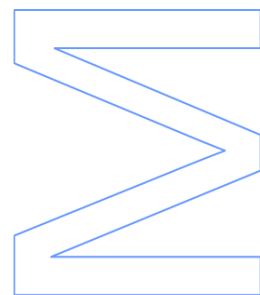




The evolution of reproductive barriers in the ongoing ecological speciation of *Littorina saxatilis*



Zita FERREIRA

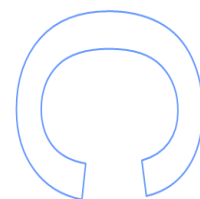
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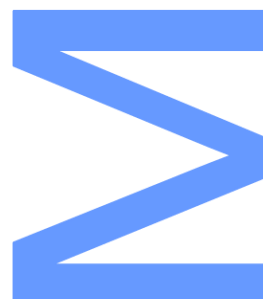




Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____/____/____



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Abbreviations

2DE: 2-dimensional electrophoresis

DNA: Deoxyribonucleic Acid

IEF: Isoelectric focusing

HY: hybrid

MS: mass spectrometry

MS/ MS: tandem mass spectrometry

pI: isoelectric point

RB : ridged and banded ecotype of *Littorina saxatilis*

RPs: reproductive proteins

RT: reproductive tissues

SDF: Sperm DNA fragmentation

SU: smooth and unbanded ecotype of *L. saxatilis*

VERL: vitelline envelope receptor for lysin

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Abstract

In exposed Galician rocky shores, two ecotypes of the periwinkle *Littorina saxatilis* are found adapted to distinct shore levels and habitats, which evolved and are maintained even in the face of gene flow.

Several pre-zygotic isolation mechanisms have been reported to restrict gene flow between these two ecotypes but, until now, no post-zygotic isolation mechanisms have been confirmed. To test for the existence of post-zygotic isolation between the Galician ecotypes of *L. saxatilis* a study comparing the fertility of males from both ecotypes and hybrids was conducted. The comparison, based on the analysis of sperm DNA fragmentation, reveals that hybrids' sperm DNA is significantly more fragmented, suggesting the existence of an incipient post-zygotic reproductive isolation.

Although reproductive proteins (RPs) have been shown to be important for the evolution and maintenance of reproductive barriers in other model systems, the role of such proteins in the speciation of *Littorina* genus is unknown. Therefore, a proteomic analysis of five reproductive tissues was carried out, aiming to identify putative reproductive proteins. With this work we have been able to identify five candidate male and two candidate female RPs.

Resumo

Littorina saxatilis é um molusco marinho para o qual estão descritos dois ecótipos na Galiza, adaptados a diferentes zonas do intertidal, e que evoluíram e se mantêm apesar da ocorrência de fluxo génico.

Vários mecanismos de isolamento pré-zigóticos foram já descritos entre estes dois ecótipos mas, até ao momento não se confirmou a existência de nenhum mecanismo de isolamento pós-zigótico. Para testar a existência de isolamento pós-zigótico entre estes ecótipos realizou-se um estudo comparativo da fertilidade dos

machos de ambos ecótipos e dos seus híbridos, através da análise da fragmentação do ADN do esperma. A fragmentação do ADN do esperma revelou-se significativamente superior nos híbridos do que nas formas parentais, sugerindo a existência de uma barreira reprodutiva pós-zigótica incipiente.

Embora se tenha demonstrado em várias espécies que as proteínas reprodutivas (PRs) podem desempenhar um papel importante na evolução e manutenção de barreiras reprodutivas, nada se sabe sobre o papel destas proteínas na especiação do género *Littorina*. Para identificar PRs neste género analisaram-se cinco tecidos reprodutivos utilizando ferramentas proteómicas. Este trabalho permitiu identificar 5 putativas proteínas reprodutivas masculinas e duas femininas.

Chapter 1: Introduction

Species can be defined in several ways (Balakrishnan, 2005), each definition focusing on a specific aspect of the speciation process. However *“no one definition has as yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species”* (Darwin, 1859). Indeed, even today there is no agreement on scientific community regarding the species definition and taxonomic categories will inevitably vary between researchers (De Queiroz, 2007), given its continuous evolution and the diversity of processes leading to speciation. Unfortunately taxonomy mostly focus on extant biodiversity, barely taking into account the dynamic process of evolution, divergence and speciation (De Queiroz, 1995). Evolutionary scientists try to uncover the evolutionary history of species and processes driving speciation (Coyne & Orr, 2004) in order to understand how species arise (Blackman & Rieseberg, 2004; Coyne & Orr, 2004; Palumbi, 1994).

