease and that of sneakers increase. The two groups that reproduce as sneaker males, “sneakers” and “large sneakers”, do switch to bourgeois males with the same body size, however “immatures” (early hatched) switch at smaller and “non-sneakers” (latter hatched) at larger body sizes than the two first groups. Therefore the data does not support the hypotheses of a fixed threshold dependent on absolute body size.

Second, individual variation may exist in the value of body size as determinant of switching point, as proposed in the threshold model (Hazel *et al.*, 1990; Emlen, 2008) which could account for variation in the tactic expressed by individuals of the same size. However, according to this hypothesis no apparent cause could justify an association between the value of the switching point and hatching date, rather a randomly varying value of switching point was to be expected. So, no support for this hypothesis is found in our data.

Third, body size, specifically relative body size, is associated to other factors, namely social factors as social dominance, and this has been demonstrated to influence the expression of the bourgeois tactic (Fagundes *et al.*, unpublished). In the natural context of this population, competition for nests is expected to be intense and the dominance hierarchy very well defined in anticipation of the breeding season. At this moment, males of the “bourgeois pathway” are the largest of their cohort and their body size is median when considering the whole population, while that of the males from the “sneaker pathway” lies at the bottom of the distribution. The hierarchical rank is tightly associated with this distribution of body size and may justify the switching of the largest individuals to

bourgeois males. During the breeding season, bourgeois nesting males leave the nest less and less as they receive eggs (personal observation in experimental conditions) and participate in less agonistic interactions but their body condition decreases (Gonçalves *et al.*, 1997; Fagundes, unpublished data) and they may abandon the nest to forage. In this way the dominance hierarchy is expected to be relaxed which may allow some of the “large sneakers” to raise their dominance rank and switch tactics. However, from 91 males sampled, only 10 were observed to switch to bourgeois during the season and only two were able to successfully reproduce as bourgeois in the same season. Males at the moment of switch, when initiating the expression of secondary sexual characters, present regressed gonads (Gonçalves *et al.*, 2008) hence they undergo a time interval during which they will be unable to reproduce. Therefore, switching tactics during the breeding season compromises the reproductive success of that season because of loss of reproductive time and is in fact an infrequent event. At the end of the breeding season many bourgeois males die after parenting. The average longevity of males from both the “sneaker” and the “bourgeois pathways” is associated with a high mortality rate at the end of the breeding season. At that moment, when the males from the “sneaker pathway” switch to bourgeois males, their size rank will be the highest because most of the older males will have died. Hence, the effect of social dominance detected in experimental conditions is compatible with the expected dynamic of the natural population and supports a threshold that is based on either relative body size or social dominance (so strongly associated that their effects are difficult to disentangle).

The life-history pathways observed are associated with the timing of hatching, supporting the “birth-date effect” hypothesis (Taborsky, 1998). The “immature” class will tend to have hatched early and reproduced directly as bourgeois males, following the “bourgeois pathway” described above. The males of the “sneaker” class would have hatch later, arriving at the first breeding season with a smaller body size and reproduced as sneakers, following the “sneaker pathway”. These males will subsequently express the bourgeois tactic in the next breeding season. Hence, date of hatching determines body size at the beginning of the breeding season which in turn translates into the expression of a given mating tactic. Contrary to the example of the Atlantic salmon, (*Salmo salar*), in which fast and slow developing life-history pathways are responsible for tactic choice, parr vs. smolt, (Rowe and Thorpe, 1990), the difference in body size in the peacock blenny seems to be due simply to the time elapsed between the hatching of the males following the two pathways as growth rates did not show a significant difference between morphotypes.

Individuals from the “bourgeois pathway” tend, however, to growth faster than those following the “sneaker pathway”. “Sneakers” mature earlier than other male classes

(average maturation age 280 days) and this early investment in reproduction may be trading-off with somatic growth, potentially due to resource allocation to sexual maturation and reproductive behaviours, as it happens in species with indeterminate growth (Henio and Kaitala, 1999). Another central trade-off in life-history theory involves a compromise between longevity and reproduction (Metcalf and Monaghan, 2003) but the data does not evidence such a trade-off. Instead, the reproductive success of males is positively correlated with longevity and this fact can be interpreted in two ways. Males of the “bourgeois pathway” switch to bourgeois with a smaller body size than do males from the “sneaker pathway” and that means that they will have to compete with larger males for access to nests. This size disadvantage may be reflected in the weak reproductive success of these males (63% of these males do not reproduce at all). This size disadvantage may also affect survival. Direct competition for access to nests exposes males, particularly smaller males, to the risk of injuries and is energetically costly which might compromise longevity. To note that the reproductive success estimated for bourgeois males of each of the pathways is in reality a conservative measure of the difference between reproductive success of the two pathways. The reproductive success of the “bourgeois pathway” is overestimated by our calculation because some of the eggs tended by these males are sired by sneakers that are not included in this pathway. Contrary, the eggs tended by the bourgeois males of the “sneaker pathway” are all eggs of males of that pathway, however our calculations do not include the eggs fertilized by these sneakers in the nests of males of the “bourgeois pathway”. Therefore, the reproductive success of bourgeois males of the “sneaker pathway” is an underestimation of the success of whole pathway. In this way, our estimations are conservative of the difference between the reproductive successes of these pathways. Such differences in the reproductive success of the two pathways further support the argument exposed above defending the expression of alternative reproductive tactics in this population as condition dependent (Brockmann, 2001; Shuster and Wade, 2003). Moreover, the low reproductive success of the ‘bourgeois pathway’ indicates that hatching at the start of the breeding season constitutes a constraint and selection to restrict the breeding season is to be expected.

Nevertheless, environmental condition at the beginning of the breeding season may favour eggs survival, or over winter survival, more than at the end of the season. The data supports this hypothesis given that the frequency of males early hatched was higher than that of males later hatched (50 vs. 41 respectively). If such a differential survival of eggs or juveniles exists, it will constitute a selective force acting in the opposite direction, *i.e.*, expanding the breeding season towards its beginning. Moreover, despite constituting a

constraint to males, hatching early is usually advantageous for females, and particularly if the environmental conditions are optimal for survival early in the season. Female fecundity is generally associated with body size (Bagenal, 1973; Jonsson, 1985). Females would then benefit from hatching earlier because that would allow them to grow more until they start reproducing and consequently would increase their reproductive success. Therefore different selective forces may be acting in females and males in opposite directions maintaining such an extended breeding season.

In summary, two life-history pathways were identified that are associated with date of hatching, rendering this study the first to confirm the “birth-date effect” on expression of alternative reproductive tactics.

The two main life-history pathways followed by males are:

1. “Bourgeois pathway”: early hatched, immature males switch to bourgeois males at age 0+ (March to June) and reproduce as such during their lifetime.
2. “Sneaker pathway”: later hatched males will reproduce as sneakers in their first breeding season, switching to bourgeois after the end of that season (October to November), at age 1.

Early hatching constitutes a constraint, since the “bourgeois pathway” presents a lower reproductive success than the “sneaker pathway”. This is most likely due to the fact that in the “bourgeois pathway” switching to bourgeois morphotype occurs at smaller body sizes, which should constitute a competitive disadvantage in acquisition of reproductive resources, for instance nests. Nevertheless, such early hatched males may be maintained in the population, *i.e.*, the breeding season may not contract, if considering opposite selective forces that favour the early hatching of females.

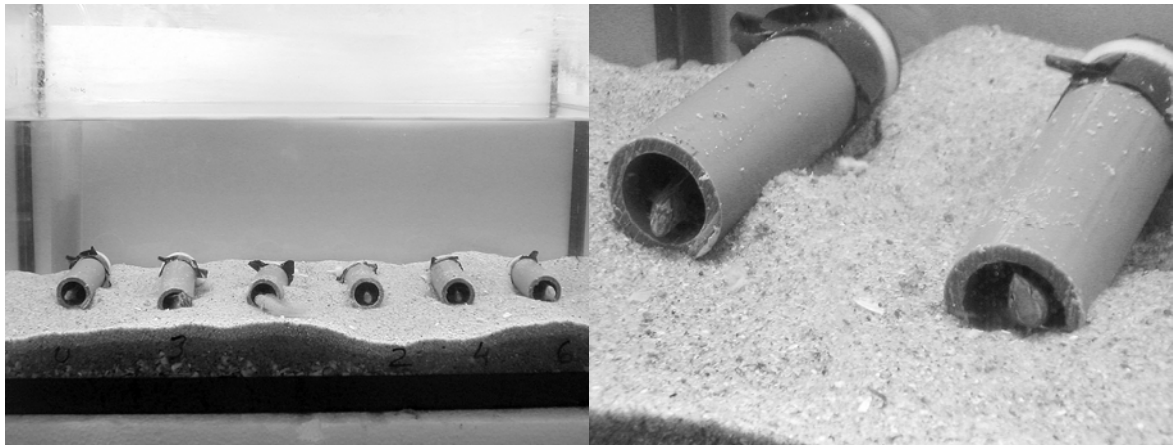
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CHAPTER IV

Social cues in the expression of sequential alternative reproductive tactics in young males of the peacock blenny, *Salaria pavo*

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ABSTRACT

The peacock blenny presents sequential alternative reproductive tactics. Younger and smaller males may reproduce as sneakers: female mimics that fertilize eggs in the nests of bigger and older nest-holder males. In experiment 1 we tested the effect of social dominance and density on the expression of reproductive tactics by immature males. Dominant males showed larger growth of secondary sexual characters (anal gland and head crest), larger testicular glands, more nesting behaviour and higher 11-ketotestosterone and testosterone levels, traits typical of nest-holders. 11-ketotestosterone mediated the effect of social dominance on the growth of head crest, but not on the remaining traits. This suggests a transition of dominant immature males to nest-holders. Gonadal development was independent of social dominance and marginally inversely related to cortisol levels. Sexual maturation and expression of reproductive phenotypes are thus somewhat independent and controlled by different mechanisms. In the second experiment we tested the effect of exclusion of male-male competition on tactic switching by sneakers. Nest-holder's removal did not promote the switching of sneakers to nest-holders, suggesting that sneakers are refractory to changes in social environment. In conclusion, dominance promoted the expression of nest-holder's traits in immature males but not sexual maturation, suggesting the conjugation of different mechanisms in reproductive tactic expression.

INTRODUCTION

Alternative reproductive tactics (ART) are characterized by distinct sets of reproductive behavioural traits, which generally are associated with differences in morphological and physiological traits. Typically, bourgeois males invest in obtaining and defending resources by being territorial, attracting females and in the case of teleost fish, very often providing parental care. Parasitic males use other behaviours like sneaking or female-mimicking, in order to exploit the reproductive investment of bourgeois males and fertilize eggs during spawning events (Taborsky, 1997).

In a descriptive approach without considering the underlying mechanisms of tactic determination, ART may be classified as fixed, sequential or reversible. In the first case each individual expresses only one reproductive phenotype throughout its life; in the second case individuals expressing one tactic switch irreversibly to another tactic at some life stage; while in the latter case individuals switch back and forth between tactics (see Brockmann, 2001). For any of the three types of tactics mentioned the expression of the tactics may be dependent on environmental cues (Gross, 1996; Shuster and Wade, 2003; Emlen, 2008), in which case assessment of these cues, whether internal or external, allows the individual to adjust its phenotype to the characteristics of the surrounding environment and/or to its own state. Detailed studies of ART in arthropods have demonstrated that body size (European earwigs, *Forficula auricularia*: Tomkins, 1999; Tomkins *et al.*, 2004), population density (mite, *Sancassania berlesei*: Radwan *et al.*, 2002; Tomkins *et al.*, 2004) or other individual or environmental (social or not) conditions (e.g. age and body condition in the horseshoe crab, *Limulus polyphemus*: Brockmann, 2002) play an important role as cues for tactic choice. However, studies within vertebrate species are still scarce (exceptions are the influence of feeding and growth opportunity in Atlantic salmon, *Salmo salar* L.: Rowe and Thorpe, 1990; rainfall in the tree lizard, *Urosaurus ornatus*: Knapp *et al.*, 2003; and male-male competition in the black goby, *Gobius niger*: Immler *et al.*, 2004).

Mechanisms underlying tactic expression should be able to convert environmental cues into appropriate reproductive behaviours and morphological traits such as secondary sexual characters (SSC). Among vertebrates, androgens and corticosteroids are major endocrine candidates for a mediation role in this process of translation between environmental conditions and phenotype expression. In fish, androgens and particularly 11-ketotestosterone (KT) are known to promote the development of SSC (Borg, 1994; Jalabert *et al.*, 2000) and to influence the expression of reproductive behaviour (Liley and Stacey, 1983; Borg, 1994; Oliveira and Gonçalves, 2008). Moreover, social environment

modulates androgen levels in teleost fish (Oliveira, 2004; Hirschenhauser and Oliveira, 2006) and, in *Oreochromis mossambicus*, androgens have been demonstrated to mediate an effect of social dominance on development of sexually dimorphic characters (Oliveira and Almada, 1998). Therefore, we expect that dominance may raise androgen levels and subsequently promote the development of SSC and other morphological and behavioural traits typical of the bourgeois tactic. In other words, we expect androgens to mediate the effect of social dominance on the choice of reproductive tactic.

More attention has been given recently to the possible mediation role of corticosteroids between social environment factors, such as social dominance, and reproductive phenotype expression (Knapp *et al.*, 2003; Leary *et al.*, 2006; Rodgers *et al.*, 2007). Despite variable responses of corticosteroids to social status, in some fish species subordinate individuals present higher cortisol levels (coho salmon, *Oncorhynchus kisutch*: Ejike and Schreck, 1980; rainbow trout, *Oncorhynchus mykiss*: Winberg and Lepage, 1998; an opposite example is the cooperatively breeding cichlid, *Neolamprologus pulcher*: Buchner *et al.*, 2004). In addition, cortisol has been demonstrated to influence the onset of sexual maturation in common carp, *Cyprinus carpio*, independently of 11KT levels (Consten *et al.*, 2002; Goos and Consten, 2002) and thus may play a role in mediating a possible effect of social status on the expression of the reproductive phenotype. Corticosteroids have also been proposed to interact with reproductive endocrine axis by suppression of androgens receptors, competing with binding globulins or steroidogenic enzymes, or by modulating androgens production itself (Knapp, 2004). In such cases, and if androgens do mediate the effect of environment on tactic expression, the effect of social factors on reproductive phenotype expression may be modulated by levels of corticosteroids, *i.e.* the effect of social factors on the expression of the reproductive phenotype may be moderated by corticosteroids (*sensus* Baron and Kenny, 1986). Thus, complementary hypotheses can be tested. One in which a subordinate status causes high cortisol levels, which in turn may suppress reproduction and /or expression of SSC and therefore it may be conceptualized as a mediation effect; and a second hypothesis, in which cortisol levels, though not determined by social status, interfere with the effect of the latter on the expression of the traits associated with the different reproductive tactics. This second hypotheses may be conceptualized as a moderation effect of cortisol.

The aim of our study is to test the influence of some social cues on the expression of reproductive tactics and the endocrine mechanisms underlying that expression. The peacock blenny is a particularly interesting model system for the study of phenotypic plasticity since it expresses different reproductive tactics sequentially, *i.e.* the same

individual may express two different tactics during its life thereby changing its reproductive phenotype. In this species, sneakers (the parasitic tactic) are younger and smaller males who reproduce by fertilizing part of the eggs inside the nests defended by nest-holders (Almada *et al.*, 1995; Gonçalves *et al.*, 1996). Sneakers deceive nest-holders through female mimicry, displaying female-like courtship towards nest-holder males and not expressing SSC. They have larger testes than nest-holders, considering differences in body size (Gonçalves *et al.*, 1996) and undeveloped testicular glands. The larger testes have been interpreted, in the context of sperm competition, as a way to overcome the disadvantages of a less proximate access to the females when these spawn inside the nest-holders nests. The testicular gland, a gonadal accessory gland, is the major source of androgens in this species (Reinboth and Becker, 1986; Oliveira *et al.*, 2001a) and plays a role in sperm maturation (Lahnsteiner *et al.*, 1990; Lahnsteiner and Patzner, 1990). The absence of testicular glands in sneaker males suggests differences in the sperm characteristics of the two reproductive tactics as is the case with *Gobius niger* (Rasotto and Mazzoldi, 2002; Locatello *et al.*, 2007). Nest-holders are older and larger males that establish and defend nests and provide parental care to the eggs that females lay in their nests (bourgeois tactic). Nest-holders express SSC: a head crest and pheromone producing anal gland (Fishelson, 1963; Papaconstantinou, 1979; Patzner and Seiwald, 1987); and they have higher levels of both testosterone (T) and KT than sneakers (Oliveira *et al.*, 2001a; Gonçalves *et al.*, 2008).

According to an ongoing mark-recapture study, males typically live 3 years and they were found to reproduce following one of three different pathways: 1) become sneakers and reproduce as such in their first breeding season, switching to nest-holders subsequently; 2) post-pone reproduction in their first breeding season and invest in somatic growth, reproducing as nest-holders in the next season; 3) differentiate SSC and breed as small nest-holders already in the first breeding season (although very small nest-holders are not very common in nature). All individuals that have achieved the nest-holder reproductive tactic maintained that phenotype throughout the subsequent breeding seasons.

Contrary to what has been the norm, in which species with fixed alternative tactics are usually tested in early stages of development (*e.g.* Tomkins, 1999; Radwan *et al.*, 2002) and species with sequential tactics have been tested only during the switch between tactics (*e.g.* Immler *et al.*, 2004), here we explore possible mechanisms of tactic determination both at the onset of reproduction and later on, at the moment of tactic switch. Of special interest is the analysis of the expression of the initial tactic, thus from juvenile immature individuals to a reproductive morph, and the endocrine mechanisms possibly involved, which, at this life stage, have been overlooked in species with

sequential alternative tactics and very much so in vertebrates. Hence, we have conducted two experiments with complementary goals:

Exp. 1. Social groups of immature males with different densities were formed and subsequently the social status of each individual was determined. Tactic-specific traits like SSC, gonad characteristics and nesting and courtship behaviours were measured to evaluate whether males reaching the first breeding season decide their first reproductive tactic (sneaker vs nest-holder) on the bases of density and/or social dominance; and if so, whether steroid hormones, usually involved in sex differentiation, mediate or moderate the effects of social dominance or density on the expression of these tactic-specific traits. We expect social dominance to promote the expression of the bourgeois tactic through higher levels of androgens and/or lower levels of cortisol; and density, as a potential signal of the intensity of male-male competition, to raise the incidence of the parasitic tactic.

Exp. 2. Groups composed of 1 nest-holder, 1 sneaker and 1 female were used as control. From the groups of the “male removal” treatment, the nest-holder was removed at the end of 1 week, leaving only 1 sneaker and 1 female in each tank for another week. Tactic-specific morphological and behavioural traits were measured to evaluate whether young males which have already entered the sneaker tactic, are able to quickly switch to a nest-holder tactic, should an opportunity appear by removal of the local nest-holder. We expected parasitic males to be able to adopt a bourgeois tactic, given a free nest, the lack of male-male competition and the impossibility of executing the parasitic tactic in the absence of bourgeois males.

MATERIAL AND METHODS

General procedures and sampling

Both immature (Exp. 1) and sneaker (Exp. 2) males of *Salaria pavo* were captured during low tide in the intertidal area of Ilha da Culatra, a barrier island situated in Ria Formosa Nature Park (36°59'N, 7°51'W), in the South coast of Portugal. Immature fish were captured prior to the beginning of the breeding season and sneakers were collected during the breeding season. Immature fish were identified as individuals without SSC, that presented a male genital papilla and that did not release milt when pressing the ventro-lateral surface of the abdomen. According to the same ongoing mark-recapture study mentioned above, immature males that in the months of April and May present the same morphological characteristics as those used in Exp. 1 can reproduce in the future as

sneakers or directly as nest-holders. Furthermore, of this group of individuals those who later reproduced as sneakers did not differ from those who became directly nest-holders, in either size of SSC relative to body size (head crest $t_{31}=0.93$, $P=0.36$; anal gland $t_{31}=0.70$, $P=0.49$) or body size itself ($t_{31}=1.4$, $P=0.17$; body size measured as total length was however truncated to the left of 5.2cm which is the minimum size of marked individuals in the mark-recapture study and corresponds to the percentile 9 of the total length of subjects of the experiment).

Sneaker males were morphologically identical to immature males but released milt when checked for. In previous studies, where fish were captured and dissected for gonadal inspection, we have verified that this is an appropriate criterion to distinguish sneakers from immature males (Carneiro *et al.*, 2003). After capture, fish were transported to the aquarium facilities of the Instituto Superior de Psicologia Aplicada in Lisbon and housed in a stock aquarium (Length: 120cm x width: 40cm x height: 50cm) with sand and shelters. The light/dark cycle and the temperature in the stock tank, as well as in the treatment aquaria, were 13L:11D and $24.5\pm 1^{\circ}\text{C}$. Fish were fed once a day with chopped mussel's flesh. Fish remained in the stock aquarium for at least 1 week before being assigned to a treatment aquarium. Just before the start of the experiment fish were anaesthetized with MS-222 (PHARMAQ Ltd, dilution 1:10000) and measured for total (TL) and standard length, and maximum head height. These morphological variables were measured with a 0.1mm-precision calliper. The length and width of the anal gland and of the genital papilla were measured with a 20x magnifier to the nearest 0.1mm (SZ-PT Olympus stereoscope). The body weight was measured on a 0.001g-precision scale. Fish were introduced in treatment aquaria immediately after recovering from anaesthesia.

In the last day of the experiment, after the last observation fish were sacrificed with an overdose of MS-222 (dilution 3:10000). Blood samples were collected immediately after the anaesthesia, from the caudal vein, with a 1 ml heparinised syringe and a 25 gauge needle. The blood sample was centrifuged at 3000 rpm for 10 min and the plasma was stored at -20°C until further processing. The same morphological variables were measured as in the beginning of the experiments, as well as length, width and weight of testes and testicular glands, and eviscerated body weight.

Experimental treatment

Exp. 1: Influence of density and social dominance on the expression of the reproductive tactics in immature males

This experiment was conducted between mid April and May of 2003 and 2004 and individuals were subjected to the experimental treatment during 30 days. The

experimental period included about two weeks of pre-breeding season and extended another two weeks into the breeding season. Young males from which sperm can be stripped are present in the field at the start of the breeding season suggesting that the decision process of which tactic to adopt occurs either during or before this early period. Thus, the period in which the experiment was carried out was selected in order to maximize the probability of including a potential sensitive period and a period during which the reproductive tactics can be expressed and identified. The one month duration of the experimental period allowed the subjects, (immature males at the start of the experiment) to express their life-history decisions regarding the current breeding season. This decision could be to differentiate SSC and breed as a nest-holder, to invest only in spermatogenesis and breed as a sneaker male, or to post-pone breeding and allocate resources into growing until the next breeding season.

Immature fish were introduced in their treatment aquaria simultaneously, just after the morphological measurements. The high density treatment consisted of 6 immature males per aquarium and the low density treatment of 3 immature males per aquarium (length: 50cm x width: 24cm x height: 30cm). The aquaria, with sandy substrate, had 1 shelter per fish. These shelters were made of grey PVC tubes (length: 10cm, opening diameter: 2cm) with one end closed by an opaque dark plastic film. The PVC tubes were distributed perpendicular to the main axis of the aquaria with a distance of 6.5cm between them, in the high density treatment, and of 13cm, in the low density treatment. These tubes were placed with the opening facing the observer to facilitate the behavioural observations. In each replicate fish were matched for size resulting in a coefficient of variation of the individuals TL in each aquarium of less than 5%. Five replicates of high density and 9 of low density treatments were conducted.

Exp. 2: Influence of nest-holder male removal on the expression of the reproductive tactics in sneakers

This experiment was conducted during the peak of the breeding season between June to August of the years 2003 and 2004. Experimental procedure lasted two weeks and was divided in two different but continuous periods. In the first period of 7 days both treatments consisted in maintaining one nest-holder, one female and one sneaker in each tank (replicate). In the second period the 'control' treatment was maintained unaltered but the nest-holder was removed from the 'male removal' treatment, leaving only one sneaker and one female in each tank during the remaining 7 days of the experiment. The duration of the experiment was selected based on the results of previous studies conducted on a related species, the *Parablennius parvicornis*, which demonstrated a differential growth of SSC within one week after androgen implantation (Oliveira *et al.*, 2001b).

The treatment aquaria had the same dimensions and conditions as the aquaria of the first experiment. Three shelters were placed in each tank: two PVC tubes, as the ones just described for the first experiment but larger (length=15cm; diameter=4cm) and one half of a small flowerpot. The two PVC tubes were placed in opposite corners of the aquaria. One of the tubes was occupied by a nest-holder male guarding eggs. The positions of the different shelters were randomized between aquaria.

After the morphological measurements described above in 'General procedures and sampling', one sneaker was placed in each aquarium. When sneakers were added to the aquaria, one nest-holder male was already established in each aquarium and the females, that were moved from aquarium to aquarium, had already laid eggs in their nests. During the whole of the experimental period females were rotated daily between aquaria to simulate the natural environment of a promiscuous system in which nest-holders receive the visits of many females. Ten replicates of the control treatment and 10 of the 'male removal' treatment were conducted.

Behavioural observations

In both experiments behavioural observations started the day after the individuals were introduced in the treatment aquaria. Each morning focal observations (Martin and Bateson, 1993) of 20 min each were conducted in each aquarium. Observations started 30 min after the lights went on, from 9:30 to 14:00 approximately, and the time of the start of the observations of the different aquaria was randomized.

For the first experiment, a total of 28 observations per replicate were conducted, during the 30 days of the experiment. For the second experiment 12 observations per replicate were conducted, 6 before and 6 after the removal of the nest-holder male from the 'male removal' treatment.

During focal observations, the behaviours of all the individuals present in the aquarium were registered and subsequently analyzed using The Observer software (Noldus Information Technology, Wageningen, The Netherlands). Agonistic behaviours: charges, bites, chases and flights were treated as events for which frequencies were registered; while dorsal fin erection and ventral presentation were treated as states for which durations, as well as frequencies were recorded. Ventral presentation, a submissive behaviour, was defined as the individual laying on its flanks immobile or swimming very slowly while presenting its abdomen to another fish. A nearly identical behaviour, dorsal presentation, has been described for another blenny as inhibiting aggressive behaviour (Santos and Barreiros, 1993).

The outcome of the agonistic interactions was used to assess social dominance. Individuals were classified as dominants, intermediates or subordinates according to a simulation statistic procedure that allows the identification of individuals that won or lost more agonistic interactions within their social groups than would have been predicted by chance (Almada and Oliveira, 1997). If the number of victories was higher than expected by random distribution (one-sided tests) individuals were considered dominants; individuals with a number of defeats higher than expected were considered subordinates; and individuals whose defeats and victories were not different from random expectations were classified of intermediate status. Only 5 of the 57 individuals tested in Exp. 1 were classified as intermediates, 2 in the low density treatment and 3 in the high density treatment. Because the use of such small groups would compromise the statistical analyses, the intermediate group was excluded. A social interaction was considered to occur when any behaviour was directed to an individual, irrespectively of the reaction of the receptor of the behaviour. The end of the interaction was determined as the absence of behaviour of the intervenients for more than 3 seconds. Defeats were determined by fleeing behaviour or ventral presentation.

Courtship behaviour was also registered as a state and two types were considered: male-like or female-like courtship displays. Male courtship display consists of jerking the body laterally while protruding the head from the shelter/nest, other courtship behaviours like zigzag swimming, in which the male leaves the shelter and circles the female, are less frequent in this population. The female courtship display is characterized by rapid movement of the opercula, fanning the pectoral fins and the waving of the dorsal and caudal fins while the body is usually vertical with the belly exposed to the male (Patzner *et al.*, 1986). Courtship displays were only analyzed as such if they occurred in a courtship context, namely when one of the intervenients was in the nest. The context is important because, in this species, female-like courtship behaviour may also be expressed in agonistic context as a submissive behaviour. The position of the individuals in relation to the nest was also registered as a state. Detailed behavioural descriptions for this species can be found in Fishelson (1963), Patzner *et al.* (1986) and Uiblein (1989).

Steroid extraction and quantification

Plasma samples were extracted for the free, sulphate and glucoronide steroid phases for the subsequent quantification of KT, T and cortisol using the methods described in Canário and Scott (1989) and Scott and Canário (1992). Steroid residues were dissolved in 1mL phosphate buffer 0.1 M, pH 7.6, containing gelatine (1 g / L), and stored again at -20°C until radioimmunoassay (RIA) was conducted. The 3 extracted steroid phases were

mixed in equal parts and RIAs were carried out to quantify the hormonal concentration in the mix. The antibody for KT was kindly donated by D. E. Kime and its cross-reactivities were described in Kime and Manning (1982). The antibody for T was purchased from Research Diagnostics (RDI-TRK2T2, Concord, USA), and the cross-reactivities provided by the supplier are 16% for 5 α -Dihydrotestosterone, 1% for androstenediol, 0.4% for androstenione, <0.1% for androsterone, <0.1% for dehydroepiandrosterone, <0.1% for progesterone, 0.001% for estradiol and 0.001% for cortisol/pregnenolone. For the quantification of cortisol, the antibody supplied by Interchim (20-CR50, Montluçon, France) was used and its cross-reactivities are 5.7% for 11-desoxycortisol, 3.3% for corticosterone and less than 0.7% for cortisone, according to the supplier. The analyses of each hormone were conducted in a single assay per hormone and the intra-assay coefficients of variation were 3.8% for KT, 5.9% for T and 1.3% for cortisol.

Because of the small size of our study species, the plasma volume obtained was occasionally lower than optimal for the radio-immunoassay. With lower plasma volumes, an increase in variation of the quantitated androgen levels is expected and *a posteriori* outlier tests detected, in fact, upper outlier values (exceeding 3rd quartile + 1.5 interquartile range) of androgen levels in plasma samples with volumes smaller than 10 μ L. Therefore, plasma volumes lower than 10 μ L were excluded from the analyses.

General statistical procedures

General linear mixed model analysis was used to detect the effects of density and social dominance in Exp. 1. The goal of this experiment was to test the effects of social dominance, an individual level variable, and of density, a group (replicate) level variable. Because individuals are nested in replicates, social dominance values within each replicate are not independent of each other. General linear mixed models allow the analyses of these different organizational levels and compose appropriate degrees of freedom and consequently significance levels, under restricted maximum likelihood estimation. Both social dominance and density were introduced as fixed and replicate as random effects. A covariate, total length, was also introduced as a fixed effect in order to control the effect of the density and dominance on SSC for body size. Additional covariates as gonad area and eviscerated body weight were used to analyse the effects on testicular gland area and gonad weight, respectively.

Additionally, univariate ANCOVA with the same covariates mentioned above and repeated measures ANOVA, were conducted on Exp. 2 data. These analyses were run on SPSS

14.0 (statistical package for the social sciences) for Windows (Chicago: SPSS Inc.). Significance levels were 0.05 and statistical tests were two-sided.

Post-hoc power analysis was conducted using G*power 3.0.10. for Windows XP (Faul *et al.*, 2007). The power ($1-\beta$) of ANOVA tests to detect small, medium and large effect sizes was calculated on F-tests section according to the significance level stipulated, the sample size and the characteristics of experimental design of each test. Conventional effect size values tested were based on Cohen (1977, 1988) and for ANOVA are considered respectively large, medium and small if f is 0.4, 0.25 or 0.1.

For the computation of the areas of morphological variables, the anal gland was considered as having a triangular shape, the genital papilla, a rectangular shape and both the testes and the testicular glands an elliptical shape. The relative size of the testicular gland (testicular gland index- TGI) was calculated as the sum of the area of both testicular glands/the sum of the area of both testes. The gonadosomatic index (GSI) was calculated as the weight of both testes/eviscerated body weight and the condition factor (K_{TL}) as $[\text{body weight}/(\text{total length})^3] \times 100$.

Variables that did not meet the assumptions of parametric statistics were either logarithm (log) or square root transformed, as it will be mentioned in the results section.

Behavioural variables, as courtship displays, which presented very low frequencies were analyzed by simulation statistics using the software ACTUS (Analysis of Contingency Tables Using Simulation, Estabrook and Estabrook, 1989). Goodness-of-fit simulation tests were conducted using ADERSSIM software (Almada and Oliveira, 1997).

Assessment of mediator and moderator role of steroid hormones on the effect of social dominance on tactic expression

To assess possible mediation and moderation roles of hormones or body condition on the effect of social dominance on the expression of the reproductive tactic, multiple regression analysis was used (MacKinnon *et al.*, 2002; Frazier *et al.*, 2004). Social dominance was operationalized as a continuous variable to be entered in the regression model by using the dominance index proposed by Barlow and Ballin (1976):

$$\text{dominance index} = [\text{number of victories}/(\text{number of victories} + \text{number of defeats})]$$

The use of this second method to assess social dominance (in addition to the simulation procedure described above) was necessary for using different statistical procedures and is justified by the fact that the two methods are mutually consistent, that is individuals

classified as dominants or subordinates by the simulation statistics procedure differ significantly in their dominance indexes [values of dominance index computed for dominant (average=0.90), intermediate (average=0.49) and subordinate (average=0.17) individuals; ANCOVA: $F_{1,54}=172.08$, $P<0.001$].

A mediation effect (Figure 1A and B) is the effect of an independent variable on an outcome variable that is accomplished by a third variable, the mediator. The independent variable causes the mediator which in turn determines the value of the outcome variable. A mediation effect is present when the following conditions are met: 1. the independent variable (X) predicts the outcome variable (Y) and thus $Y=a+bX$, where $b \neq 0$ (Figure 1A, b is the unstandardized coefficient of regression and represents the total effect of X on Y); 2. X predicts the mediator (Me) and thus $Me=a'+cX$, where $c \neq 0$ (Figure 1B); and 3. Me predicts Y, when controlling for X, which implies that $Y=a''+b'X+dMe$, where $d \neq 0$ (Figure 1B, b' represents the direct effect of X on Y). The indirect or mediated effect, i.e. the effect of X on Y via Me, is represented by $c*d$ (Figure 1B) and as a consequence of the above conditions $c*d \neq 0$.

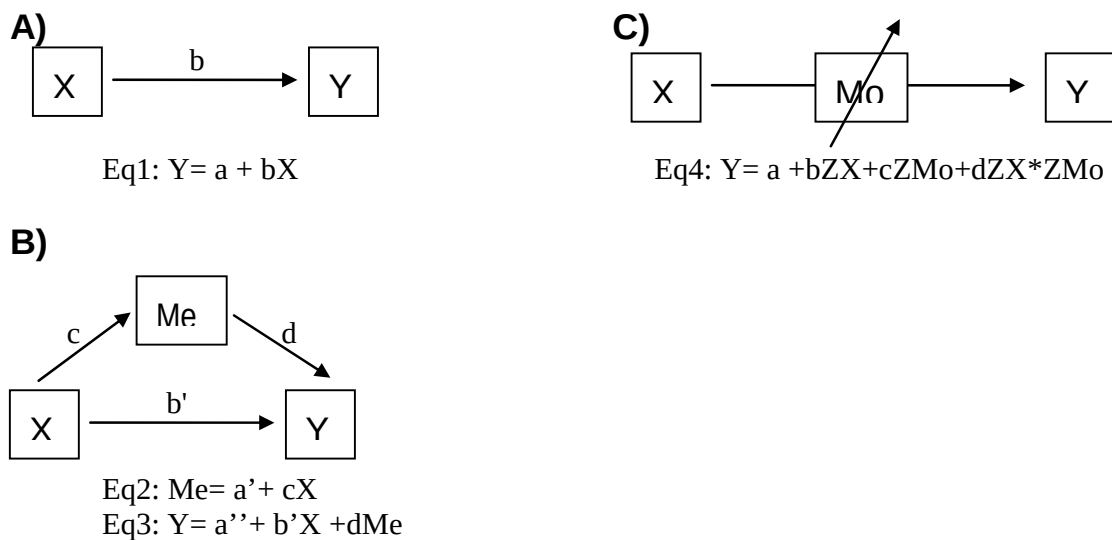


Figure 1. Mediation (A and B) and moderation effect (C). A) the total effect (b, the unstandardized coefficient of regression from equation 1) of X on Y. B) the direct effect (b', the unstandardized coefficient of regression from equation 3) of X on Y and the indirect effect ($c*d$, the product of the regression coefficients c and d, from equations 2 and 3 respectively). C) The moderation effect in which Mo varies the effect of X on Y as represented by the effect of the interaction of X and Mo (d from equation 4).

Our analysis of the mediation effects follows the recommendations of MacKinnon *et al.* (2002). Because in this study we intend to test the effect of the mediator variables on a set

of outcome variables, it is more economical to first test $c \neq 0$ (Figure 1B) through simple regression analysis and subsequently use a test of collapsibility (Cloog *et al.*, 1992) to analyze the significance of the indirect effect (the mediation). This method is based on the fact that the indirect effect, $c*d$, is equivalent to $b-b'$ (Figure 1A and B). The method uses the statistic test $(b-b')/[\rho_{XMe} \sigma_{b'}]$, where ρ_{XMe} is the correlation coefficient between X and Me and $\sigma_{b'}$ is the standard error of b' , and this statistic follows a t_{n-3} distribution.

A moderator effect is the interaction between an independent (X) and a moderator (Mo) variable. The effect of X on Y, the outcome variable, depends on the level of Mo. Mo does not cause Y but it changes the relation between X and Y (Figure 1C). To test a simple moderator effect all the variables except Y should be standardized in order to diminish multicollinearity between X and Mo. The interaction term is usually the product $X*Mo$. The effect is tested by a hierarchical multiple regression in which both X and Mo are introduced in the model in a first step, in general simultaneously, and the interaction term is added in the second step of the analysis. The moderator effect is accepted if the fit of the data improves with the addition of the interaction term, *i.e.* if $F\text{-change} \neq 0$ (Frazier *et al.*, 2004).

Ethical note

The number of individuals captured and sacrificed was kept to a minimum and biological material collection for complementary studies was maximized. After capture fish were always anaesthetized before manipulation and recovered from anaesthesia in a small isolation aquarium. In the first experiment fish were introduced in the experimental aquaria together with other individuals and were monitored for the first hours. In Exp. 1 individuals were matched for size within each replicate in order to limit the intensity of the aggression. In Exp. 2 vigilance was more strict due to the introduction of a small sneaker into an aquaria with a nest-holder already established. In some cases it was necessary to insert a transparent partition after the introduction of the sneaker to protect him from the male and female aggression. One replicate was aborted in the first experimental day, due to the extreme aggression of the nest-holder.

The experimental procedure followed the Portuguese legislation for the use of animals in experimental research.

RESULTS

Exp. 1: Effect of social dominance and density on tactic expression in immature males

Within the 5 high density replicates, 3 presented 1 dominant individual and 2 replicates presented 2 dominant ones; within the 9 low density replicates, 7 presented only 1 dominant individual and in 2 replicates there were 2 dominant individuals. The analyzes of frequency of the different social dominance categories (dominant vs. intermediate vs. subordinate males) in the 2 density treatments, through simulated contingency tables, did not reveal any difference between treatments in terms of proportion of individuals with different social status (ACTUS simulation statistic, contingency table of density treatment x social status: $\chi^2_{2,57} = 1.995$; in 1000 simulation 389 were equal or exceeded the calculated chi-square). Dominant males occupied preferentially 1 (47% of the males), 2 (35% of the males) or 3 nests (17% of the males, all in the high density treatment), as tested on ADERSSIM software by chi-square goodness-of-fit tests in which the number of seconds spent inside the nests were compared to values expected from a uniform distribution of each dominant male by the different nests.

Despite the fact that in each replicate, individuals were matched for size, the individuals that became dominant within each replicate were already significantly larger (TL) at the beginning of the experiment. No other differences (in head crest or anal gland) were detected at the beginning of the experiment between individuals that would later become dominant and subordinate. At the end of the experiment dominant in comparison to subordinate males showed larger head crests and anal glands relative to body size, but no difference was detected in the size of genital papilla. Dominant males had larger testicular glands than subordinates relative to the area of testes but the weight of testes controlled for eviscerated body weight did not differ between dominants and subordinates (Table 1).

In summary, dominant and subordinate males differed in all the morphological traits that distinguish the bourgeois from the parasitic tactic except in the size of testes with dominant individuals approximating the bourgeois phenotype. No effect of density was detected besides a tendency for a larger development of the anal gland in the low density treatment (Table 1). Nevertheless the low number of groups (n=5 vs n=9) pertains a high statistical test power for both density and the interaction density x dominance effects.

It should be mentioned that most individuals were still sexually immature (with very low GSI: average=0.18% $\pm$ 0.042%; N=57) at the end of the experiment and that there were no

individuals with SSC fully developed as found in the reproducing nest-holders in nature. At the end of the experiment, 4 individuals expressed the sneaker tactic (identified by the production of large amounts of sperm as could be perceived by the release of milt when pressing the abdomen and by the large testes with only a vestigial testicular gland). These 4 sneakers were all subordinate males.

The behaviour of dominant males was similar to the behaviour of nest-holder males in that they occupied nests for a longer period of time and participated in more agonistic interactions than subordinates, which was interpreted as nest defence and will be referred to as nesting behaviour. Furthermore, density and dominance status interacted to influence both participation in agonistic interactions and time in nest, the latter presenting only a marginally non-significant interaction effect. The positive effect of social dominance on time spent in nest was stronger in the low density treatment but the effect on the number of agonistic interactions was stronger in the high density treatment (Table 1). The frequency of female-like courtship, that sneakers usually display when approaching the nests, was however very low and did not differ between dominant and subordinate males (ACTUS simulation statistic: $\chi^2_{1,52} = 0.85$; in 1000 simulation 377 were equal or exceeded the calculated chi-square). The single effect of density on behaviour of the immature males was not significant (Table 1).