Since the publication of Darwin's famous book in 1859, several models have been proposed to explain the processes driving speciation (Butlin *et al.*, 2006; Hendry & Day, 2005; Turelli *et al.*, 2001). One of the most polemic questions on speciation is related with its geographic context (Coyne & Orr, 2004; Kirkpatrick & Ravigné, 2002): does speciation require geographical isolation? Based on the spatial distribution of organisms during their divergence process, three models were described: allopatric, parapatric and sympatric. Allopatric speciation is the most common and widely accepted model (Butlin *et al.*, 2008; Coyne & Orr, 2004; Rolán-Álvarez, 2007; Turelli *et al.*, 2001) and occurs when geographic isolation precludes gene flow between two populations allowing their divergence over time due to drift and/or selection and ultimately leading to speciation.

By contrast, non-allopatric speciation occurs when gene flow is maintained during the divergence period (Butlin *et al.*, 2008; Rolán-Álvarez, 2007). Non-allopatric

speciation includes parapatric and sympatric models. Speciation is classified as parapatric when gene flow during the divergent process is higher than 0 (allopatric speciation) and lower than 0.5 (sympatric speciation; Coyne & Orr, 2004). Parapatric speciation can occur in a narrow hybrid zone (Jiggins & Mallet, 2000; Reid, 1996) due to the existence of environmental differences across the distribution area although this speciation model can cause population divergence under distinct biological scenarios (Coyne & Orr, 2004; Gavrillets *et al.*, 2000). Parapatric divergence represents a good alternative scenario to allopatric divergence in the process of speciation of coral-reef fishes (Rocha *et al.*, 2005) as these species usually live in highly fragmented habitats, often distributed as a metapopulation, with subpopulations connected through gene flow due to dispersal during the pelagic larval stage (Rocha & Bowen, 2008). Hybridization speciation is another example of parapatric speciation (Jiggins & Mallet, 2000). This kind of speciation is frequent in plants (Buerkle & Rieseberg, 2001; Rieseberg & Willis, 2007), and despite being much less frequent, it also happens in animal taxa.

Among the different speciation models none is as controversial and polemic as the sympatric model. Only over the last decade the occurrence of sympatric speciation started to be widely accepted by biologists (Coyne & Orr, 2004; Lessios, 2007; Ödeen & Florin, 2000; Via, 2001). The resistance of the scientific community to sympatric speciation was due to the almost absence of clear examples and to the special conditions required for divergence to occur in sympatry (Ödeen & Florin, 2000; Via, 2001). Indeed as sympatric speciation occurs within a panmictic population, it requires strong natural and/or sexual disruptive selection to restrict gene flow between two groups of individuals. Sympatric speciation will only occur when the strength of selective forces driving divergence overcomes the homogenizing effect of effective gene flow (Gavrillets *et al.*, 2008). These conditions are expected to be rare in nature and because of this, allopatric speciation is considered the null model that is necessary

to reject in order to prove sympatric speciation (Johannesson, 2001). To consider sympatric speciation as the most likely process of divergence of two species, it is required that these occur in sympatry and are the closest sister species and that the existence of an allopatric phase of divergence is highly unlikely (Coyne & Orr, 2004). As some hybridization (introgression) can make two species look genetically closer than they originally were (Coyne & Orr, 2004), it is always possible that putative examples of sympatric speciation resulted from allopatric divergence followed by a secondary contact. Accordingly, to reject allopatric speciation is almost impossible and until recently, this was a big obstacle for the acceptance of sympatric model. Despite of this, mathematical simulations have shown that sympatric speciation is possible under certain biological conditions (Gavrilets, 2003), such as sexual conflict (Gavrilets & Waxman, 2002) and assortative mating coupled with disruptive selection (Gavrilets, 2003). More recently, with the evolution of the genomic tools, a better and more empirical knowledge has started to be gathered on speciation in the face of gene flow (Smadja & Butlin, 2011), originating some new theories about its causes (Nosil & Feder, 2012).

Sympatric speciation is expected to occur more often when the same phenotypic character causes both disruptive natural selection and assortative mating (Crow *et al.*, 2010; Rice, 1984). The divergence of *Rhagoletis pomonella* (Bush, 1969; Feder *et al.*, 1988) and the palm trees *Howea's* (Savolainen *et al.*, 2006) are clear examples of such process.

As knowledge about speciation increases, authors tend to abandon the geographical classification and focus on the mechanisms that leads to speciation events (reviewed by Smadja & Butlin, 2011). Among those mechanisms are sexual and natural selection (Kirkpatrick & Ravigné, 2002) associated with reinforcement (Servedio & Noor, 2003).

Speciation is the emergence of reproductive barriers between individuals descending from an initial single species (Coyne & Orr, 2004). Reproductive barriers usually do not arise all at once; by restricting gene flow, the first barrier to evolve facilitates the evolution of additional isolation mechanisms that further contribute to speciation. Reproductive barriers may be classified as pre-zygotic or post-zygotic. Pre-zygotic barriers prevent the formation of hybrid zygotes and may act either before or after mating. Premating isolation prevents the copula of individuals from two distinct forms. These barriers include factors affecting the choice of a sexual partner, a complex behaviour based upon rituals that may rely on several different kinds of stimuli: visual, sonorous, tactile and/or chemical. The courtship traits and associated preferences are some of the causes of reproductive isolation in animal taxa (Jiggins & Mallet, 2000; Ritchie, 2007). Multiple examples can be found across taxa; from the nuptial parade of birds such as *Chlamydotis undulate* (Gaucher *et al.*, 1996) or *Sula nebulosii*; (Velando *et al.*, 2005) to the pheromone trail left by some female insects to attract males [species of the genus *Yponomeuta* and *Heliothis* (Smadja & Butlin, 2009); among others].

Pre-mating barriers can arise when a trait determining the habitat or host choice is under divergent selection (Johannesson, 2001; Knowlton *et al.*, 1997; Lessios, 2007; Rundle & Nosil, 2005). Pre-zygotic barriers may also act after mating but before fertilisation; these barriers are called post-copulatory (or post mating)-pre-zygotic. Post-insemination pre-zygotic mechanisms are difficult to study and are often indirectly determined (Birkhead & Pizzari, 2002; Klug *et al.*, 2010). Post-copulatory sexual selection exerted by female cryptic choice from promiscuous species can influence the evolution of multiple phenotypes as for example the size of the sperm or its motility (Snook, 2005). Female cryptic choice can happen until fecundation. In feral fowl, *Gallus gallus domesticus*, for example, a female can eject the sperm of a male that she does not want to sire the offsprings (Pizzari & Birkhead, 2000). Bretman *et al.*, (2004)

demonstrated that females of the cricket *Gryllus bimaculatus* select the sperm of unrelated males over the related ones. A similar mechanism of female cryptic choice has been reported for the sympatric populations of *Drosophila yakuba* and *D. santomea* (Matute, 2010) in São Tomé Island, but not in the allopatric populations, suggesting that reinforcement may play an important role in the evolution of such phenotypes. *D. yakuba* females from sympatric population deplete *D. santomea*'s sperm faster than that of conspecific males (Matute, 2010).

Post-zygotic isolation results from the reduced viability and/or fertility of the hybrids either due to extrinsic or intrinsic factors (Turelli *et al.*, 2001). Extrinsic isolation results from a reduced hybrid ability to fit into the parental and/or available habitat (Agrawal, Feder, & Nosil, 2011; Coyne & Orr, 2004). In some cases, sexual selection against hybrids may also be an extrinsic reproductive barrier if sexual displays are environmentally mismatched (Keller & Gerhardt, 2001; Rundle & Nosil, 2005).

Intrinsic post-zygotic isolation is the best studied and therefore the best known type of post-zygotic barriers (Coyne & Orr, 2004). Intrinsic isolation can result from Dobzhansky-Muller incompatibilities (DMI; Coyne & Orr, 2004; Nosil & Schluter, 2011). The DMI result from fixation of derived alleles in two or more different loci. Fixed mutations at different loci that evolved independently in two divergent lineages may be detrimental when brought together in the same individual (hybrid; Coyne & Orr, 1998; Giraud *et al.*, 2008). The simplest model considers two populations derived from a common ancestor with two monomorphic loci AA and BB. Each of the daughter populations fixes a mutation in a different locus so that one population has the genotype aaBB and the other the genotype AAbb. If the mutations a and b are not (fully) compatible, an individual with the genotype AaBb (hybrid) will display reduced fitness (Coyne & Orr, 2004). The Dobzhansky-Muller model can explain sub-lethal and condition-dependent hybrid dysfunction (Fitzpatrick, 2008a, 2008b) as well as hybrid sterility and inviability. Those hybrid dysfunctions are expected to accumulate over