Dominant individuals presented higher circulating levels of KT and T but not cortisol and there was no effect of density on steroid circulating levels (Table 1).

Possible mediation of the effect of social dominance on the expression of the different traits that characterize each tactic, by steroids or body condition (condition factor, K_{TL}) was tested. Social dominance, the independent variable measured as dominance index and therefore as a continuous variable, could predict the hypothesized mediators, androgen levels ($\log KT = 0.04 + 0.36Di$, $r^2 = 0.24$, $p = 0.01$, $N = 30$; $\log T = 0.72 + 0.46Di$, $r^2 = 0.30$, $p < 0.01$, $N = 30$). The results of the collapsibility test (Table 2), show the significance of the decrease of the effect of social dominance on the outcome variables by the introduction of the mediator (total effect – direct effect, $b - b'$, Figure 1), which is equivalent to the mediated or indirect effect ($c \cdot b$, Figure 1). These results show that only the mediated effect of social dominance on head crest development through KT circulating levels was significant. Neither cortisol plasma levels ($\log \text{cortisol} = 0.82 + 0.22Di$, $r^2 = 0.03$, $p = 0.35$, $N = 30$) nor body condition ($K_{TL} = 0.01 + 0.00Di$, $r^2 = 0.40$, $p = 0.10$, $N = 57$) were associated with social dominance, therefore a possible mediation effect is excluded *a priori*.

Table 1. Results of experiment 1. General linear mixed model analysis of the effects of density and social dominance on the morphological, gonadal, endocrinological and behavioural variables of immature males of the peacock blenny. Social dominance, density and the covariate, total length, were introduced as fixed effects, and replicate as random effects. *, ** and *** indicate significant F-test values at the significance levels 0.05, 0.01 and 0.001 respectively; 1 and 2 represent marginally non-significant F-test values, respectively P = 0.06 and P = 0.09 (two-sided tests).

	Density	Dominance status	Estimated difference (dominant males – subordinate males)	Density x Dominance status
Pre-experiment values	TL	$F_{1, 11.932}=0.035$	$F_{1, 36.004}=21.160^{***}$	$F_{1, 36.004}=0.658$
	Maximum head height	$F_{1, 11.152}=0.583$	$F_{1, 40.896}=2.990^2$	$F_{1, 37.506}=0.283$
	Anal gland width	$F_{1, 5.407}=2.701$	$F_{1, 24.818}=0.579$	$F_{1, 22.945}=2.849$
	Maximum head height	$F_{1, 10.863}=0.358$	$F_{1, 41.755}=13.559^{**}$	$F_{1, 35.677}=0.100$
Post-experiment values	Anal gland width	$F_{1, 11.766}=3.374^2$	$F_{1, 43.737}=3.874^1$	$F_{1, 37.689}=0.001$
	Genital papilla length	$F_{1, 9.331}=1.119$	$F_{1, 42.316}=0.482$	$F_{1, 36.033}=2.194$
	Testicular gland area	$F_{1, 10.621}=0.006$	$F_{1, 36.562}=11.052^{**}$	$F_{1, 37.637}=0.372$
	Gonad weight	$F_{1, 11.543}=0.681$	$F_{1, 43.245}=0.024$	$F_{1, 36.369}=1.548$
Experiment values	KT (log)	$F_{1, 4.397}=0.114$	$F_{1, 18.052}=6.832^*$	$F_{1, 18.052}=1.381$
	T (log)	$F_{1, 5.516}=0.152$	$F_{1, 18.057}=7.938^*$	$F_{1, 18.057}=0.970$
	F (log)	$F_{1, 8.651}=0.152$	$F_{1, 18.027}=3.166$	$F_{1, 18.057}=0.268$
	Nr. of interactions (square root of) Time in nest	$F_{1, 11.532}=0.030$	$F_{1, 36.125}=36.583^{***}$	$F_{1, 36.125}=10.709^{**}$
		$F_{1, 10.721}=1.174$	$F_{1, 36.048}=66.612^{***}$	$F_{1, 36.048}=3.756^1$

Table 2. Test of mediation effect of KT and T between social dominance, measured as dominance index, and the outcome variables. The outcome variables are head crest relative size=maximum head height/TL, anal gland relative size= anal gland maximum width/TL, genital papilla relative size=genital papilla length/TL, testicular gland index= Σ area of both testicular glands/ Σ area of testes, GSI (log transformed), total number of agonistic interactions in which subjects participated and total time spend in nest. Unstandardized coefficients of regression are represented by b for the regression between dominance index and each of the outcome variables and by b' for the multiple regression between dominance index and each of the outcome variables when corrected for the mediator (logKT or logT). $(b-b')/|\rho XMe \sigma b'|$ is the statistic test for collapsibility (Clogg *et al.*, 1992) which follows a t_{n-3} distribution (n=30). The significant mediating effects are represented with asterisks.

Mediator (Me)	KT (log transformed)			T (log transformed)	
Outcome (Y)	b	b'	$(b-b')/ \rho XMe \sigma b' $	b'	$(b-b')/ \rho XMe \sigma b' $
Head crest relative size	0.020	0.011	2.629**	0.015	1.151
Anal gland relative size	0.059	0.056	0.236	0.071	-0.850
Genital papilla relative size	0.020	0.017	0.472	0.016	0.567
Testicular gland index	17.665	15.108	0.533	12.457	0.953
Log(GSI)	0.070	0.076	-0.052	0.232	-1.238
Nr. of interactions	301.699	254.603	0.921	231.111	1.210
Time in nest	238.358	237.925	0.022	211.823	1.178

Additionally, possible moderator effects of cortisol and body condition (again K_{TL}) of the effect of social dominance on tactic choice were tested. When testing these potential moderators (Table 3), the introduction of an interaction factor in the multiple regression model improved the fit of the data only for the test of K_{TL} as a moderator of the effect of social dominance on SSC (Figure 2). Hence the effect of dominance on SSC was dependent on the level of K_{TL} , supporting a moderation effect. For individuals above average body condition social dominance predicted the expression of the head crest (maximum head height/TL = $0.16+0.03Di$, $r^2=0.63$, $p<0.01$, $N=17$) and of the anal gland (anal gland width/TL= $0.13+0.11Di$, $r^2=0.47$, $p<0.01$, $N=17$) but for males with body condition below the average,

Table 3. Test of moderation effect of cortisol circulating levels and body condition (measured as condition factor) on the effect of social dominance, measured as dominance index, on the outcome variables: head crest relative size=maximum head height/TL, anal gland relative size= anal gland maximum width/TL, genital papilla relative size=genital papilla length/TL, testicular gland index= Σ area of both testicular glands/ Σ area of testes, GSI (log transformed), total number of agonistic interactions in which subjects participated and total time spend in nest, measured in minutes. r^2_{adj} represents the adjusted coefficient of determination of the regression of each of the outcome variables on dominance index and each of the moderators (plasma cortisol or body condition = [body weight/(total length)³] $\times$ 100), and $r^2_{adj_{int}}$ includes also the interaction term (dominance index $\times$ moderator) as a independent variable. The independent terms of the regression are all normalized. The significant change in the fit of the data is given by F_{change} and is represented by as asterisk.

Moderator (Mo)	cortisol			body condition		
	r^2_{adj}	$r^2_{adj_{int}}$	F_{change}	r^2_{adj}	$r^2_{adj_{int}}$	F_{change}
Head crest relative size	0.326	0.330	1.147	0.345	0.422	8.036**
Anal gland relative size	0.234	0.252	1.672	0.143	0.253	8.973**
Genital papilla relative size	0.055	0.035	0.446	0.015	0.053	3.193
Testicular gland index	0.269	0.242	0.043	0.112	0.116	1.246
Log(GSI)	0.081	0.050	0.133	0.133	0.119	0.140
Nr. of interactions	0.230	0.275	2.652	0.144	0.139	0.710
Time in nest	0.596	0.595	0.950	0.491	0.482	0.102

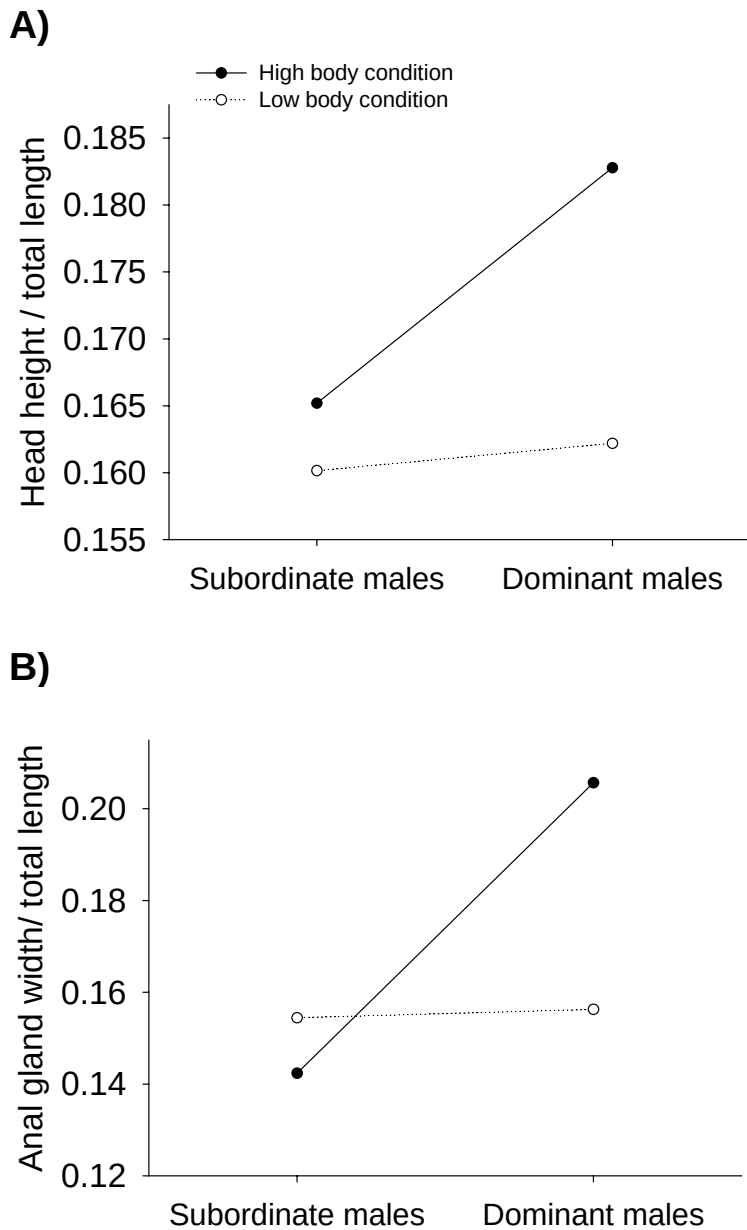


Figure 2. Effect of social dominance on SSC expression: A) relative size of head crest and B) relative size of anal gland, as moderated by body condition ($K_{TL} = [\text{body weight}/(\text{total length})^3] \times 100$). Dominant and subordinate males are defined here as average dominance index $+1\text{SE}$ and -1SE , respectively. The same is true for the categorization of body condition.

No moderation effect of cortisol was to influence the effect of social dominance on the expression of any of the variables indicative of the reproductive tactic. However, cortisol was marginally negatively correlated to GSI ($r=-0.35$, $p=0.06$, $N=30$, both variables were log transformed) and both androgens were positively correlated with the number of agonistic interactions in which the subjects participated (for KT: $r=0.39$, $p=0.03$, $N=30$; for T: $r=0.45$, $p=0.01$, $N=30$, T and KT were log transformed).

Exp.2: Effect nest-holder male removal on tactic expression in sneakers

The removal of the nest-holder male had no effect on the development of the sneakers in spite the exclusion of male-male competition. The effect of treatment on the size of SSC controlled for body size did not differ between the start and the end of the experiment (Table 4) with subjects from both treatments maintaining the typical morphology of a sneaker male. The removal of the nest-holder males did neither affect the size of the testes of the sneakers nor their testicular gland size when corrected for eviscerated weight and testes area respectively (Table 4).

Sneakers engaged in nesting behaviour more intensively after removal of nest-holder male as suggested by a marginally non-significant interaction effect between time and treatment on time spent inside the nests and number of agonistic interactions (Table 4) respectively. However, the duration of courtship behaviour directed to the female and received from the female did not vary significantly with the removal of nest-holder male (Table 4). Due to the very low number of blood samples that could be obtained it was not possible to analyse the effect of nest-holder removal on the level of circulating steroids in sneakers. However, and as in Exp. 1, T was correlated with the total number of agonistic interactions in which the individual participated ($r=0.82$, $p=0.02$, $N=7$; T was log transformed).

The second experiment was designed with a sample size ($N=20$) within the common range of sample sizes used in studies of phenotypic plasticity in vertebrates (e.g., Immler *et al.*, 2004) and in endocrine studies of the studied species. These earlier studies have detected significant differences in sex steroids between nest-holder and sneaker males (e.g. Gonçalves *et al.*, 2008). However, the power of the statistical tests used in our study turned out to be low even for a large effect of experimental treatment on the development of SSC and on behavioural variables (for RM ANOVA, large effect: $f=0.4$, $1-\beta=0.4$; medium effect $f=0.25$, $1-\beta=0.2$), despite detection of some significant differences. The analysis of gonad weight and testicular gland development presented high power to detect a large effect (for ANCOVA: $f=0.4$, $1-\beta=0.8$) but for a medium effect size, the power dropped again to low values (for ANCOVA: $f=0.25$, $1-\beta=0.4$).

Table 4. Results of experiment 2. Univariate ANOVA results of the effect of treatment (male removal vs. control) on the morphological and gonadal variables of sneakers of the peacock blenny. Repeated measures ANOVA of the effects of treatment on behaviour. * indicates significant F-test values at the significance levels 0.05 and ¹ indicates p=0.08 (two-sided tests). Only the relevant results of the Repeated measures ANOVA, the interaction treatment x time, are presented but main effects were tested simultaneously.

		Treatment: male removal vs control
Pre-experiment values	Maximum head height	$F_{1, 17}=0.063$
	Anal gland width	$F_{1, 17}=0.004$
Post-experiment values	Maximum head height	$F_{1, 17}=0.045$
	Anal gland width	$F_{1, 17}=0.525$
	Genital papilla length	$F_{1, 17}=0.201$
	Testicular gland area	$F_{1, 17}=0.159$
	Gonad weight	$F_{1, 17}=0.092$
		Interaction treatment x time
Experiment values	Nr. of interactions (square root of)	$F_{1, 18}=4.54^*$
	Time in nest	$F_{1, 18}=3.33^1$
	Courtship to female	$F_{1, 18}=0.385$
	Courtship from female	$F_{1, 18}=0.409$

DISCUSSION

Exp. 1: Effect of social dominance and density on tactic expression in immature males

In the first experiment in which size matched immature males were grouped in different densities, the emerging social hierarchy predicted the morphological development, androgen circulating levels and nesting behaviour of immature males but not their gonadal maturity (GSI). Initial relative body size was associated with social status achieved by the end of the experiment. Body size difference is an important determinant of social dominance in fish (Whiteman and Côté, 2004; Jacob *et al.*, 2007), therefore the dominance structure was most likely a consequence of experimental procedure. Dominant males developed towards a nest-holder phenotype in that they expressed higher plasma levels of androgens, larger SSC and testicular glands, and more nesting behaviour (*i.e.* they spent more time in nests and participated in more agonistic interactions) than subordinate males. Typical behaviour of nest-holder males involves establishing and defending a nest, for which a dominant status is advantageous. Other studies have reported an association of social dominance with expression of different reproductive phenotypes, namely in a cichlid species, *Haplochromis burtoni* (Francis *et al.*, 1993) and in the sex-changing fishes: the bluebanded goby, *Lythrypnus dalli* (Reavis and Grober, 1999) and the bluehead wrasse, *Thalassoma bifasciatum* (Godwin *et al.*, 1996). In addition body size has been confirmed to contribute to the choice of the reproductive tactics in different life stages of diverse species (*e.g.* the beetle *Onthophagus Taurus*, Emlen, 1997; Emlen and Nijhout, 2001; the european earwings, *Forficula auricularia*, Tomkins, 1999). However, the influence of social dominance on the expression of a reproductive tactic has not been addressed specifically as in the present study of the peacock blenny. Nevertheless, because social dominance was associated with relative body size and despite the effort of matching individuals per size (the coefficient of variation was lower than 5% within each group), the effects of social dominance and of relative body size were not possible to dissociate. In this species, even small initial differences in relative body size seem to determine the establishment of male dominance, despite studies in other fish species reporting that larger individuals tend to win contests only when body size differences are substantial (Prenter *et al.*, 2008).

Social dominance could predict androgen levels and SSC in immature males and these data fitted a mediator role of KT for the effect of social dominance on the development of the head crest. Therefore the data support the hypothesis that higher dominance status will promote higher KT levels, which in turn will facilitate the development of the head

crest in immature males. It had already been demonstrated that the administration of KT promotes development of SSC, namely of the anal gland, in sneakers of the peacock blenny (Oliveira *et al.*, 2001b), however, and against expectations, in this experiment we could not detect a similar effect on the development of the anal gland of immature males of the same species. The analysis of mediation effects, as all path analyses, requires high sample sizes. Data from only 30 subjects were available for this analysis and that may have compromised the power of the test to detect an effect that we expected to be small given the incomplete development of SSC observed.

In addition, a moderation effect of body condition was detected on the expression of SSC, that is, the influence of social dominance on SSC's development was differentiated according to body condition: When body condition was high, more dominant males presented larger SSC than subordinates. But when body condition was low, the expression of SSC was compromised and head crests and anal glands were not dependent on social dominance. One could expect that the more dominant males would have monopolized food resources and inhibit feeding of subordinate males (Koebele, 1985). However, even if such monopolization of food resources occurred in our experiment, no consequences were detected in body condition since body condition was unrelated to social dominance. Therefore, the data do not support an effect of social dominance on SSC expression through the mediation of body condition but rather support a moderator effect of body condition, which renders dominance a condition not sufficient for the expression of the bourgeois tactic, at this stage.

In addition, our data did not support a simple mediation role of cortisol on the effect of social dominance on reproductive tactic expression since cortisol was independent of social dominance, nor a moderator effect of this hormone.

Despite the fact that the experiment has ended during the breeding season, at the end of the experiment only four individuals had reached sexual maturity and none had fully developed SSC. These four mature males were all subordinates and all expressed the sneaker tactic (did not present SSC and released milt when pressing the ventro-lateral surface of the abdomen). Thus, within the subordinate group, which presented the less developed SSC, both sneakers and immature males could be found; while the dominant group was constituted by males that, despite the fact of being immature, showed the largest SSC and thus developed towards a bourgeois phenotype. Although hormonal levels of sneakers (of which a distinctive feature is a larger GSI) could not be compared with those of other groups due to low sample size, GSI was marginally inversely correlated with circulating levels of cortisol. Hence, in this experiment the onset of

reproduction was associated with lower levels of cortisol but not associated to androgen levels or social dominance. This result is in accordance with previous studies in pubertal male common carp, *Cyprinus carpio*, showing that cortisol retards gonad development (Consten *et al.*, 2002; Goos and Consten, 2002). The expression of different reproductive tactics may therefore be envisioned as a combination of two independent factors: social dominance which determines the expression of SSC through the mediation of androgens, namely KT; and the onset of sexual maturation, hypothetically influenced by levels of cortisol. This hypothesis would fit our data and explain the morphological types found in nature. It would predict that dominant individuals would develop SSC and mature as soon as cortisol levels are sufficiently low to allow this. It would also predict that subordinate males would restrict growth of SSC and, depending on cortisol levels either reproduce as sneakers or postpone reproduction. Additionally, captivity might have increased stress levels and consequently cortisol levels of the experimental subjects, thus leading to a lower number of mature individuals at the end of the experiment than expected.

In nature, immature males preferentially occupy areas of hard substrate with crevices less optimal for nest establishment. In these areas sneakers and nest-holders occur less frequently (T. Fagundes and R.F. Oliveira, unpublished data). Our experiment simulated such a social environment and a translation of the results obtained to field conditions suggests that dominant immature males will develop SSC faster and possibly migrate to reproductive areas (where nest-holders and sneakers are found preferentially). On the other hand, the young individuals present in reproductive areas will be subjected to a strong dominance from larger nest-holder males which will inhibit the development of the traits typical of nest-holders allowing sneakers to continue to use a female-mimicking tactic. Although, according to our data social dominance did affect the development of SSC, we were not able to determine an environmental cue for the onset of reproduction.