time, as divergence further increases between two lineages (Bolnick & Near, 2005; Gavrillets, 2003) with the subsequent increase of DMI (Fitzpatrick, 2008b). Although a large number of loci can interact in the hybrid genetic background (Nosil & Schluter, 2011), two non-compatible loci are enough to cause DMI (Gavrillets, 2003). The cumulative effect of many incompatibilities may result in sterility and inviability even if each incompatibility *per se* only has small effects on hybrid fitness (Orr & Turelli, 2001).

Several reproductive proteins (RPs) are required for reproductive processes from gametogenesis to fertilisation (typically associated with pre-zygotic-post-mating reproductive barriers; Swanson & Vacquier, 2002a), pregnancy and, in mammals, lactation (Speakman, 2008). Although many RPs express exclusively in reproductive tissues (Findlay *et al.*, 2008; Panhuis, Clark, & Swanson, 2006), others may have several functions as it is the case of SP-40,40 or clusterin (Wong *et al.*, 1994), a protein that is common in human blood and that is also found in the seminal plasma at much higher concentrations (Kirszbaum *et al.*, 1989).

RPs are especially interesting from an evolutionary point of view as most of them have been shown to evolve rapidly and under positive selection (Clark *et al.*, 2009; Clark *et al.*, 2006; Marshall *et al.*, 2010; Swanson *et al.*, 2004). RPs have been intensively studied in marine invertebrates (reviewed in Turner & Hoekstra, 2008) such as abalones (*Haliotis*; Lee *et al.*, 1995; Swanson & Vacquier, 1998; Vacquier, 1998; Yang, *et al.*, 2000) and sea urchins (*Echinometra*, *Strongylocentrotus*; Geyer & Palumbi, 2003; Levitan & Ferrell, 2006; Palumbi & Metz, 1991). In sea urchin, most of the research focused on the male protein *bindin* that mediates the attachment of the sperm to the egg required for fertilization (Lessios, 2007). In *Haliotis* spp a pair of interacting proteins has been intensively studied: the male lysin and its female receptor, the vitelline envelope receptor for lysin (VERL). Like *bindin*, lysin and VERL are involved in the process of binding sperm to egg but they also induce acrossome reaction (Swanson & Vacquier, 1997). Those three proteins, found on the surface of

gametes membranes, evolve faster than non-reproductive proteins (Panhuis *et al.*, 2006; Swanson & Vacquier, 2002b). RPs from *Drosophila* and crickets have also been shown to evolve fast, being on average twice as divergent as those expressing in non-reproductive tissues (Marshall *et al.*, 2010; Swanson, Clark, *et al.*, 2001; Swanson *et al.*, 2004). Similar observations have been reported for the Mammalian zona pellucida egg coat proteins ZP2 and ZP3 and the oviductal glycoprotein – OGP (Swanson, Yang, *et al.*, 2001), which bind the sperm and induce the acrossome reaction. Other RPs influence the receptivity of the females to remate (*Drosophila's* Acps; Wolfner, 2009) or prevent females from being fertilized by other males (Dean *et al.*, 2009; Kingan *et al.*, 2003). The properties and features of RPs make them potential candidates for speciation genes, as these may cause the evolution of reproductive isolation barriers.

Several proteomic methods have been used for studying individuals and species proteins. These allow to study proteins expression and to identify proteins at a large-scale (Aebersold & Mann, 2003). Although the genome of an individual is the same during all its life, the proteome is continuously changing, adjusting itself according to the different situations, developmental stages and circumstances that an organism faces during its lifetime. Thus proteomic studies are snapshots of protein expression at a given moment and under certain conditions (Görg *et al.*, 2004; López, 2005). Therefore, proteomic studies are required for fully understand the complexity of biological systems, as they allow to simultaneously analyse a multiplicity of proteins (López, 2007) . Moreover proteomics makes possible to quantitatively describe protein expression and to study how expression is affected by biological disturbances (López, 2005). Accordingly, proteomic analyses may provide a valuable contribution to evolutionary and speciation studies (Diz *et al.*, 2012; Diz & Skibinski, 2007; Enard *et al.*, 2002).