Density is another characteristic of the social environment known to affect the expression of alternative tactics in other species (in the mite *Sancassania berlesei* density at rearing affects the choice of male reproductive tactic, *e.g.*, Radwan *et al.*, 2002; Tomkins *et al.*, 2004). In our first experiment the effect of density did not result in consistent changes as was found for social dominance, despite a tendency for males of the low density treatment to develop larger anal glands. Low density may signal weak male-male competition and thus contribute to the development of the bourgeois tactic, of which SSC are characteristic. No other effect of density was detected, but due to the low power of the tests conducted, a small to medium effect of density on tactic expression can not be excluded. In our experiment, dominant immature males defended 1 or 2 nests more intensively and chased other males away from all other nests in the aquarium. In the field,

nests sites are aggregated having a short distance between them (similar to the distances used in the present experiment). It is likely that such nest-site proximity may render social dominance a cue in tactic choice that is stronger than density, because it allows dominant males to control much of the resources irrespectively of the local male-to-nest ratio, which would depend on density.

Regarding behaviour, no association between KT or T and male nesting behaviour was detected. These results agree with previous data which showed that neither KT, nor T nor E2 promoted territorial and/or nesting behaviour in sneaker males (Oliveira *et al.*, 2001b; Gonçalves *et al.*, 2007). On the other hand the administration of arginine vasotocin (AVT) promoted female-like courtship behaviour in sneakers (Carneiro *et al.*, 2003), thus suggesting that other neuromodulators than androgens are playing a major role in the expression of tactic-specific reproductive behaviours. Finally, T was associated with the number of agonistic interactions in which immature males participated, in both experiments. Because our hormonal and behavioural data are purely correlative no causal effect can be inferred from these results and, in fact, theory supports a bidirectional relation between androgens and aggressive behaviour (Wingfield *et al.*, 1990; Oliveira, 2004).

In conclusion, the expression of ART in immature males reaching their first breeding season may be under control of diverse mechanisms. Our data show that high social dominance/ relative body size promotes SSC's expression, nesting behaviour, testicular gland development and elevates circulating levels of androgens (KT and T). Additionally the effect of social dominance on SSC's expression is dependent on body condition (condition factor). The data also support an androgen (KT) dependent mechanism through which social dominance could be translated in the development of one of the most prominent SSC, the head crest. No association between social dominance and gonadal development was found but rather a marginally inverse correlation between cortisol circulating levels and GSI. This dissociation between expression of SSC and sexual maturation is fundamental to the expression of the female mimicking tactic. Thus, we propose that several mechanisms, rather than a single one, determine the changes in reproductive tactics: dominance and androgens facilitating SSC's development, while cortisol might hypothetically play a role in determining the onset of sexual maturation.

Exp.2: Effect nest-holder male removal on tactic expression in sneaker males

In the second experiment, the removal of nest-holders did not induce tactic switching in sneakers despite allowing sneakers to display nesting behaviour. No significant changes

in SSC, testicular development or testicular gland were detected. The absence of an effect of the nest-holder removal may indicate that sneakers are not using social cues to switch tactics and this is supported by field data. In fact, in nature males only reproduce as sneakers in their first breeding season and all sneakers reproduce as nest-holders on their second breeding season, which suggests that this transition is probably not influenced by social cues but may be age or size dependent (T. Fagundes, D. Gonçalves, J. Saraiva and R.F. Oliveira, unpublished data). However, a long-term mark-recapture field study also shows that the large majority of individuals switch from sneakers to nest-holders outside the breeding season (T. Fagundes, D. Gonçalves, J. Saraiva and R.F. Oliveira, unpublished data) suggesting that once males adopt the parasitic tactic, they do not easily switch their reproductive mode within the same breeding season. This may be due to the fact that in this population a considerable number of floater males (non-nesting males with developed SSC) are present. Because these males are larger than sneakers they have an advantage over sneakers in potential nest takeovers. Again, field data indicate that when a nest becomes vacant during the breeding season it is taken-over either by floaters or by other nest-holders but never by a sneaker that would subsequently change tactic (T. Fagundes, M. G. Simões, D. M. Gonçalves, R.F. Oliveira, unpublished data).

Experimental duration was determined on the bases of previous studies (Oliveira *et al.*, 2001b) in which androgen manipulation affected development of SSC within one week. It is possible that a translation from environmental cues to physiological and subsequently morphological traits may require a longer time interval than a direct effect of androgen manipulation on SSC. Hence the timing, as well as the short duration of this second experiment (7 days of experimental treatment) may have prevented the full effect of the treatment to be detected.

In summary, data of the first experiment show that in immature males high social dominance/relative body size promotes the expression of the bourgeois tactic (larger SSC and testicular gland, expression of nesting behaviour and higher levels of KT and T), and support a simple mediation of KT as the mechanism translating social dominance into a typical trait of bourgeois tactic, the head crest. Moreover, the absence of an effect of social dominance on sexual maturation suggests the possibility that the expression of the reproductive tactics may be controlled by diverse mechanisms. This allows for the existence of female mimicking males that lack SSC but are nevertheless sexually mature. In the second experiment, the removal of the nest-holder male influenced nesting behaviour performed by sneakers but no other changes in behaviour, morphology or testicular characteristics were detected suggesting that the switching of sneakers to nest-

holder males is either inhibited during the breeding season or that it is not influenced by social cues, but probably age or absolute size dependent. The extent to which the social environment might induce the expression of a reproductive tactic is thus more prominent in immature males and less so in sneakers.

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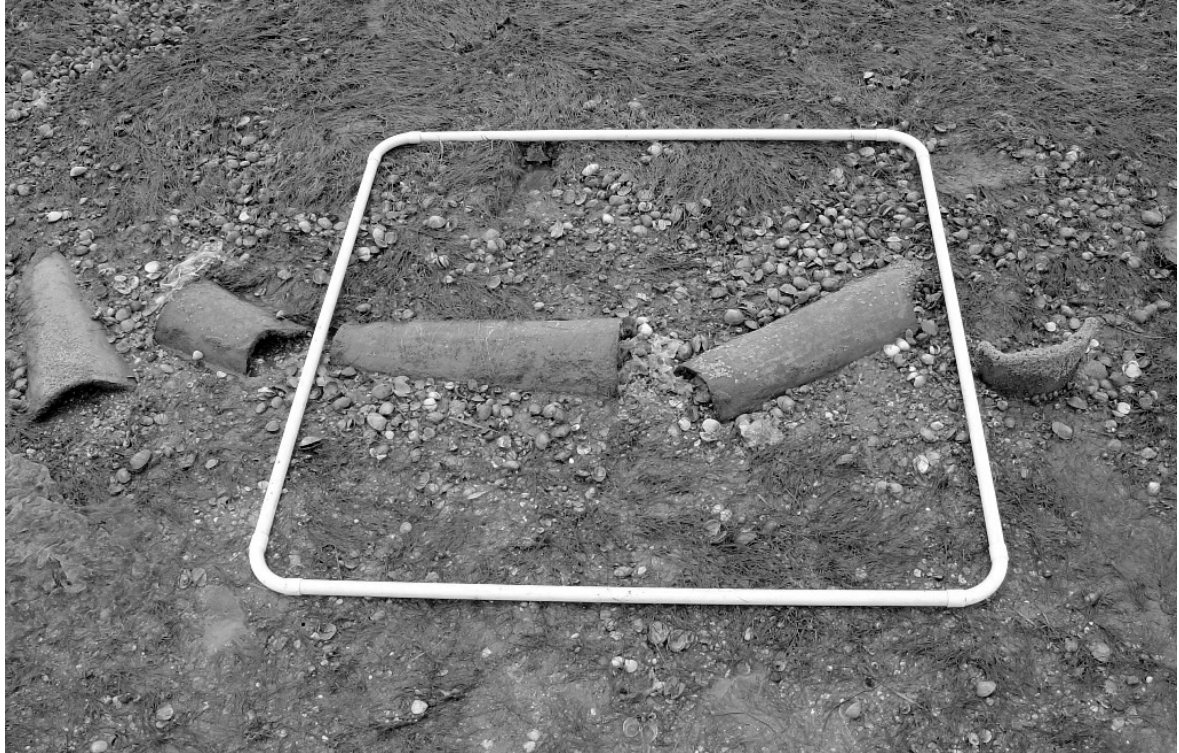
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CHAPTER V

Seasonal and tidal variation of microhabitat use by the peacock blenny, an intertidal fish with alternative reproductive tactics

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ABSTRACT

This study describes seasonal and tidal variation in the microhabitat use of a population of the peacock blenny, *Salaria pavo* (Risso, 1810) occurring in a mudflat area at Culatra island, southern Portugal. This population is characterized by the presence of different life history pathways in males related to alternative reproductive morphotypes. As a consequence, sexually active males with well-developed (M+) and with underdeveloped (M-) secondary sexual characters can be found. Most individuals were captured in areas with shelters in the form of artificial debris such as tiles and bricks. Population density was highest outside the breeding season due to recruitment of the younger classes. Larger M+ males preferentially occupied microhabitats suitable for nest establishment and had a constant distribution pattern throughout the year and the tidal cycle. Sexually active M- males (sneakers) showed a similar distribution to that of M+ males, of which they are reproductive parasites. Immature M- males, similar in body size to sneakers, occupied preferentially permanently immersed microhabitats with shelters not suitable for nesting. Juveniles also favoured the use of this microhabitat while disfavoured the use of immersed potential nests, where the density of the large M+ males was higher. Outside the breeding season, juveniles, together with females, show differences in microhabitat distribution according to tidal cycle. The occupation rate of intertidal potential nests on vegetation by juveniles was lower than expected during ebbing. Females, who favoured the use of permanently immersed shelters and of potential nests on intertidal vegetation, varied the use of microhabitat with season. The use of permanently immersed potential nests by females was higher during the breeding season than outside it and the occupation of potential nests on intertidal vegetation showed the opposite pattern. This fact suggests that areas with immersed potential nests are mainly reproductive areas, whereas intertidal areas with vegetation and potential nests are also used for other purposes. When outside the breeding season, females used this latter microhabitat more intensely during the flood but avoided it during high water. Therefore females are using other areas as high water refuges but favour the use of this microhabitat during their active period. The more stable distribution of male classes poses no particular considerations for sampling procedures. However, the more dynamic use of different microhabitats by females and juveniles according to tide or season should be accounted for.

INTRODUCTION

Intraspecific differential distribution has been demonstrated in some teleost species (*Apletodon dentatus*, Gonçalves *et al.*, 2002; *Poecilia reticulata*, Croft *et al.*, 2006), but more rarely studied in intertidal ones (e.g. *Lipophrys pholis*, *Coryphoblennius galerita*, *Gobius cobitis*, Faria and Almada, 2001; *Lipophrys pholis*, Monteiro *et al.*, 2005). Such asymmetrical distribution may be caused by different environmental and nutritional requirements, predation vulnerability, activities and social interactions and it is usually described according to sex- (reviews by Ruckstuhl and Neuhaus, 2005; Wearmouth and Sims, 2008) and size-classes (review by Werner and Gilliam, 1984).

In coastal lagoons, a diversity of microhabitats can be found in relatively confined areas. They range from sedimental zones to highly productive seagrass beds, to semi-terrestrial salt marsh vegetation. In addition to the diversity of microhabitats available, further environmental variation is provided by tidal cycles. The intertidal habitat is one of the most variable environments (Barlow, 1999) and microhabitat characteristics may change within hours. To minimize such extreme environmental variation, intertidal fish can either engage in tidal migrations thereby keeping their tidal position or stay put and use pools and emerged shelters to remain protected from desiccation as the tide recedes. In addition to coping with environmental variation, individuals may execute different activities at different tide levels and therefore, microhabitat use may vary according to tidal cycle (Gibson, 1999; 2003). They may also engage in activities that do not permit them to perform tidal migrations, as for example, territoriality and parental care (e.g. *Lipophrys pholis*, Faria and Almada, 2001). Because different activities are carried out in different periods of the year, particularly in temperate areas, seasonal differences in microhabitat distribution might be expected in addition.

Here, we characterised the microhabitat distribution of a teleost fish the peacock blenny, in a coastal lagoon population with male alternative reproductive tactics. Besides sex-classes, species with sequential alternative reproductive tactics classically present different male morphotypes that represent different life-history stages. These males differ in their reproductive, territorial, parental and even feeding activities. Morphology and body size also vary between reproductive tactics, in addition to variation in behaviour. All of these factors have been proposed as causes of differential microhabitat use (Ruckstuhl and Neuhaus, eds; Wearmouth and Sims, 2008). Hence, we analyzed the temporal and tidal dynamics of microhabitat distribution for different gender and different stages of the male life-history (Table I). This allowed discriminating differences in microhabitat use between male alternative reproductive tactics, adding a further level of resolution to the

analyses of microhabitat use. Further understanding of differential habitat use will contribute to improving sampling procedures and to better define conservation strategies for intertidal species.

MATERIALS AND METHODS

Study area

The study was conducted at Culatra Island (36°59'N, 7°51'W, Parque Natural da Ria Formosa, southern Portugal). The area is characterized by an intertidal mudflat with sediment mostly constituted by silt and sand. The formation of the flat is strongly promoted by local clam growers which continuously add layers of sand to the higher parts of the mudflat to cultivate the clams. Artificial debris, like bricks and stones, border individual areas of clam fields and are used by the study species for shelter and to establish nests. At the lower limits of the intertidal and in the subtidal areas small seagrass beds are present and additionally, algae mats composed of *Ulva* spp can be found in the area.

Study species

The peacock blenny, *Salaria pavo* (Risso, 1810) is an intertidal benthic resident species (Zander, 1986) abundant in the rocky shores of the Mediterranean sea (Fishelson, 1963) but also found on the Atlantic, from France to Maroco (Zander, 1986). Males of the population studied inhabit a coastal lagoon in Parque Natural da Ria Formosa (Portugal). They present alternative reproductive tactics which are sequential: younger males may reproduce as sneakers, while older males reproduce as nest-holders defending a nest and providing parental care to eggs. Sneakers mimic females and try to fertilize eggs when females spawn inside the nests of nest-holders (Gonçalves *et al.*, 1996). In alternative to reproducing as sneakers, young males may postpone reproduction and invest in growth (Oliveira *et al.*, 2001a). Thus, different life-history trajectories are present, in which young males may: 1. reproduce as sneakers and switch to nest-holders males subsequently; 2. postpone reproduction and reproduce later as nest-holder males; or 3. reproduce in the first breeding season already as nest-holder males.

The classification of the reproductive classes was based on body size, sex, development of secondary sexual characters and presence of milt (Table 1). Individuals with less than 4.8cm of total length were classified as juveniles, since 4.8cm corresponds to size at

maturity (unpublished data) and it is the minimum size at which males and females can reliably be visually distinguished under these sampling conditions. Males and females were classified according to the genital papilla: the genital papilla of females presents an atrium that precedes the opening of the papilla, while the male papilla is tubular and protuberant.

Table 1. Classification of the different reproductive classes and their distinctive characteristics. Sneakers and immature males can only be distinguished during the breeding season by testing milt release, outside the breeding season both classes have reduced gonads and do not produce milt therefore are not discernible. The body size of individuals captured is indicated in parenthesis (average $\pm$ Sd).

Reproductive class		Secondary sexual characters (head crest and anal gland)	Genital papilla	Body size (used for the classification of juveniles only)	Release of milt
M+	Male	YES	Male-like	Large (10.2 $\pm$ 1.7cm)	NO
M-	Sneaker*	NO	Male-like	Small (6.8 $\pm$ 1.0cm)	YES*
male	Immature male*	NO	Male-like	Small (7.4 $\pm$ 1.7cm [#])	NO
Female		NO	Female-like	Small (6.9 $\pm$ 1.5cm [#])	NO
Juvenile		NO	Not identified	$\leq$ 4.8cm of TL (4.0 $\pm$ 0.5cm)	NO

* only during the breeding season
values truncated to the left of 4.9

As mentioned above, males of *S. pavo* may reproduce as nest-holders, which are large males with secondary sexual characters (namely a head crest and an anal gland) or as sneaker which are smaller males that do not have secondary sexual characters. A third group of males is constituted by immature males who are morphological similar to sneakers but not sexually mature. Both immature males and sneakers lack secondary sexual characters but, during the breeding season, can be distinguishing by the developmental state of their testes. While immature males have vestigial and immature gonads, sneakers have large, active gonads. In practice, sneakers are identified by the release of milt when their abdomen is pressed, whereas immature males do not release milt (Carneiro *et al.*, 2003). Outside the breeding season sneakers and immature males are not discernible, since at that time, none is sexual active and both have non functional gonads. Following the categorization used in the *Parablennius parvicornis*, outside the breeding season, males will be identified only by the presence of secondary sexual characters. M- males are males who lack SSC, these may include sneakers and immature

males, whereas M+ males present SSC. Within the breeding season, the M- males category will be further subdivided into sneakers and immature males.

Field methods

Data was collected in daytime during two periods of the year: at the peak of the breeding season (May-July 2005) and outside the breeding season (end of September-November 2005).

Random stratified sampling was used to evaluate the distribution of the different reproductive classes through the different microhabitats. Strata corresponded to microhabitats which were defined according to the type of substrate, the presence of vegetation, the presence and type of shelter and the emersion/submersion cycle (Table 2). Within each sampling stratum, quadrats were randomly assigned and the same quadrat was sampled during four different tide levels (Table 3) in random order in different days, both inside and outside breeding season, in a repeated measures design. Quadrats were sampled using a throw trap of 1x1x1m of 3.5mm mesh. Within the trap, fish were anaesthetised with clove oil (c. a. 150ml of a solution 10 ethanol: 1 clove oil) and captured with two hand nets. Captured fish were sexed and their total length measured. They were allowed to recover from anaesthesia before being returned to the sampling point. High tide and subtidal sampling were carried out snorkelling or diving, letting the trap fall from above the sampling point. For these sampling points, capture of fish followed the procedure just described but a clove oil solution was used that was approximately three times more concentrated. Contrary, when emerged, *i.e.*, in intertidal areas during low tide, quadrats were sampled without the use of anaesthetic.

The use of throw traps as the sampling method was considered to be the most effective and consistent for the characteristics of the species and study area. This sampling method has been tested before and has been considered a relatively efficient and accurate method of estimating fish density in different environmental condition including a range of diverse depths and vegetated areas (Chick *et al.*, 1992; Jordan *et al.*, 1997; Rozas and Minello, 1997).

Table 2. Classification of the different microhabitats and their distinctive characteristics. The intertidal area is exposed to air twice a day; the subtidal or low intertidal areas are permanently immerse but the latter may be shortly exposed in spring tides. * measurements of potential nests sampled (average $\pm$ SD): width= 3.65 ± 0.25 cm; height= 2.67 ± 0.80 cm; depth= 21.44 ± 6.82 cm.

Microhabitat	substrate	vegetation	Submersion cycle
Intertidal Mobile Substrate (MSit)	Sand no shelters	No	intertidal area
Immerse Mobile Substrate (MSi)	Sand and/or empty small shells no shelters	No	subtidal or low intertidal area
Intertidal Vegetation (Vit)	Sand and/or mud no shelters	Seagrass, (<i>Zostera noltii</i>)	intertidal area
Immerse Vegetation (Vi)	Sand and/or mud no shelters	Seagrass (<i>Zostera</i> spp. and/or <i>cymodocea</i> spp.) and/or algae (<i>Ulva</i> spp.)	subtidal or low intertidal area
Intertidal Hard Substrate (HSit)	small stones or debris the form irregular cavities on sandy area	No	intertidal area
Hard Substrate on Intertidal Vegetation (HSitv)	Debris (usually bricks or tiles)	Seagrass (<i>Zostera noltii</i>)	intertidal area
Immerse Hard Substrate (HSi)	small stones or debris the form irregular cavities on sandy area	No	subtidal or low intertidal area
Intertidal Potential Nests (PNit)	Debris composed of bricks with regular cavities* with one opening	No	intertidal area
Potential Nests on Intertidal Vegetation (PNitv)	Debris composed of bricks with regular cavities* with one opening	Seagrass (<i>Zostera noltii</i>)	intertidal area
Immerse Potential Nests (PNI)	Debris composed of bricks with regular cavities* with one opening	No	subtidal or low intertidal area

Table 3. Classification and procedure description of sampling the different tide levels

Tidal level	Sampling time
Low water	Between 1hour before and 1 hour after the low tide had achieved its minimum level or, in intertidal areas, when the sampling point was exposed
Rising tide	Between 15 and 45 min after the sampling point had been covered by water or, in subtidal areas, between 1h:15min and 1h:45min after the low tide had achieved its minimum level
High water	Between 1hour before and 1 hour after the high tide had achieved its maximum level
Ebbing	Between 15 and 45 min before the sampling point was exposed or, in subtidal areas, between 1h:15min and 1h:45min before the low tide had achieved its minimum level

Data analysis

A repeated measures ANOVA was carried out by means of SPSS (Release 14, 2005, SPSS Inc., Chicago, USA). The analysis was conducted on the population density (variable transformed by $\log[X] + \log[X+1]$) to analyse the distribution of the species through the microhabitats and the effects of season and tide, as well as the interactions between these factors. In four of the microhabitats (immerse and intertidal mobile substrate and immerse and intertidal vegetation) the species was rare or not present and these microhabitats were excluded from this analysis. The values reported are Greenhouse-Geisser corrected.

The associations between reproductive classes, seasons, microhabitats or tide levels were analysed by means of ACTUS (analyses of contingency tables using simulations; Estabrook and Estabrook, 1989) to identify changes in microhabitat distribution, population composition and microhabitat segregation. ACTUS was applied to the sum of captured individuals. This software evaluates the significance of the chi-square statistic of independence, when contingency tables have too low frequencies of observations for the chi-square test to be applied in a standard way. For each of the 1000 simulated tables produced, where the distribution of observations is random but respects the total frequency of each line and each column, a chi-square statistic is calculated. Hence, obtaining a distribution of test values to which the chi-squared value of the observed data will be compared, to assess its significance. Furthermore, the simulated tables produce a distribution of expected cell values under the null hypothesis of independence of factors that allows identifying which cells fall outside the confidence interval, *i.e.* which cells deviate from the population distribution. The distribution of pairs of reproductive classes was compared to analyse microhabitat segregation. Because the same test was applied

to the same set of data repeatedly, a progressive Bonferroni correction was used (Legendre and Legendre, 1998) with consequent alteration of significance level.

In order to detect deviations from uniform distribution of each reproductive class, a goodness of fit test was calculated for each of the classes by means of ADERSSIM software (V.C. Almada ©, ISPA, Lisbon, Portugal). This software compares the observed frequency of individuals on each microhabitat with its expected frequency, by comparing the chi-square observed with the distribution of chi-square values obtained by 1000 simulated tables. Therefore the procedure is similar to that of ACTUS and, equally, ADERSSIM identifies cell that deviate from the expected frequencies. The expected frequencies were here determined as: (number of quadrats sampled in microhabitat) x (total number of individuals captured) / (total number of quadrates sampled). Thus, expected frequencies were corrected for sampling effort.

Except where explicit, tests were 2-sided and the significance level was established in 0.05.

RESULTS

General microhabitat use

As a consequence of the mesh used for the nets during sampling, the individuals that were collected during our sample procedures had a total length larger than 3.1 cm (percentile 5 of juveniles, the smallest class = 3.2cm). The size distribution of individuals captured is presented in table 1, according to reproductive class.

S. pavo was absent or rare on microhabitats without shelter, *i.e.*, without hard substrate or potential nests (Figure 1). In fact, no individual was captured on intertidal mobile substrate (MSit) and density values were very low on immerse mobile substrate (MSi average= 0.25m^{-2}) and immerse (Vi average= 0.14m^{-2}) or intertidal vegetation (Vit average= 0.02m^{-2}). In the subtidal zone of the lagoon, adjacent to the mudflat and sampled until -3.5m ZH, which presents no shelters, no individuals were captured or observed. The few individuals found on MSi and Vi were captured in the small channels that drained the water from the mudflat to the lagoon and that were covered with empty shells, (mainly common cockle shells, *Cerastoderma edule*). Only juveniles and some small females (TL<6.2cm) were present in these microhabitats.

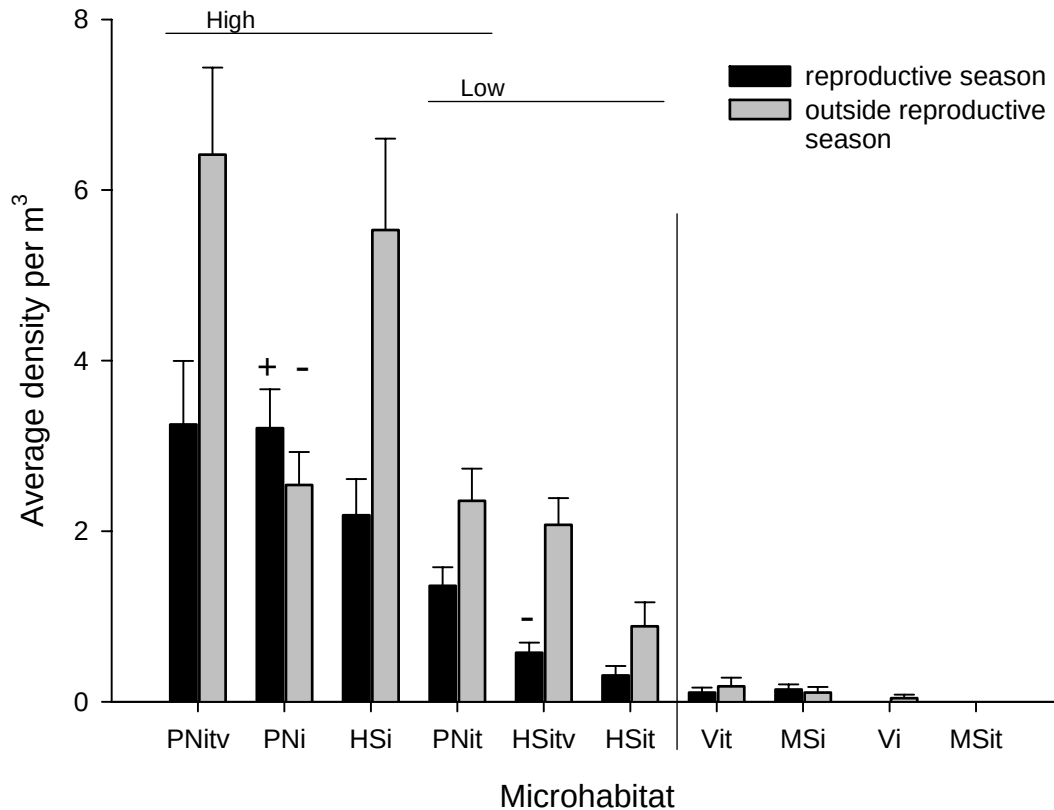


Figure 1. *Salaria pavo* density per m^3 (average + SE) in the different microhabitats during and outside the breeding season. The interaction effect of microhabitat distribution x season, as tested by ACTUS is represented by + and – when density is respectively above and below expected values of equal distribution in both seasons. The two extreme density homogeneous groups identified by tukey's test are identified as high and low. ANOVA RM on density values - microhabitat: $F=6.046$, $df=5$, $df\text{ error}=44$, $p<0.001$; season: $F=27.834$, $df=1$, $df\text{ error}=44$, $p<0.001$; season x microhabitat: $F=2.901$, $df=5$, $df\text{ error}=44$, $p=0.024$. PNitv - potential nests on intertidal vegetation; PNi – immersed potential nests; HSi – immersed hard substrate; PNit – intertidal potential nests; HSitv - hard substrate on intertidal HSvit - hard substrate on vegetation; HSit – intertidal hard substrate; Not included in the RM ANOVA: Vit – intertidal vegetation; MSi – immersed mobile substrate; MSit – intertidal mobile substrate; Vi – immersed vegetation.

Excluding the four microhabitats mentioned above, which presented null or very low densities of *S. pavo*, the repeated measures ANOVA showed that the distribution of the individuals of this species through the different microhabitats is not uniform, that the population density is higher outside than inside the breeding season and that there is a significant interaction between season and microhabitat use (Figure 1 includes statistical

results). Post-hoc Tukey's test revealed the following homogeneous subsets: the lowest density group ($p=0.228$) includes intertidal hard substrate (HSit), hard substrate on intertidal vegetation (HSitv), intertidal potential nests (PNit); the intermediate density group ($p=0.301$) consists of HSitv, PNit, immersed hard substrate (HSi) and immersed potential nests (PNi); and the highest density group ($p=0.171$) comprises PNit, HSi, PNi and potential nests on intertidal vegetation (PNitv). Despite the high degree of overlap of these groups, the highest and the lowest density groups constitute rather distinct groups which have in common only PNit, the microhabitat with an intermediate density of *S. pavo*, not different from the any other microhabitat in terms of population density. Contrary, HSit and PNitv show the most extreme densities (Figure 1).

In concordance with the significant interaction effect of microhabitat x season detected by the RM ANOVA, the simulation analyses revealed that the population distribution is not independent of season and thus microhabitat distribution changed over time (ACTUS season x microhabitat: $n=900$, $df=8$, $\chi^2=47.026$, $p>0.001$; Figure 1 illustrates the groups that were identified as significantly different from expected values). During the breeding season, the number of individuals present on HSitv was significantly lower than expected by chance, while on PNi it was higher than expected during the breeding season.

Intraspecific microhabitat use

The general distribution of each reproductive class through the different microhabitats was analyzed. The analysis of the distribution of sneaker and immature males was restricted to the breeding season when these classes were distinguishable while for the remaining classes the analyses was conducted on the sum of the frequencies of both seasons. For each reproductive class an ADERSIM test was conducted where the number of individuals observed on each microhabitat was compared with the frequency expected from a uniform distribution corrected for sampling effort. On each test, only the microhabitats where the reproductive class in question was present were included in the analysis. No reproductive class presented a uniform distribution. All male classes were found to be absent from microhabitats without shelters. M+ males and sneakers presented higher than expected frequencies on the two microhabitats with potential nests: PNitv and PNi. Lower than expected frequencies were detected on microhabitats with other types of hard substrate: on HSit and HSitv (M+ males, $n=225$, $df=5$, $\chi^2=273.793$, $p<0.001$, Figure 2a; sneakers, $n=44$, $df=5$, $\chi^2=48.816$, $p<0.001$, Figure 2b). Differently, immature males showed higher than expected frequencies on HSi. Negative deviations from expected values were detected on HSit ($n=26$, $df=5$, $\chi^2=36.176$, $p<0.001$, Figure 2c.).

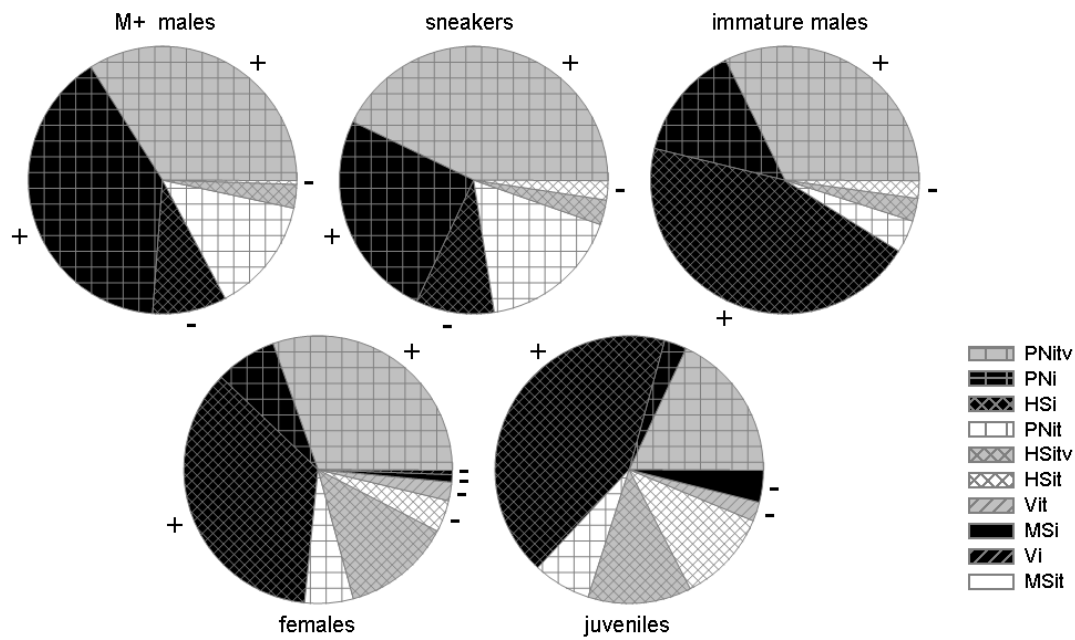


Figure 2. Microhabitat distribution of each reproductive class, corrected for sampling effort of each microhabitat. Positive and negative deviations from a uniform distribution of each class through the different microhabitats marked by + and – according to ACTUS results. See Fig 1 for microhabitat abbreviations.

Females were absent only from MSit and had higher frequencies than expected on HSi and PNitv and lower frequencies on HSiT and on the microhabitats with no shelters, MSi, Vi and Vit (ADERSIM, $n=243$, $df=8$, $\chi^2=338.972$, $p<0.001$, Figure 2d). Similarly to females, juveniles had higher than expected frequencies on HSi and lower on MSi and Vit but, unlike any other class, lower frequencies on PNi ($n=157$, $df=7$, $\chi^2=146.389$, $p<0.001$, Figure 2e.). Juveniles were absent from MSit and Vi.

Pair wise comparisons in the use of microhabitat by reproductive classes were analysed on ACTUS in order to detect microhabitat segregation. Additionally, microhabitat segregation within male classes (M+, sneakers and immature males) was analysed during the breeding season. M+ males and juveniles have the most contrasting distributions (ACTUS M+ x juveniles: $n=382$, $df=7$, $\chi^2=166103$, $p<0.001$; Figure 3a). Even after progressive Bonferroni correction ($\alpha'=0.009$), M+ males showed higher occupation rates than expected by chance on PNi and PNitv and lower ones on MSi, HSi and HSiT in comparison to juveniles. Juveniles showed the opposite pattern. M+ males and females also showed extensive microhabitat segregation in their general distribution (ACTUS M+ x females: $n=468$, $df=8$, $\chi^2=136.606$, $p<0.001$; Figure 3a). The distribution of M+ males and females diverged ($\alpha'=0.010$) in HSi and HSiTv, where the occupation rate of females was

higher than expected and that of M+ males was lower than expected; and on PN_i, which showed the opposite pattern.

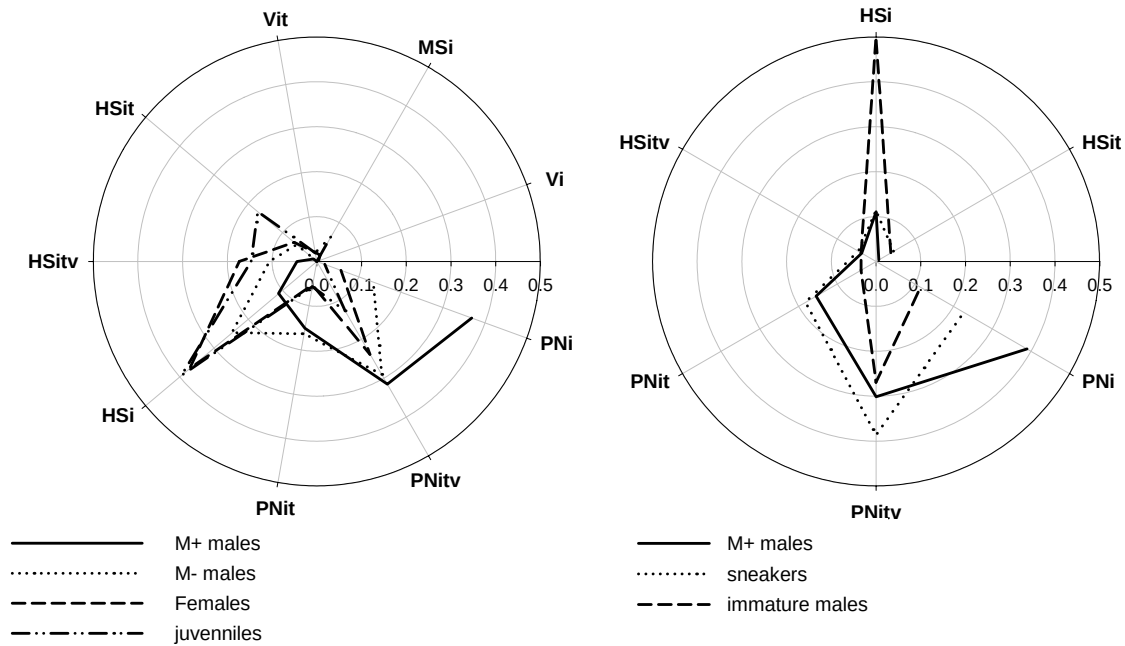


Figure 3. Comparison of the microhabitat distribution of a) each reproductive class b) males reproductive classes during breeding season.

Additionally, lower than expected occupation rates of females were observed in PN_{it} and of M+ males on HS_{it}. M- males and juveniles also segregated in the use of microhabitat (ACTUS M- males x juveniles: $n=432$, $df=7$, $\chi^2=79.374$, $p<0.001$; Figure 3a). They showed complementary distributions ($\alpha'=0.013$) in MS_i where M- males had lower occupation rates than expected and juveniles higher. Juveniles also had lower than expected frequencies in all the microhabitats with potential nests, PN_i, PN_{it} and PN_{itv}, and higher frequencies on HS_{it}. M+ and M- males segregated (ACTUS M+ x M- males: $n=500$, $df=5$, $\chi^2=55.281$, $p<0.001$; Figure 3a) on PN_i and HS_i ($\alpha'=0.017$). In the first microhabitat the frequency of M+ males was higher than expected and that of M- males was lower; in HS_i the opposite pattern was found. Females and M- males segregated (ACTUS M- males x females: $n=508$, $df=8$, $\chi^2=45.174$, $p<0.001$; Figure 3a) only on HS_i which presented a lower than expected occupation rate of females and a higher one of M- males ($\alpha'=0.025$). Females and juveniles showed the most parallel distribution but still juveniles showed higher than expected occupation rates on HS_i and lower on PN_{itv} (ACTUS M- males x females: $n=508$, $df=8$, $\chi^2=45.174$, $p<0.001$; Figure 3a).

During the breeding season, as expected, M+ males and sneaker revealed a matching distribution (ACTUS M+ males x sneakers: $n=180$, $df=5$, $\chi^2=6.368$, $p=0.264$; Figure 3b). In contrast, M+ and immature males diverged (ACTUS M+ x immature males: $n=163$, $df=5$, $\chi^2=27.882$, $p<0.001$; Figure 3b) in the use of HSi, which presented higher occupation rate of immature males than expected ($\alpha'=0.025$). Similarly, sneakers and immature males segregated (ACTUS sneakers x immature males: $n=70$, $df=5$, $\chi^2=13.893$, $p=0.008$; Figure 3b) in HSi with a heavy occupation rate of immature males but lower than expected frequencies of sneakers ($\alpha'=0.05$).

Seasonal variation

Besides the difference in density and in microhabitat use, the composition of the population was also different between the two sampled seasons in terms of reproductive classes. The proportion of individuals from the different reproductive classes showed significant seasonal variation (ACTUS season x reproductive class, applied to the sum of number of individuals: $n=900$, $df=3$, $\chi^2=95.961$, $p>0.001$; Figure 4). During the breeding season, the proportion of M+ males was higher while that of M- males and juveniles was lower than outside the breeding season,

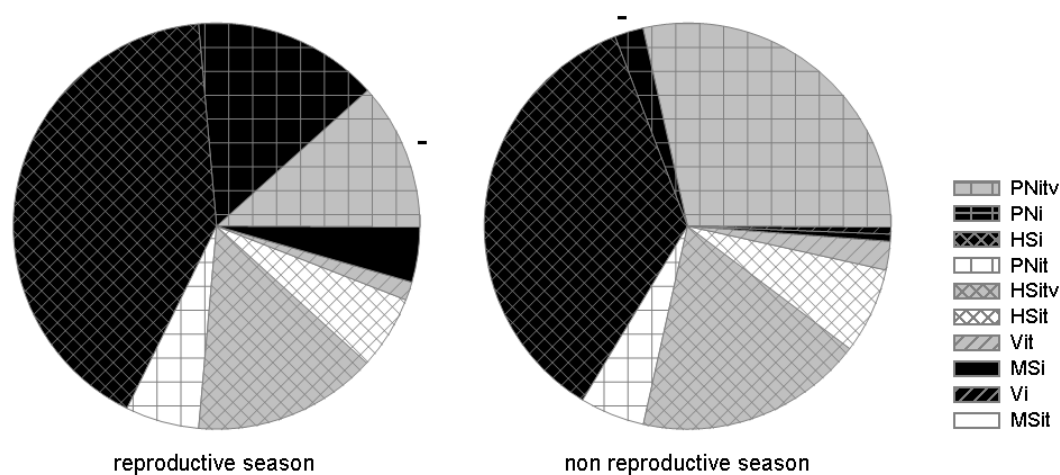


Figure 4. Total number of individuals from the different reproductive classes captured during and outside the breeding season with an equal sampling effort. Deviations from a similar distribution between sampled season are marked by + and – according to ACTUS results. Overall differences between seasons in the number of captured animals is marked with *** ($p<0.001$).