Proteomic techniques like 2DE can provide a genetically independent data set for reconstructing a phylogeny (Goldman *et al.*, 1987; López, 2005; Navas & Albar, 2004).

However the most important aspect of proteomics is that no prior knowledge of the protein sequence is required as it can be determined *a posteriori* by mass spectrometry (López, 2007). 2DE involves two successive electrophoreses: the first separates the proteins by their isoelectric point (Görg *et al.*, 2009; Görg *et al.*, 2004), while the second separates proteins according to their relative molecular mass. 2DE is able to resolve up to 10 000 proteins spots simultaneously (Görg *et al.*, 2004). The technique allows detecting and quantifying proteins amounts as low as 1 ng per spot (Görg *et al.*, 2004; López, 2007). However, and despite its interesting applications, 2DE resolution is limited by the physical boundaries of the gel itself. Proteins with extreme isoelectric points (< 3 or > 10) and/or mass < 15 or > 200 kDa cannot be resolved through 2DE (Wu & MacCoss, 2002). To analyse those proteins gel-free protein separation approaches, such as shotgun proteomics were developed. This technique relies on triptic digestion of the sample, followed up by a mass spectrometry analysis (Old *et al.*, 2005). Although these techniques are different and each has its own advantages and limitations (Rabilloud, 2002), they complement each other. Mass spectrometry allows the identification of proteins (Wu & MacCoss, 2002). It requires a proteolytic digestion of the sample, resulting in a complex peptidic mixture compatible with the mass spectrometer. This methodology can be applied both to a solution extracted from a tissue and to a spot excised from a gel after bidimensional electrophoresis (2DE).

The model organism: *Littorina saxatilis*

As previously mentioned, few examples of non-allopatric speciation are widely accepted by the scientific community. One of the best documented examples of such process is the evolution of ecotypes of *Littorina saxatilis* (Olivi, 1792; Butlin *et al.*, 2008; Quesada *et al.*, 2007; Rolán-Álvarez *et al.*, 2004), an intertidal gastropod species. *L. saxatilis* is a dioecious and polygamous species with internal fertilization (Cruz *et al.*, 2004) for which ecotypes have been described and studied in three areas of the

northern Atlantic rocky shores (Sweden, England and Spain; Butlin *et al.*, 2008; Pérez-Figueroa *et al.*, 2007). In each one of these areas *L. saxatilis* displays a specific morph (or ecotype) pair (Butlin *et al.*, 2008). Despite its independent evolution in the distinct areas, the ecotype pairs show similar morphological adaptations (Johannesson, 2003; Quesada *et al.*, 2007; Rolán-Álvarez, 2007). The ecotype living in the less exposed areas of the shore is larger with a thick shell but a relative narrow aperture (Butlin *et al.*, 2008). The ecotype living in the most exposed areas of the shore is small, with a thin shell and a wide aperture. The Spanish ecotypes are called ridged and banded (RB) and smooth and unbanded (SU). The RB ecotype lives preferentially on the upper shore associated to the barnacle belt (*Chthamalus* spp; Conde-Padín, *et al.*, 2008; Johannesson *et al.*, 1995; Rolán-Álvarez, 2007). RB is the largest ecotype; its size and thick shell allows the individuals to resist predator attacks while the small aperture minimizes water loss during the long periods of desiccation to which organisms are exposed at this shore level (Butlin *et al.*, 2008; Johannesson *et al.*, 1993; Johannesson *et al.*, 1995). The SU ecotype can be found at the lower shore, on the mussel belt (Rolán-Álvarez, 2007). Its small size and strong muscular foot allows SU to resist dislodgement by the heavy wave action (Smith, 1981). This strong muscular foot is associated to a relative wider aperture area than that observed in RB (Carvajal-Rodríguez *et al.*, 2005). The most striking morphological difference between RB and SU is their size as SU is about half the RB's size (Rolán-Álvarez, 2007). These two ecotypes of *L. saxatilis* co-exist in some exposed Galician shores, and can meet and reproduce (producing fertile hybrids) at mid-shore, where barnacles and mussels overlap forming a patchy microhabitat (the typical width is about one to five meters; Galindo *et al.*, 2009; Pérez-Figueroa *et al.*, 2005; Rolán-Álvarez *et al.*, 1999; Rolán-Álvarez, 2007).