Only within the female class, was variation in microhabitat distribution found to be significantly associated with season. The occupation rate of females in PNitv was lower during the breeding season than outside it, and in PNi, the occupation rate of females was lower outside the breeding season than during it. (ACTUS season x microhabitat, $n=243$, $df=8$, $\chi^2=24.763$, $p=0.001$; Figure 5).

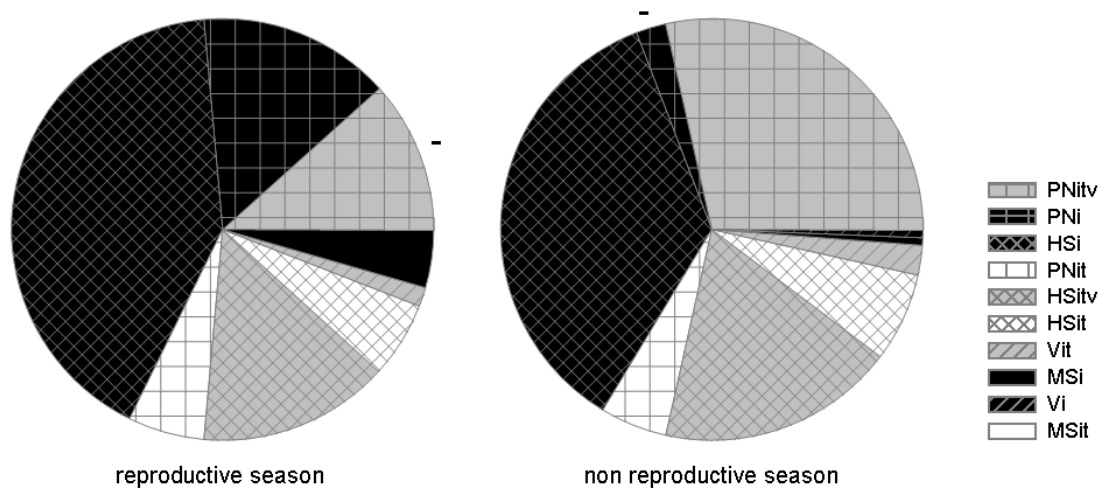


Figure 5. Total number of females captured during and outside the breeding season with an equal sampling effort. Deviations from a similar distribution between sampled season are marked by + and – according to ACTUS results. See Fig 1 for microhabitat abbreviations.

During the breeding season, when comparing microhabitat composition in terms of reproductive classes, only two microhabitats were not significantly associated with any class of individuals: Vit and PNit. However, the first was hardly used by the individuals of this population. Outside the breeding season, all the microhabitats showed deviation for the general composition of the population (Table 4).

Tidal variation

A closer analyses of the data revealed that only outside the breeding season the use of microhabitat was associated with tide level; during the breeding season the occupation rates of the different microhabitats were constant throughout the different tide levels (ACTUS tide x microhabitat: $n=306$, $df=21$, $\chi^2=22.475$, $p=0.353$). Outside the breeding season, the very few individuals that used the microhabitat Vit were all found during high waters and thus the occupation rate of this microhabitat was higher than expected at that tide level.

Table 4. Total number of individuals of each reproductive class captured in the different microhabitats, during and outside the breeding season. MSit - mobile substrate on intertidal area, MSi - mobile substrate constantly immerse, HSit - hard substrate intertidal on area, Hsvit - hard substrate on vegetated intertidal area, HSi - hard substrate constantly immerse, PNit - potential nests on intertidal area, PNvit - potential nests on vegetated intertidal area, Pni - potential nests constantly immerse, Vit – vegetation on intertidal area, Vi - vegetation constantly immerse.

microhabitat	Reproductive season					Outside reproductive season			
	M+ males	sneakers	immature males	females	juveniles	M+ males	M- males	females	juveniles
Msit	0	0	0	0	0	0	0	0	0
Msi	0 ⁻	0	0	3	5 ⁺	0	0	1	5 ⁺
Hsit	1 ⁻	2	1	4	7 ⁺	1 ⁻	13	12	20 ⁺
Hsvit	5	2	1	10 ⁺	4	5 ⁻	26	32	19
Hsi	15 ⁻	5	13 ⁺	28 ⁺	7	10 ⁻	50	62	55 ⁺
Pnit	21	8	1	4	4	13	36 ⁺	9 ⁻	5 ⁻
Pnvit	41	17	7	8 ⁻	3 ⁻	30	56	50	17 ⁻
Pni	53 ⁺	10	3	10	0 ⁻	30 ⁺	24	4 ⁻	3 ⁻
Vit	0	0	0	1	2	0	0	4	1
Vi	0	0	0	0	0	0	0	1	0

The occupation rate of HSit was also higher at high waters but during the flood it was lower than expected. In contrast, PNitv presented higher occupation than expected during the rising tide and lower during the high tide (ACTUS tide x microhabitat: n=594 df=24, $\chi^2=77.369$, p=0.001).

To test the association between tide level and the distribution of each reproductive class, ACTUS analyses were conducted independently for each reproductive class and each sampling season. This analysis showed that, within the breeding season, the distribution of each reproductive class was constant throughout the different tide levels, as expected (ACTUS tide x microhabitat: n=32, df=18, p>0.05). However, outside the breeding season, females and juveniles showed differences in their distribution according to tide level. The occupation rates of PNitv by females was higher during flood and lower during high waters but contrary, at higher waters, the occupation rate of Vit was higher than at the remaining tide levels (ACTUS tide x microhabitat, applied to the sum of females captured outside the breeding season: n=175, df=24, chi-square=41.645, p<0.01, Figure 6a). Juveniles only showed lower occupation rates than expected on PNitv, during ebbing (ACTUS tide x microhabitat, applied to the sum of juvenile individuals captured outside the breeding season: n=125, df=21, chi-square=38.905, p<0.05, Figure 6b). M+ and M- males, whether sneakers or immature males, presented a distribution that was never significantly associated with tide level.

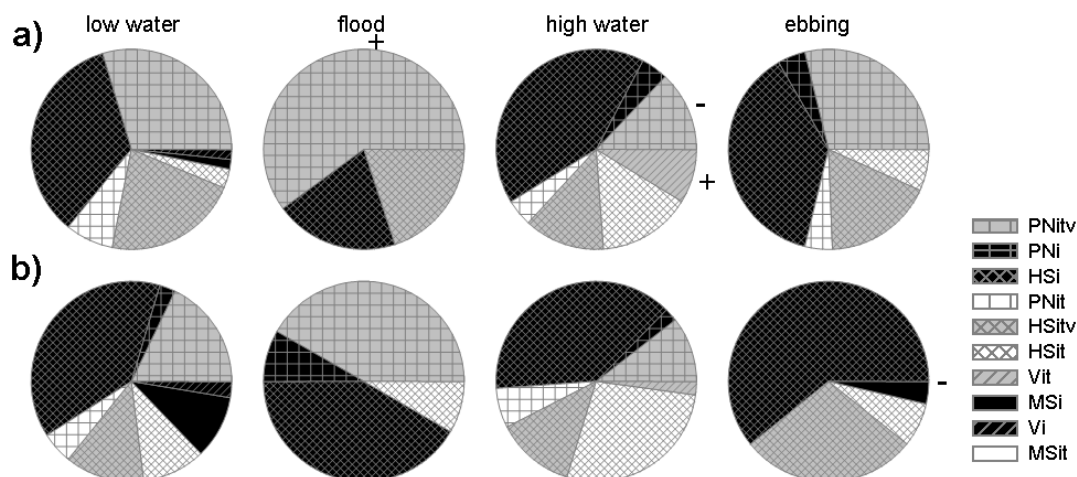


Figure 6. Microhabitat distribution of a) females and b) juveniles according to tide level and outside the breeding season, corrected for sampling effort of each microhabitat. Positive and negative deviations from a similar microhabitat distribution between the different tide levels for each reproductive class are marked by + and – according to ACTUS results. See Fig 1 for microhabitat abbreviations.

DISCUSSION

General microhabitat use

This study was designed to characterise the intraspecific variation in microhabitat preferences of *Salaria pavo* in a coastal lagoon in the south of Portugal. In general, the species was absent or rare in microhabitats with no shelters (MSit, MSi, Vit, Vi) at all sampled seasons and tide levels. Furthermore, the species was not found in the sampled subtidal zone of the lagoon (until -3.5mZH). This might be due to the lack of shelters in our study area at these depths. However, Orlando-Bonaca and Lipej (2007) also reported the presence of this species only above 1 meter of depth, in the Mediterranean Sea, despite the presence of rocky substrate below this depth. Thus the distribution of the species is likely restricted to the intertidal and upper subtidal zone. On the intertidal area of Culatra Island, the density was found to be highest on microhabitats with potential nests composed of human-born debris that are fit for nest establishment, due to their regular cavities with only one entrance; and particularly in areas with high capacity to retain humidity, like permanently immersed areas or intertidal areas with vegetation. The importance of the particular structure of substrate was reinforced by the fact that the

microhabitat with the highest density, PNitv, presented significantly higher density than its most similar microhabitat, HSitv. These two intertidal areas with vegetation differed only in substrate structure, the first being composed of regular structures as just described and the latter consisting of irregular debris or tiles that form large cavities with two opposite large openings. The known association between body size and the dimensions of shelters used by blennioid fish (e.g. *Lipophrys spp.*, Kotrschal, 1988) could not explain the higher density found on microhabitats with potential nests, since these consisted of cavities of homogenous dimensions, and diverse reproductive classes, varying greatly in body size, were represented there. Hence, it is likely its function as substrate for reproductive activity that justifies the elevated density of PNitv.

Seasonal variation and intraspecific microhabitat use

In addition to spatial variation, the species also showed temporal variation in microhabitat use. Between the reproductive and non-breeding season the overall population density increased and its composition changed: the relative frequencies of the younger classes, namely juveniles and young males (M- class), raised and, contrarily, that of M+ males was reduced. This indicates that recruitment of new individuals into the mudflat is initiated in between the two sampling points of this study. Despite producing benthic eggs, the larvae of *S. pavo* are pelagic. Nothing is known so far about larvae migration in this population but the settlement occurs approximately at day 27 after hatching (TL $\approx$ 1.4cm; Fishelson, 1963). A high mortality of M+ males at the end of the breeding season should contribute to this shift in population composition as well, for not only the proportion but also the absolute number of M+ males diminished between the two sampled seasons. M+ males when defending a nest and providing parental care rarely leave their nests (Gonçalves and Almada, 1997) and that restrains their feeding activity. The general condition of these males has been observed to decrease drastically as the breeding season progresses (Gonçalves and Almada, 1997) and a high mortality rate is observed at the end of the season predominantly in the older age classes (T. Fagundes, M. G. Simões, D. M. Gonçalves, R.F. Oliveira, unpublished data).

Moreover, the use of microhabitats was generally different between the two sampled periods. Occupation rates of PNi were higher during the breeding season than outside it and those of HSitv were lower. Two factors may have accounted for these changes in *S. pavo* distribution: differential use of microhabitat by females in the two sampled seasons, and modification of population composition. M+ and M- males did not alter their distribution throughout the sampled period (June to November). M+ males and sneakers

followed the general distribution pattern of the species, with a preferential use of microhabitats with potential nests, either immersed or on intertidal areas with vegetation (PNi and PNitv), and show lower occupation rates of HSit and HSitv. During the breeding season, the proportion of M+ males was significantly higher, while the proportions of M- males and juveniles were significantly lower than those expected if the distribution would have been equivalent in both sampled seasons. In fact, the higher proportion of M+ males in the population during the breeding season justifies the more intense use of PNi during that sampling period, since M+ males occupy preferentially this microhabitat. For the same reason, the lower occupation rate of HSitv during the breeding season is most likely due to a higher proportion of M+ males who disfavour the use of this microhabitat. The distribution of immature males, however, differed from this pattern: they showed preferential use of HSi. When comparing the distribution of these male classes, immature males differed from M+ males and sneakers in having higher occupation rates of HSi. Thus, sneakers and M+ males showed similar distributions, which reflect the use of a restricted nesting area by sneakers, as reported earlier (Gonçalves *et al.*, 2003). M+ males attract and court females, while sneakers parasitize these encounters, fertilizing the eggs that the females lay within the nest of M+ males. The fidelity of sneakers to a few particular nests, which hypothetically maximizes their reproductive opportunities, should explain the identical distribution observed for these classes. It also raises the question of whether a transitional state exists between pure sneaker behaviour and a satellite condition (Gonçalves *et al.*, 2003), in which nesting males accept the presence of other smaller males in exchange of benefits, like nesting defence as has been found in a related species *Parablennius parvicornis* (Santos, 1985). Differently, immature males showed partial microhabitat segregation relative to the remaining male classes. The similar body size of sneakers and immature males excluded effects due to predation pressure and feeding requirements, in terms of size of food items, as the cause for this segregation. In fact, what distinguishes these classes is their reproductive activity: the species shows diverse ontogenetic pathways, and while sneakers mature early, immature males postpone reproduction and invest on growth (Oliveira *et al.*, 2001a). Hence, contrary to sneakers, immature males do not have the reproductive benefit of remaining in areas occupied by M+ males. Immature males should prefer rich food areas to optimize body growth, and should avoid agonistic interactions that, not only could compromise their survivorship but also demand allocation of energy towards social behaviours. Thus, these two classes have most likely different activity budgets that dissociate their microhabitat use. This microhabitat segregation of male reproductive classes may play an important role in the life-history pathways followed by the males of this species. The development of the young males of this population has been shown to be sensitive to social cues,

particularly social dominance (Fagundes *et al.*, in prep.), and the observed segregation implies differences in social environment. Our descriptive data does not allow inferring a causal relationship between this segregation and life-history pathway decisions but the hypothesis calls for further investigation.

Contrary to males, females do change their distribution with season. Besides the preferential use of PNitv, females also show a positive deviation from a uniform distribution on HSi and a negative deviation on HSi, MSi, Vi and Vit. This general distribution pattern is altered during the breeding season, when the occupation rate of PNitv by females is, against expectations, lower than outside the breeding season; and outside the breeding season when the occupation rate of PNi is reduced in comparison to the breeding season. This fact suggests that PNitv, contrary to PNi, may play an important role in other activities besides reproduction. M+ males presented high densities on PNitv, particularly during the breeding season when they show stronger territoriality (Oliveira *et al.*, 2001b). Immerse potential nests provide nesting places, shelters and protection from dehydration, and probably from predation, but the presence of vegetation on PNitv, constitutes, additionally an important food source. In this microhabitat, vegetation is typically composed of seagrass beds, known for their high productivity (McRoy and McMillan, 1977) and invertebrate richness and abundance (Orth *et al.*, 1984) and the diet of *S. pavo* is mainly composed of benthic invertebrates (Zander, 1986). Thus, females would benefit from using this microhabitat throughout the year but the presence of many territorial males during the breeding season may exclude females from this area allowing their presence only to mate.

Juveniles, which showed no seasonal changes in the use of microhabitat, also used preferentially HSi but showed low occupation rates in PNi and microhabitats with no shelters (MSi and Vit).

Microhabitat segregation was also evident in the general distribution of reproductive classes, not only between M+ and immature males during the breeding season. In general M+ males privileged the use of the microhabitats with potential nests (PNitv and PNi) and disfavoured those with other types of hard substrate. In contrast, juveniles and females showed the opposite pattern but females shared with males an important use of PNitv. Females and juveniles showed the most parallel distribution but still juveniles presented higher than expected occupation rates on HSi and lower on PNitv.

Tidal variation

Changes of microhabitat distribution associated with tidal cycle were detected only on juveniles and females, and only outside the breeding season. During the breeding season the diverse reproductive classes maintain their distribution throughout the tidal cycle. Outside the breeding season, the only exception to a constant distribution of juveniles was PNitv that juveniles avoided during the ebbing tide. Females, on the other hand, showed lower occupation rates of this microhabitat during high waters and higher rates during the flood. This population has been observed to reduce activity during high waters, with individuals inhibiting swimming behaviour and remaining inside crevices (personal observations), possibly to avoid predation, since high water level allows the access of larger fish species to the intertidal area. Owing to the reduction of activity during high waters and to air exposure during low waters, individuals of this species were expected to become more active in the intertidal areas during ebbing and flooding. Therefore, PNitv represents an important microhabitat for female activity during the flood, of which foraging may constitute a significant component due to the presence of seagrass beds. Contrary to expectations, the same did not hold for ebbing tide. It is possible that the timing of the sample was too close to low waters and that some of the individuals may have already sought refuge in other microhabitats, since the presence of M+ males may hinder the access of females to these refuges.

Little is known about intrasexual microhabitat distribution of teleost fish and of intertidal fish in particular. Here, we identify microhabitat segregation that extends beyond sex and age classes and involves intrasexual differential distribution according to behaviour morphs (reproductive tactics). The differential use of microhabitats by this population stresses the importance of considering a representative sample of the microhabitats available to the individuals for estimation of population parameters. This applies to other fish populations, particularly to intertidal species, due to the intrinsic environmental variation of that habitat, consequence of zonation, and which is further complexified by cyclic changes in water level. Curiously, tidal cycle was not determinant in the distribution of any of the male classes. However, outside the breeding season, females, which are known for their greater mobility (Gonçalves *et al.*, 2003), and juveniles, to a smaller degree, varied their distribution according to tidal cycle. Nevertheless, data suggests that low waters may give a fair representation of microhabitat distribution of the population and of the different reproductive classes, since no deviation from general distribution was detected in either sampling season. However, considering that low water is not an active period, it may not faithfully represent the social context in which individuals are inserted. Maximum activity should occur during flood and ebbing, but these differ from low waters in

microhabitat distribution. Particularly, females use relative more PNitv during the flood and juveniles less during ebbing tide. Therefore, outside the breeding season, due to concentration of females in PNitv at flooding, parameters like local sex ratio, for instance, will be biased towards males. Seasonal variance should also be accounted for due to phenomena like recruitment and male mortality which affects both population density and composition.

The highest densities of this species were found on microhabitats with potential nests, PNitv in particular. Sampling these microhabitats is essential due to the presence of M+ males and sneakers which are less frequent in other microhabitats; however females and juveniles are underrepresented in this microhabitat. Within the high density group of microhabitats, PNit is perhaps the better mirror of population composition during the breeding season as it is not associated with any particular reproductive class or tide cycle.

In summary, the microhabitat distribution of the population is not uniform and we have found discrepancy in microhabitat use by the different sexes and life-history stages of this population, which include male alternative reproductive tactics. Microhabitats without shelters show the lowest values of density but mudflat channels covered with empty shells may constitute a refuge for juveniles. The areas with bricks, suitable for nest establishment, particular those on vegetated areas, showed the highest densities and were preferentially used by all reproductive classes. However, while M+ males and sneakers used additionally immersed potential nests, immature males, juveniles and females, used immersed hard substrate with ample opportunity for shelter but not appropriate for nesting. This revealed microhabitat segregation between different classes of males that have similar body sizes but differ in reproductive activity (immature males vs. sneakers). Females also used preferentially hard substrate on intertidal vegetation. Moreover, the distribution of females varied with season and, outside the breeding season, it was associated to tidal oscillation, as was the distribution of juveniles. M+ and M- males did not change distribution according to season or tidal cycle. The alteration of the composition of the population from the breeding season (May to July) to the sampling point outside the breeding season (October and November) indicates the occurrence of recruitment into the mudflat and reflects the mortality of M+ males from one period to the next, and it is most likely the main reason for the change in the general microhabitat distribution of the population rather than changes in the use of the microhabitats by each of the reproductive classes. This dynamic should be considered when designing sampling procedures.

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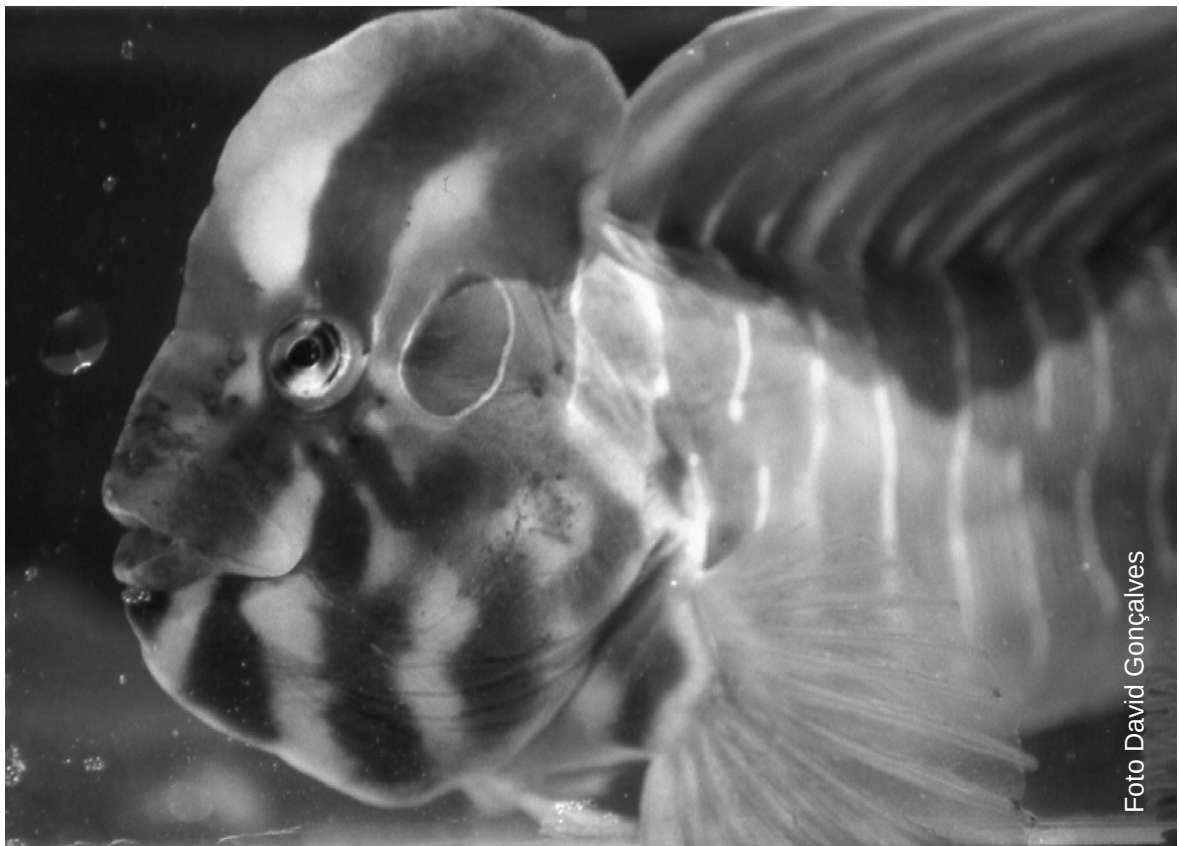


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GENERAL CONCLUSIONS

General conclusions

Sex role reversal

In sex reversed species females compete among themselves for the access to males, exhibit mate attraction behaviours such as courtship, and may develop secondary sexual characters. Males of these species provide more parental care to their progeny than females and are selective in their mate choice (Rosenqvist 1990; Andersson, 1994). All these features have been described for the population of the peacock blenny of the Ria Formosa, except that females do not possess secondary sexual characters, rather males do. Hence, sex role reversal has been identified in this population (Almada *et al.*, 1995).

In the area where the population is located, nests are scarce and aggregated (Almada *et al.*, 1994) allowing some males to monopolize these reproductive resources (Saraiva *et al.*, 2009) which prevents other males from reproducing. Additionally, occupied nests have been reported to be saturated with eggs (Almada *et al.*, 1995). Both of these factors restrict the reproductive rate of males and consequently the operational sex ratio is expected to be biased towards females therefore limiting the spawning opportunities of the females and leading to female intrasexual competition (Almada *et al.*, 1995; Saraiva *et al.*, 2009). Female-female indirect competition may occur when females are searching for males and trying to access them before nests are filled with eggs. But females have also been reported to directly and aggressively compete among themselves (Almada *et al.*, 1995).

Males, on the other hand, provide parental care to eggs which is generally assumed to be non depreciable (Blumer, 1982; Lazarus and Inglis, 1986). However, in this population eggs hatch in approximately 10 days (Fishelson, 1963; von Westernhagen, 1983; Ros *et al.*, 2009) and the breeding season extends from May to September, allowing the production of many sequential broods. Males do not leave the nests while providing parental care (Almada *et al.*, 1994), supposedly to avoid nest takeovers by other males. This comes at a cost: male condition decreases steeply (Gonçalves and Almada, 1997; Fagundes, unpublished data) and a peak of male mortality is observed at the end of each breeding season (Fagundes, unpublished data). Therefore, the more eggs a male will have, the higher the costs of parenting due to a longer permanence in the nest that prevents them from foraging. Owing to this high investment in reproduction, males are expected to be selective towards females. Different authors have recently emphasized the effects of the costs of reproduction as the main organizing force of mating systems rather than potential reproductive rates or availability of potential mates, as measured by

operational sex ratio (Kokko and Managham, 2001; Simmons and Kavarnero, 2006). Reproductive costs are a more upstream cause of these parameters and may account for other factors such as predation risk not often considered in the studies of mating systems despite its known negative effect on mate choice (Forsgren, 1992; Berglund, 1993, Johnson and Basolo, 2003) and male courtship (Candolin, 1997), for instance. Male mate choice may emerge, for example, if males that choose for larger females increase their fitness, seen that larger females lay more (Patzner, 1985; Berglund and Rosenqvist, 1990) and/or larger eggs with higher survival rates (Pitman, 1979, Kraak and Baker, 1998; Heath *et al.*, 2003). And in fact, in this population, males aggressively reject courting females (Almada *et al.*, 1995) suggesting active male mate choice.

In summary, females are subjected to both intrasexual and intersexual competition, conducting to intense sexual selection. However, it is males that show secondary sexual characters.

Male secondary sexual characters and male-male competition

The competition between males for access to nests is expected to be intense due to the scarcity of this resource, and consequently males will also be subjected to strong sexual selection (Almada *et al.*, 1994; Saraiva *et al.*, 2009). The sexual characters of these males, the head crest and the pheromone producing anal gland are not used for actual fighting though they must play an important role in communication. Recent studies have demonstrated that pheromones are also important signals in the context of intrasexual competition, as in the case of the Mozambique tilapia (*Oreochromis mossambicus*) in which males use chemical odorants to signal social status to other males (Barata *et al.*, 2008a) and in the butterfly *Pieris napi* where males use pheromones to assess male density (Mellström and Wiklund, 2009). Because in the peacock blenny the head crest is associated with social dominance (Chapter IV), it may constitute an indicator of dominance or fighting potential and hence be under sexual selection through intrasexual competition. Supporting the hypothesis that, in this population, the male secondary sexual characters are preserved through intrasexual competition is the fact that differences in the extent of development of these characters were observed between different populations of this species that differ mainly in nest availability and are therefore subjected to different regimes of male-male competition (Saraiva *et al.*, 2009).

Male secondary sexual characters and female mate choice

Secondary sexual characters may also be maintained by intersexual selection. However, it is conventionally considered that competition in one sex, that in excess, will drive selectivity in the other and therefore competition and mate selectivity should be mutually exclusive (Trivers, 1972). Thus, in sex role reversed species, females are not expected to be selective.

Contrary to this expectation, the mating system of the peacock blenny appears to support the model by Kokko and Monaghan (2001) that predicts that selectivity of one sex is not a consequence of competition in the opposite sex, as an economic model of offer and demand, but it is driven from the cost/benefit relationship of selectivity, where in fact parental investment plays a major role. In their model, female selectivity was promoted by the low costs of mate searching (including high encounter rate of both sexes), high costs of breeding and variance in male quality. Production of eggs is always an investment considerably higher for females than for males, despite the fact the anisogamy in fish is not so accentuated as in birds, for instance. Females of the peacock blenny may produce more than their weight in eggs in a single breeding season (Gonçalves and Almada, 1997), a considerable investment which requires a high foraging effort that increases predation risk. On the other hand, egg survival is totally dependent on paternal care (Barata *et al.*, 2008b) but males may abandon a nest or cannibalize eggs (Gonçalves and Almada, 1997) and therefore females risk losing part of their investment. Thus, if the costs of searching for “better” males are low, females are to benefit from being selective (Johnstone *et al.*, 1996), despite female-female competition. Female mate choice may evolve as long as the benefits of finding a high quality male are higher than the costs of selectivity, including lost mating opportunities via female-female competition.

In this population, nest aggregation suggests that female mate sampling might be a low-cost activity in terms of energy, time or even predation risk, hence favouring female selectivity. In fact, females of the peacock blenny do not mate randomly, supporting the hypothesis that females are choosy. Females mate preferentially with larger, courting males that have larger secondary sexual characters (head crest and anal gland), larger genital papilla and more eggs in the nest (Chapter I). Some of these traits may be associated with indirect benefits, like body size which may reflect age and so high viability (Turner, 1993; Andersson, 1994) but most indicate capacity of providing parental care and thus direct benefits: 1. Larger broods are a direct proof of good parental care (Kraak and Videler, 1991) and the larger the brood, more intense the dilution effect raising the probability of survival of eggs in case of predation or cannibalism (Rohwer, 1978);

2. larger males will more effectively defend a nest against conspecifics (Sheppey and Ribbink, 1985) and potential egg predators (Keenleyside *et al.*, 1985) than smaller ones; 3. courtship has been proposed as an honest indicator of parental care quality (Knapp and Kovach, 1991); 4. the anal gland has been demonstrated to produce antimicrobial mucus that may enhance egg survival (Giacomello *et al.*, 2006) and the pheromone it produces have been shown to attract females (Barata *et al.*, 2008b; Serrano *et al.*, 2008); 5. finally, the head crest accompanies the decline of body condition as the breeding season progresses (Fagundes and Oliveira, unpublished results) suggesting a function as an energetic reserve which may indicate the capacity to provide parental care, instead of abandoning the nest to forage, for instance. Therefore, the population presents mutual mate choice and female selectivity may additionally contribute to the maintenance of secondary sexual characters in males of this population, despite the reversal of sex roles.

Females minimize the cost/benefit relationship of mate choice by using a “one step decision tactic” with complete recall when searching for mates (Chapter I). This consists of a sequential assessment of males in which females choose mates on the bases of a threshold that is adjustable to the standard quality of males found and to the costs of sampling additional males, which in turn depend on male spatial dispersion (Janetos, 1980; Real, 1990). Although females using this sampling tactic usually mate with the last or the second last male visited, in this population they are able to return to any male previously visited which may be a consequence of the low costs of sampling males when these are spatially aggregated. Costs of sampling are crucial determinants of mate selectivity to the point that they may eliminate it. An example is the pipefish, *Syngnathus typhle*, whose males, usually selective, mate randomly in the presence of predator (Berglund, 1993). These costs may be high in circumstances of intense intrasexual competition due to the risk of losing mates to competitors. However, aggregation of males allows females to visit and compare several males in a short period of time and space, keeping sampling costs low.

Male reproductive costs and brood size manipulation

The number of eggs is a possible criterion of female mate assessment. This prone to diverse interpretations because if a male is preferred for some reason other than the number of eggs it self, it will mate more often and therefore receive more eggs. In that case, brood size would be a consequence of mate choice based on other cues, rather than a criterion of mate assessment per se. Nevertheless, this study (Chapter II) supports an effect of the number of eggs on female choice as expected from similar experiments in other blenny species (Kraak and Groothuis, 1994). In this study males with experimentally

enlarged broods received more eggs and males with reduced broods received fewer eggs, than before brood size manipulation. These results strongly suggest that the number of eggs in the nests modulated female spawning decisions. However, instead of a runaway process in which the number of eggs would raise indefinitely, with time brood size returned to initial non-manipulated values indicating that brood size is adjusted to a stable size that should optimize current vs. future reproductive effort. In the peacock blenny, parental care consists of nest and egg defence and cleaning and fanning the egg mass, thus costs can be either direct energetic costs, if more eggs require more fanning as demonstrated in *Aidablennius sphynx* (Kraak and Videler, 1991), or costs related to the survival of eggs considering that density of fish eggs may increase infections (Takahashi *et al.*, 2004) and reduce oxygenation. Even though most nests have been observed to be saturated with eggs at the peak of the productive season (Almada *et al.*, 1995), the space occupied by eggs varied from year to year and with the body size of the parental male (chapter II; Fagundes, unpublished results). Temperature is negatively associated with oxygen dissolved in water, therefore in warmer years expenditure in fanning is expected to increase to compensate for low oxygen levels in water, also smaller males have been observed to expend more on fanning (Lissåker and Kvarnemo, 2006). Therefore, even when nests are not saturated, the costs of parenting may limit the reproductive rate of males and thus determine sex role reversal and explain male mate choice. Hence, despite being restricted to stationary eggs, parental care in the peacock blenny is not non-depreciable has classically assumed for fish (Blumer, 1982; Lazarus and Inglis, 1986).

The data did not support the expected negative correlation between androgens and brood size of which parental care is a function (reviewed in Ziegler, 2000; Hirschenhauser and Oliveira, 2006). Brood size and testosterone, but not 11-ketotestosterone, showed even a positive correlation. Opposite to the correlation in control males, males with experimentally reduced brood size increased in testosterone levels while males with enlarged broods reduced their testosterone levels. Since androgens are classically associated with sexual behaviours in vertebrates in general (Nelson, 2005) and in fish (Borg, 1994; Oliveira and Gonçalves, 2008), this may provide a mechanism which underlies the regulation of brood size, through the regulation of sexual behaviours such as courtship.

Therefore, a large asymmetry in male reproduction is observed due to three reasons. 1. competition for resources: many small males are not able to acquire a nest and consequently are excluded from the conventional form of reproduction. 2. reproductive costs: the reproductive success of nesting males depends on the capacity of providing parental care to eggs, namely how many eggs males are able to care for simultaneously in the nest and for how long males are able to continue in the nests caring for sequential

(but overlapped) broods. 3. Female mate choice: female selectivity accentuates the asymmetry in reproductive success between males who signal good and worse parental care competence. Nevertheless, since females have their own reproductive success restricted by the male's capacity of receiving eggs, it is unlikely that females will refuse mating opportunities, as supported by an adjustable threshold mate choice tactic. Instead an asymmetry in female quality, and consequently in egg quality, is expected to occur between better and worse parental males.

Considerations on sex roles and measures of sexual selection

Sex role reversal has been thought of as driven by asymmetrical intensities of sexual selection acting on females more intensively than on males (Vincent *et al.*, 1992). However, in this population the occurrence of male alternative reproductive tactics suggests intense sexual selection acting also on males, driven from the lack of nest sites. Therefore, both males and females are subject to intense sexual selection. We may understand this apparent incompatibility of theoretical perspective and the empirical analyses if we consider that, in this system, sexual selection is acting at different moments and via different components. The limiting factor here is nesting opportunities and this ultimately limits reproduction in both sexes but at different points in time. Reproduction in the peacock blenny is absolutely dependent on nesting. In a first, non-reproductive moment, male-male competition occurs for establishment of nests and is independent of the opposite sex. Potential nest sites are scarce and aggregated allowing for monopolization by a small number of larger and thus more dominant males (Saraiva *et al.*, 2009). This competition is so intense that many males (20% of recaptured males, Fagundes and Oliveira unpublished results) never establish a nest. Scarcity of nests will therefore lead to intense male-male competition, which should contribute to the maintenance of secondary sexual characters of males (Saraiva *et al.*, 2009), create asymmetries in male reproduction and drive the emergence of alternative reproductive tactics. Usually intrasexual competition is seen as the interaction between individuals of the same sex for access to reproductive resources in which the opposite sex is included and most often the solely considered resource. In most circumstances, intrasexual competition is analysed simply as the competition for access to the scarce opposite sex (or gametes of the opposite sex). This is reflected in any of the parameters classically used to describe or quantify sexual selection from the parental investment perspective, to operational sex ratio, to potential reproductive rates and back again to reproductive costs. As Ahnesjö *et al.* (2001) have noted, such parameters can be good predictors of mate competition but do not account for resource competition. Therefore, they describe

generally well the reproductive moment, the second moment, in which the two sexes are actually interacting. At this moment, receptive individuals of both sexes have to have mutual access and whatever factor will determine their receptivity and availability, will also determine how competitive and selective the different sexes will be. In the peacock blenny, male's high parental investment (sensu Trivers, 1972) and high breeding costs (sensu Kokko and Monaghan, 2001) would in fact determine sex role reversal and so would potential reproductive rate (Clutton-Brock and Parker, 1992) and operational sex ratio (Emlen and Oring, 1977) be expected to. However, these parameters do not reflect the whole dynamic of the system, they only account for mate competition. Because the first, pre-reproductive moment of intrasexual competition is rarely limiting, it is usually neglected without greater consequences or it is reflected later in mate choice (of which resources are commonly a criterion of selection). However, in populations like this one, the pre-reproductive moment imposes a regime of very strong intrasexual competition that is independent of the later reproductive moment. It is not the fact that the males compete initially, in a pre-reproductive moment, that determines the sex roles, as it is not operational sex ratio, at the reproductive moment, that determines the strong component of male-male competition. If there were fewer males in the population, as much as nesting sites, no intrasexual competition would occur at this point and the system's dynamic would be satisfyingly described by the parameters mentioned above. No alternative reproductive tactics would have emerged and most likely also no secondary characters of males would have been maintained, or at least would not be so exuberant. Females would equally compete for mating opportunities driving the reversal of sex roles due to the low number of nesting males, nest space and costs of parenting that limit brood size. Therefore, strong sexual selection is not exclusively a product of the scarcity of one sex. If reproductive resources (other than females) are rare, strong intrasexual competition for those resources in one sex will in nothing influence the sex roles, that is, the selectivity and competition in the other sex, rather the reproductive resources themselves will. In this population it is not one sex that limits the reproduction of the other sex but the reproductive resources that limit reproduction in both sexes. Therefore, it seems important to analyse the dynamic of a mating system at different moments and consider different components: a pre-reproductive moment, in which intrasexual competition is independent of the opposite sex (resource competition), and a reproductive moment that is generally well described by the quantifying parameters of sexual selection (mate competition). A third post-copulatory or post-mating moment in which sperm competition and female cryptic choice occur must equally be considered (Andersson and Simmons, 2006; Fedina and Lewis, 2008). Moreover, this separation in different moments implies that females should not be interpreted as a resource but rather as an active player in the shaping of the

mating systems. Finally, due to their independence, the forces acting at these distinct moments can not be added or balanced; their effects have to be considered independently.

Alternative reproductive tactics

Whenever the variation in mating success among males is high, i. e., strong sexual selection, there is the potential for alternative behaviours to emerge (Taborsky, 2001; Shuster and Wade, 2003). By exploiting the investment of others, less competitive males are able to overcome their disadvantage and reproduce. Very commonly the disadvantage is related to body size, which prevents the acquisition of reproductive resources by small males in competition with larger males (Taborsky, 1994). This is, for example, the case with the Shell-Brooding Cichlid Fish *Lamprologus callipterus* (Sato *et al.*, 2004) and the horned beetle, *Onthophagus acuminatus* (Emlen, 1997). Also in the peacock blenny the smaller and younger males are those that use the parasitic tactic (sneakers), while the older and larger reproduce as bourgeois, also named nest-holders or parental males (Gonçalves *et al.*, 1996). In the peacock blenny ARTs are flexible, which means that the same individual may express both tactics during its lifetime; and present a fixed sequence (*sensu* Brockman, 2001; Oliveira *et al.*, 2005; Taborsky *et al.*, 2008), that is, one tactic is followed by the other in a fixed order and once expressing the later tactic, the individuals can not revert to the initial one (Chapter III). Therefore the reproductive tactics in the peacock blenny are expressed sequentially by the same individual: first the sneaker tactic and later the bourgeois tactic, and the switching from the parasitic to the bourgeois tactic is unidirectional. This confirms the hypothesis raised by Gonçalves *et al.* (1996) of an ontogenetic sequence in male reproductive tactics when describing the age patterns of the two tactics.

Life-history pathways

When ARTs are expressed in ontogenetic sequences the life-history approach is essential to understand their pattern of expression. In the peacock blenny such an approach allow the identification of more then one life-history pathway. In this population not all males express both reproductive tactics. Some males never reproduce as sneakers and mature directly as bourgeois males. Hence two alternative life history pathways are found (Chapter III):

1. “Bourgeois pathway” - males that express the bourgeois tactic at age 0+ (switch to bourgeois tactic from Mars to June) and reproduce as such in their first breeding season

and subsequent seasons. Males from this pathway hatch early in the season and switch to the bourgeois morphotype before or at the beginning of their first breeding season, with an average body size of 8.8cm. On average, they reproduce as bourgeois males during 1 breeding season (to a maximum of 3 seasons) and die at the end of that first season. The reproductive success of these males increases with age and size but their survivorship rate beyond the first breeding season is very low. Therefore only very few males achieve substantial reproductive success.

2. “Sneaker pathway” - males that reproduce as sneakers in their first breeding season switching to bourgeois after the end of that season (October to November), at age 1. In subsequent seasons they continue to reproduce as bourgeois males. Males that follow the “sneaker pathway” hatch late and their average body size is 5.8cm at the moment when males from the other pathway switch to the bourgeois tactic. They reproduce as sneakers during their first breeding season and switch tactics at the end or after that season, with an average body size of 9.5cm, reproducing as bourgeois males in all the subsequent seasons. Similarly to the “bourgeois pathway”, they may reproduce as bourgeois for a maximum period of 3 breeding seasons but on average they live only 2 years and reproduce as bourgeois in one breeding season, the second. Their reproductive success is much higher than that of the bourgeois pathway (Chapter III).