The Spanish ecotypes of *L. saxatilis* represent an example of incipient speciation. The reproductive isolation barriers documented until now are mostly pre-zygotic, mainly

pre-copula (Rolán-Álvarez, 2007). This species reproduces all year round (Reid, 1996). Males find potential partners by following mucous trails but, unlike other species of the *Littorina* genus, *L. saxatilis*' females do not release a gender-specific cue in their trails (Johannesson *et al.*, 2010). Strong assortative mating based on body size has been described with males of both ecotypes showing preference for females of a size similar to their own (Cruz *et al.*, 2004; Erlandsson *et al.*, 1999; Rolán-Álvarez *et al.*, 1999, 2004). As females of this species are highly promiscuous, sperm of several males can be deposited into the female's *bursa copulatrix* (sperm storage organ). In males of *L. saxatilis* after spermatogenesis the sperm is stored in the seminal vesicle (Buckland-Nicks *et al.*, 1999). Before ejaculation sperm crosses the prostate where it is capacitated and mixed with proteic secretion (Buckland-Nicks *et al.*, 1999). At these stages and until fertilisation, several male RPs are expected to interact with female RPs and these may be target of sexual selection due to female's cryptic choice or to intra-sexual competition. These processes may cause assortative mating between ecotypes and thus contribute to the on-going speciation. Unfortunately, nothing is known regarding the *L. saxatilis*' proteins involved in reproduction.

This work aims to increase knowledge on the mechanisms contributing to maintain the morphological and genetic differentiation between the Spanish ecotypes of *L. saxatilis*, despite of the gene flow occurring between these. We will test for the occurrence of post-zygotic reproductive isolation in this model system due to reduced hybrid male fertility. Male fertility can be inferred from the percentage of sperm showing DNA fragmentation (SDF; Evenson & Wixon, 2006). Accordingly we will use SDF as an indicator of sperm viability and compare the degree of SDF observed in hybrids with that observed in pure RB and SU individuals

Reproduction is a complex process that depends on a set of specific proteins (RPs). The high density of *L. saxatilis* individuals (Carballo, *et al.*, 2005), associated

with the high promiscuity observed in females of this species (Panova *et al.*, 2010) make RPs good candidates for playing a role in the on-going speciation process. Accordingly, this work aims to identify putative male and female RPs. For that, proteomic analyses will be performed in several reproductive tissues including testis, seminal vesicle and prostate in males and ovary and bursa copulatrix in females.

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Chapter2: Incipient pos-zygotic isolation between *Littorina saxatilis* ecotypes due to reduced viability of hybrids' sperm

Abstract

Very few examples of non-allopatric speciation are known and accepted by scientific community and for that reason knowledge on the evolution of reproductive barriers in such context is still scarce. *Littorina saxatilis* is one of the best known and documented examples of non-allopatric speciation. In some exposed Galician shores two ecotypes (RB and SU) of this species, adapted to distinct shore levels and habitats, can be found to occur in sympatry. Most of the reproductive barriers already described between these ecotypes are pre-zygotic. In the present work we test for the existence of post-zygotic isolation between the Spanish ecotypes of *L. saxatilis*, by comparing the fertility of hybrids (HY) with that of parental forms, through analyses of sperm DNA fragmentation. Individuals morphologically identified as RB, SU and HY were collected at mid-shore in Cabo Silleiro, where these ecotypes occur in sympatry. Sperm DNA fragmentation analyses were conducted in sperm collected from seminal vesicles. Our results reveal a significantly higher percentage of sperms with fragmented DNA in hybrids than in both pure forms. These results suggest that an incipient post-zygotic barrier exists between these two recently diverged ecotypes, which is probably contributing to their reproductive isolation.

Introduction

The intertidal snail *Littorina saxatilis* (Olivi, 1792) represents one of the best known and documented examples of on-going non-allopatric speciation. This gonochoric and polygamous species with internal fertilization (Cruz *et al.*, 2004a) can be found in a large variety of habitats across north Atlantic shores, from exposed cliffs

to macroalgae, rocky shore boulders, salt marshes and mudflats (Panova *et al.*, 2011). In Sweden, United Kingdom (UK) or northwest Spain (Galicia), two ecotypes, each adapted to different shore levels and habitats, can be found in sympatry (Butlin *et al.*, 2008; Rolán-Álvarez 2007). Although the ecotypes are believed to have evolved independently in each of these three areas, similar adaptations can be found in ecotypes from similar micro-habitats (Butlin *et al.*, 2008; Quesada *et al.*, 2007). These ecotypes result from an incipient ecological speciation process (Erlandsson *et al.*, 1999; Johannesson *et al.*, 1995; Rolán-Álvarez 2007) and provide the ideal framework to study the mechanisms that allow divergence to proceed in the face of gene flow.