A small group of males also reproduces as sneakers on their first breeding season, despite early hatched, but they tend to be smaller than males of the bourgeois pathway (7.6cm when males from the bourgeois pathway switch to the bourgeois tactic).

Although two main life-history pathways were identified in this species, a third pathway may potentially be present. This would be mostly constituted by individuals that are recruited to the breeding grounds with a relatively large body size and later than the remaining individuals, after the breeding season. Their estimated growth curves revealed that during the breeding season previous to their tagging, these males had a body size larger than the expected body size at first maturation and therefore could have had already reproduced as sneakers. On the other hand, a study of the microhabitat use in this population (Chapter V) indicates that young immature males are the ones that preferentially occupy sheltered non-breeding areas. From perspective, this potential third life-history pathway would comprise immature males that postponed reproduction in their first breeding season. However, from a life-history perspective postponing reproduction should favour somatic growth which is not evident from the analyses of the data. Thus, the identification of a third potential life-history pathway was not possible in this study (Chapter III).

“Birth-date effect”

The two main life-history pathways described above are associated with the hatching date of the males. Males from the “bourgeois pathway” hatch early in the season while those from the “sneaker pathway” hatch later. Taborsky (1998) proposed the “birth-date effect” hypothesis to explain the expression of different tactics that have an underlying determination that is dependent on body size. Individuals born earlier should achieve larger body sizes at the breeding season, which might constitute an advantage for resource defence and therefore optimal for the expression of the bourgeois tactic. Contrary, individuals born later may have achieved, by the breeding season, an insufficient body size to effectively obtain and defend resources. So, the latter would be more successful as parasite than as bourgeois males. Additionally, in temperate climates differences in environmental conditions may vary considerably during a breeding season, particularly if seasons are long. These differences may lead to divergent growth patterns which may further differentiate body size at the moment of reproduction. In the peacock blenny, the hatching date is associated with the life-history pathway followed by males but the two pathways show no clear difference in growth patterns. Hence, the association between the hatching date and the life-history pathways followed by the males supports a “birth-date” effect. Although the effect of date of birth on growth, survival (Cargnelli and Gross, 1996; Wright and Gibb, 2005) and reproductive success (Dijkstra *et al.*, 1990) had been explored by some authors in diverse taxa, its specific effect on expression of ART has not been tested before. The hypothesis is now confirmed but not in all of its premises. The effect of date of birth observed was not due to differences in growth pattern but to the time elapsed between hatching of the males of the two pathways that confer the early hatched males more time to growth.

Conditional tactics and social status

Despite the consensus that any expression of a phenotype is always the product of a gene x environment interaction, alternative reproductive tactics can be considered conditional (Gross, 1996; Broackmann, 2001; Hazel *et al.*, 1990; Tomkins and Hazel, 2007) or genetically determined (genetic polymorphism: Brockmann, 2001; mendelian strategies: Shuster and Wade, 2003). In conditional tactics, individuals have the potential to express any reproductive tactic or follow any life history pathway, and the “choice” of the alternative expressed will be taken in response to environmental factors or to the individual's state. Alternatively, if tactics are genetically determined, the genetic programming of each individual will determine its reproductive tactic “independently” of

environmental conditions. In the case of genetic polymorphisms the reproductive success of the two tactics, or in this case of the life-history pathways, should be equivalent in a life time perspective (Brockmann, 2001; Shuster and Wade, 2003). Shuster and Wade (2003) consider that this should be the most widespread situation since they defend that the behavioural plasticity exhibited by conditional strategies is a derived character that, in order to emerge, needs cues associated with fitness and a predictable environment where these cues are systematically available to the population as a whole, not only to a minority of individuals. However, conditional tactics are undeniably demonstrated in many species such as the Atlantic salmon, *salmon salar* (Rowe and Thorpe, 1990; Garant *et al.*, 2003; Aubin-Horth and Dodson, 2004) or the horned beetles, *Onthophagus acuminatus* (Emlen, 1994; Emlen, 1997).

The observed association between hatching date and the reproductive tactic expressed initially by the males of the peacock blenny suggests that the expression of the tactics is conditional. If the ability to express or not the sneaker tactic initially would correspond to a genetic polymorphism, and therefore follow the mendelian rules of heritability, the frequencies of both pathways would not differ significantly between early and later hatched males as they do (Chapter III). Moreover, such an asymmetry in reproductive success of the different pathways, as the one found over the sampled period of seven reproductive cycles, makes it seem unlikely that the different pathways represent different genetic variants. These facts demonstrate that expression of the tactics in this species is condition dependent and ultimately a consequence of hatching date.

The expression of conditional tactics is based on the concept of threshold (Rolf, 1996). If a determinate cue reaches the value of that threshold, individuals under those circumstances will express one tactic but if that threshold is not achieved, the opposite tactic will be expressed. Some (Eberhard, 1982) have proposed that the parasitic tactic is expressed in face of insufficient resources that prevent an individual to achieve a minimum condition, like body size. However, in the Atlantic salmon, the opposite pattern is observed with the fastest growing males maturing earlier as parr, the parasitic tactic.

Two major hypotheses have been proposed as general mechanisms for expression of conditional tactics. The status dependent model (Gross, 1996; Gross and Repka, 1998 a, b) and the threshold model (Hazel *et al.*, 1990; Hazel *et al.*, 2004; Tomkins and Hazel, 2007; Emlen, 2008). Both models include the threshold concept but, while in the status dependent model the threshold is assumed as fixed and thus populations are genetically monomorphic in respect to the decision mechanism, in the threshold model the value of the threshold is itself variable between individuals and represents a genetic

polymorphism. This last model has been demonstrated for the European earwig (Tomkins and Brown, 2004) and the Atlantic salmon (Aubin-Horth and Dodson, 2004) based on variance in the threshold value observed between populations or even subgroups within the same population that inhabit different environments.

The integration of two studies, one descriptive and carried out in natural conditions (Chapter III) and a second one conducted in laboratory (Chapter IV) suggests that the expression of tactics in the peacock blenny is associated with social dominance and/or relative body size. One single threshold determining the onset of the expression of the bourgeois tactic could explain the occurrence of the two life-history pathways observed. Sneakers mimic females which are smaller and duller than males and have no secondary sexual characters; therefore both morphotypes show no morphological particularity and resemble juveniles. Thus, apart from the development of testes, sneakers are not expected to undergo any morphological development when maturing. Contrary, the expression of the bourgeois morphotype involves the development of specific structures like secondary sexual characters, specific coloration and gonadal accessory glands. Since within the life-history pathways identified all males mature once reaching the breeding season, only one switching point is necessary to differentiate those who will reproduce as sneakers, whose morphotype expression depends only on sexual maturation, and those who, lying above the threshold will reproduce as bourgeois males through the onset of development of their specific traits. In the peacock blenny, relative body size is associated with dominance which was experimentally demonstrated to influence the onset of the expression of secondary sexual characters, testicular gland and nesting behaviour, all typical traits of the bourgeois males (Chapter IV). Previously to the start of the breeding season, competition for nests is expected to be very intense and the dominance hierarchy expected to be very well defined due to the high number of interactions between males. Males who reproduce directly as bourgeois males are, at this point, the largest of their cohorts but when ranking the males of the two pathways of all the cohorts, the body size of the males of the bourgeois pathway is median, while that of the males from the sneaker pathway lies at the bottom of the distribution. At the end of the breeding season many bourgeois males die after parenting. The average longevity of males from both the sneaker as the bourgeois pathway is associated with a high mortality rate at the end of the breeding season. At the moment, when the males from the sneaker pathway switch to bourgeois males, their size rank will be the highest because most of the older males from the bourgeois pathway will have died, as well as the two years old males of the sneaker pathway. Therefore, these males will be the most dominant at the moment of switching, after the end of the breeding season.

Thus, the effect of social dominance detected in experimental conditions is compatible with the expected dynamic of the natural population and supports a threshold that is based on either relative body size or social dominance (so strongly associated that their effects are difficult to disentangle). Any of these two factors allows individuals to effectively adjust their phenotype to the social environment conditions and maximizes their reproductive success. The social environment changes predictably along the year, given the constraints of the system - scarcity of nests - and its demographic characteristics: a high density and competition preceding the breeding season and a high mortality of older males at the end of the season. The high predictability of the system dynamics and its strong effect on social dominance as predictive of successful defence of nest, and consequently reproductive success, renders the emergence of a conditional tactic viable (Shuster and Wade, 2003). However, the present analyses of the field data does not allow to test the variability, or lack of it, in the threshold value of the switch to the bourgeois tactic. Consequently, despite supporting a conditional expression of the tactics, the data cannot confirm or exclude either of the two models (status-dependent vs. threshold model).

Mechanisms of expression of reproductive tactics

The experimental data on the effect of social dominance on tactic expression demonstrated that dominance did not influence sexual maturation (chapter IV). This is consistent with the mechanisms of tactic expression exposed above. Males from both pathways reach sexual maturity simultaneously (although the sneaker pathway may be understood as an early maturation pathway given that it is composed of males that are some months younger than those following the other pathway). The males from the two pathways vary in morphology, behaviour and gonad characteristics but no evidence of postponed maturity was detected in association with any of the two pathways. Therefore a separation of mechanisms determining the onset of sexual maturity and the morphological and behavioural traits is not only compatible with the expression of reproductive alternative tactics in the peacock blenny but is the pivotal mechanism allowing the existence of these tactics.

This dissociation of gonadal and behavioural / morphological traits is reflected in the proximate mechanisms underlying the expression of the alternative tactics (Oliveira *et al.*, 2005; Oliveira *et al.*, 2008). Generally, in vertebrates, androgens are involved in the development of secondary sexual characters, reproductive behaviour and spermatogenesis (Nelson, 2005; reviews for teleost fish: Borg, 1994; Oliveira and Gonçalves, 2008). However, evidence has been gathered that questions the involvement

of sex steroids in the expression of reproductive behaviour (Oliveira *et al.*, 2008). In the studied species, 11-ketotestosterone (Oliveira *et al.*, 2001), testosterone (Chapter IV) and 17 β -estradiol (Gonçalves *et al.*, 2007) were not associated with or failed to promote territorial and nesting behaviour. Female-like courtship was however inhibited by 11-ketotestosterone and testosterone in sneakers (Oliveira *et al.*, 2001; Gonçalves *et al.*, 2007). On the other hand, the forebrain neuropeptide arginine vasotocin (AVT), induced female-like courtship in sneakers and females (Carneiro *et al.*, 2003) supporting an important role for neural pathways in behavioural expression. Central neuromodulatory mechanisms allow a faster regulation than hormonal ones which suits better the rapidity of behavioural expression, particularly if we consider biochemical switches (Zupanc and Lamprecht, 2000). Nonetheless, the morphological effects of sex steroids are clearer. In the peacock blenny, as in all fish species with alternative reproductive tactics that differ in morphology, the levels of 11-ketotestosterone are higher in the bourgeois tactic than in the parasitic tactic (Oliveira, 2005). 11-ketotestosterone has been identified as the main androgen in expression of secondary sexual characters (Borg, 1994) and that has been confirmed in the peacock blenny (Saraiva *et al.*, 2010), namely in the development of the anal gland (Oliveira *et al.*, 2001). The results presented in Chapter IV support a positive effect of social dominance on the development of the head crest that is achieved through 11-ketotestosterone. Despite this, the question remains, how can sneakers achieve sexual maturation and accomplish spermatogenesis, which depends on 11-ketotestosterone (Schulz and Miura, 2002), without the expression of secondary sexual characters, that are also dependent on 11-ketotestosterone. Three hypotheses have been proposed but remain to be verified (Oliveira *et al.*, 2008): 1. the effect of 11-ketotestosterone is dependent on dose and target tissue. This concept of compartmentalization may be achieved through different sensibility of target tissues due to differences in the density of androgen receptors (Ketterson and Nolan, 1999; Oliveira *et al.*, 2005; Taborsky *et al.*, 2008). In this way, gonads may respond to 11-ketotestosterone levels and carry out spermatogenesis while the remaining tissues continue immune to it. 2. A balance between 11-ketotestosterone /testosterone may allow the development of secondary sexual characters in bourgeois males without the excessive growth of gonads and the corresponding spermatogenic activity (Oliveira, 2005). Testosterone produced by the testes acts as a feedback element on hypothalamo–pituitary unit also in fish (Khan *et al.*, 1999), inhibiting the release of gonadotrophins and therefore reducing spermatogenesis. 11-ketotestosterone, on the other hand, promotes spermatogenesis (Schulz and Miura, 2002). Since testosterone will interfere with spermatogenesis through this negative feedback but this feedback does not involve secondary sexual characters, such a mechanism could also differentiate the effect of

11-ketotestosterone on those two traits. 3. Direct enervation of steroidogenic cells in the gonads from the hypothalamus has been described by Crews (1993). This alternative pathway between the brain and the gonads may constitute an autonomous way to organize gonad activity and development independently of circulating androgen levels.

Cortisol levels have been proposed to interact with androgens due to competition for the enzymatic complex 11 β -hydroxylase and 11 β -hydroxysteroid dehydrogenase. This enzymatic complex is involved in the conversion of testosterone to 11-ketotestosterone and in the activation and deactivation of cortisol (White, 1997; Bentley, 1998). If cortisol is dependent of social status, as it happens in some fish species (e.g. the African cichlid, *Haplochromis burtoni*: Fox *et al.*, 1997; Rainbow trout, *Oncorhynchus mykiss*: Winberg and Lepage, 1998), this could constitute a mechanism translating social dominance to androgen levels and in turn reproductive phenotype expression (Perry and Grober, 2003; Knapp, 2003). However, recent work in the protogynous sandperch (*Parapercis cylindrica*) specifically addressed this hypothesis but found no support for substrate competition between corticosteroids and androgens (Frisch *et al.*, 2007). In the peacock blenny no association was found between social dominance and cortisol levels also rejecting the existence of such mechanism in this species (Chapter IV). Despite the fact that in the peacock blenny, social dominance does not determine cortisol levels, this mechanism may be valid and should be tested in other species or for other factors determining cortisol levels.

In conclusion, the population of the Ria Formosa presents flexible ARTs that are expressed in a fixed sequence. However, two pathways were identified: the sneaker pathway in which the males express first the parasitic tactic (sneakers) and later the bourgeois tactic; and the bourgeois pathway in which males reproduce directly as bourgeois. Males from both pathways mature at the first breeding season (age 0+/1) but males of the bourgeois pathway have hatched at the start of the previous breeding season while those from the sneaker pathway hatched towards the end of the season. Therefore an effect of birth-date (Taborsksy, 1998) was identified, but unlike postulated, this effect is not due to distinct growth patterns of early and late hatched individuals rather it is simply due to the fact that early hatched males are allowed more time to grow until the breeding season. An environmental effect on the expression of ARTs in this population together with the fact that lifetime reproductive success of the two pathways, estimated along 7 reproductive periods, is distinct strongly suggest that ART expression in this population is conditional. A mechanism of one single switch point based on social dominance or relative body size may underlie the determination of the two pathways. But a crucial dissociation of physiological mechanisms needs to be considered between sexual maturation and

expression of specific bourgeois morphological traits. At the first breeding season all males appear to mature but some reproduce as sneakers, without further morphological development except that of testes, and others reproduce as bourgeois with development of all the typical morphological traits. Therefore, maturation is simultaneous in both pathways but the switching point will determine the subsequent expression of the bourgeois tactic either by the immature males (bourgeois pathway) or later by the sneakers (sneaker pathway). This threshold mechanisms that converts a level, or a range of levels above a minimum value, of social dominance (or relative body size) into bourgeois traits, is mediated by 11-ketotestosterone, in the specific case of the head crest. Other mechanisms need to be explored to understand the onset of sexual maturation in this population and specifically its dissociation from the development of morphological traits. Additionally, the physiological mechanisms mediating the conversion of social dominance in the bourgeois' remaining traits, besides head crest, is still to be understood.

Notes on population sampling

In chapter V, the study of the microhabitat distribution shows that the highest densities of this species are found on reproductive areas. Moreover, M+ males and sneakers are less frequent in other type microhabitats and therefore the area monitored for the study of life-history pathways is ideal to maximize the recapture of reproductive males. Within reproductive areas, those without vegetation and subjected to circadian exposure and submersion are the most representative of population composition and therefore the most parsimonious way of evaluating demographic parameters like sex ratios. Sampling at low tide represents well the general distribution of the different sex classes of this population through the different microhabitats and is practical because fish are inactive and easier to capture.

However, outside the breeding season females tend to migrate to breeding areas during the flood, when individuals are active. Thus, sampling of reproductive areas during low tide, despite representative of population parameters, may not mirror the perception of the social context that the individuals of this population have. Low tide sampling in these microhabitats may therefore underestimate parameters like local operational sex ratio outside the breeding season.

Seasonal variance should also be accounted for due to phenomena like recruitment and male mortality which affects both population density and composition.

Prospects for future research

Both from the ecological and from the physiological perspective, the mating system of the peacock blenny still reserves questions for further research and constitutes an ideal model to approach some of the central issues of the study of ARTs. For the knowledge gathered about the system, for its simplicity and accessibility, this population provides the means for an integrative approach to ARTs. Some future questions to be addressed in this model species are here suggested:

1. Independent quantification of resource, mate and sperm competition may reveal productive in elucidating and specifying mechanisms of sexual selection that cumulatively contribute to the organization of mating systems. In the study population, when quantifying parameters, such as OSR, sampling procedures should account for asymmetrical sex class distribution across microhabitats and seasonal and tidal phases.
2. Costs of reproduction, hypothesized to limit brood size and therefore reproductive rates of males and to bias operational sex ratios, may further clarify the driving factors of sex role reversal. The mechanisms dependent on testosterone that control brood size should be explored for further understanding of the trade-off between parental and mating investment.
3. Identification of selective forces that sustain the existence of alternative life-history pathways in this population, with such different reproductive successes, may clarify the dynamics of ARTs in general and the trade-off involved in their shaping. Specifically, it may prove productive to address the following topics, in this population:
 - a) The potential trade-offs involved in early and later hatching, *e.g.*, early hatching conditions lead to low reproductive success but may favour egg or first winter juvenile survival.
 - b) Environmental resilience conferred by the existence of a shorter pathway that reproduces quicker by the, supposedly, most successful tactic, and a longer pathway that despite initial low reproductive success presents higher lifetime reproductive success. Such combination of pathways may play a role in maintaining the alternative pathways in unstable environments. To assess this hypotheses paternity and fertilization success needs to be evaluated through genetic markers. Moreover, frequency dependent dynamic assuring that, both tactics may continue to occur. Since parasitic one year old males depend on bourgeois males to reproduce, a pathway of one year old bourgeois males ensures that even in harsher years, if survival is shortened reproduction is possible for both tactics. Additionally, in such years the

reproductive success of the bourgeois tactic would be expected to be higher, contributing as a stabilizing force to the maintenance of both pathways.

Maternal effects may also be important in the optimization of trade-offs, females may determine their timing of spawning as a function of their condition and consequent egg quality managing survival vs. reproductive success of offspring. Investigation of sex ratio manipulation along the breeding season is also a promising goal, given that females should benefit from early hatching and males from later hatching.

4. For further knowledge of the model system, the possible third life-history pathway of postpone reproduction of males and how does microhabitat distribution and forms of recruitment to the mudflat influence its occurrence, should be evaluated. Would this pathways be confirmed, two thresholds should be examined: the threshold of expression of the morphological, behavioural and testicular traits of bourgeois males proposed above, and a second one determining sexual maturation.

5. Testing the hypotheses reviewed by Oliveira *et al.* (2008) regarding the dissociation of spermatogenesis and other traits dependent on 11-ketotestosterone are essential to understand the physiological mechanisms of ARTs expression, namely:

a) Compartmentalization of the sensitivity to sex steroids achieved through endocrine receptors and also of the availability of such steroids to target tissues modulated by binding globulins and steroidogenic enzymes, including those competing with glucocorticoid substrates.

b) How the 11-ketotestosterone/testosterone ratio may control spermatogenesis.

c) Direct innervations of the gonads by the hypothalamus, bypassing the need to induce spermatogenesis through circulatory levels of 11-ketotestosterone.

6. Finally, exploring the mechanisms through which social dominance is converted in secondary sexual characters, testicular gland and behaviours typical of bourgeois males, in which the mediation of 11-ketotestosterone on the development of the anal gland should be confirmed. Identifying the potential crucial role of the testicular gland, as the major product of 11-ketotestosterone which promotes SSC and inhibits female type behaviours expressed by sneakers. Evaluating other neural mechanisms as possible mediators of the effect of dominance on behaviour, including AVT when considering biochemical switches but also neurogenesis associated with structural reorganization.

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