In Spain two ecotypes of *L. saxatilis* occur in highly exposed shores: the ridged and banded ecotype (RB) and the smooth and unbanded ecotype (SU). The RB occurs in the upper shore and has a large and robust shell to protect against crab predation and a small aperture to minimize water loss and avoid desiccation (Conde-Padín *et al.*, 2007; Galindo *et al.*, 2009). Contrastingly SU is adapted to lower shore, possessing a smaller shell with a relatively larger aperture that encloses a strong muscular foot that prevents dislodgement by the heavy wave action (Conde-Padín *et al.*, 2007; Galindo *et al.*, 2009). At mid-shore, the two habitats overlap, forming a hybrid zone. Typically, Galician *L. saxatilis*' hybrid zone occupies one to five meters (Rolán-Álvarez, 2007) and is formed by patches of the habitats of both pure forms (micro-habitats; Carballo *et al.*, 2005; Erlandsson *et al.*, 1999; Galindo *et al.*, 2009; Pérez-Figueroa *et al.*, 2005; Rolán-Álvarez, 2007). In these hybrid zones the two ecotypes occasionally mate producing fertile HYs (Cruz *et al.*, 2004b; Rolán-Álvarez, 2007) usually reaching a frequency of 5-20 % (Johannesson *et al.*, 1993; Rolán-Álvarez *et al.*, 1999).

Until now, mainly prezygotic barriers were found to restrict gene flow between the Spanish ecotypes (Rolán-Álvarez, 2007), although some evidence suggest that divergent sexual selection in morphological traits can be reducing reproductive success of intermediate morphological HYs (Cruz & García, 2001). Ecological displacement

between the two ecotypes leads to their micro-geographical isolation, given the reduced dispersal usually observed in individuals from this species (Fernández *et al.*, 2005). In places where the two ecotypes meet, gene flow between them is reduced due to assortative mating. Both ecotypes of *L. saxatilis* are known to form micro-aggregates of individuals of similar size (Cruz *et al.*, 2004b) and to select their mate based on body size (Cruz *et al.*, 2004a; Rolán-Álvarez *et al.* 2004), frequently mating with others from the same aggregate (Johannesson *et al.* , 1995; Rolán-Álvarez *et al.*, 1999). No significant differences were found in relative male gonad area and colour or female fecundity, in individuals morphologically classified as HYs, SU or RB (Johannesson *et al.*, 2000), suggesting that fertility of HY individuals is similar to that of pure forms. However, the viability of sperm cells was never studied in this species. Male fertility is directly related to sperm viability which can be estimated using the percentage of sperm's DNA fragmentation (SDF). In several species SDF has been shown to be negatively correlated with fertilisation (Evenson *et al.*, 1999; Evenson *et al.*, 1994; Morris *et al.*, 2002) and birth rates (Agarwal & Allamaneni 2004) and positively correlated with abortion rates (Benchai, 2003). SDF is also negatively correlated with adult progeny viability (Fernández-Gonzalez *et al.*, 2008). Taken together, these data suggest that SDF analyses may provide important information not only on the fertility of an individual but also on the viability of its progeny, thus being particularly interesting in the context of speciation research.

In the present work we will test for the occurrence post zygotic isolation between the two Galician ecotypes of *L. saxatilis* due to reduced fertility in HY males. Male HY fertility will be inferred from analyses of SDF conducted in individuals morphologically classified as HYs, RB and SU.

Methodology

Individuals were sampled in September 2010 at *Cabo Silleiro*, in the northwest coast of Spain (42°6'N, 8°53'W). Several individuals were collected simultaneously at the hybrid zone (mid-shore level) and morphologically classified as SU, RB or HY (Carballo *et al.*, 2005). Individuals showing intermediate shell features between the two ecotypes (banded but smooth, ridged but unbanded or at least two broken bands) were classified as HY (Johannesson *et al.*, 1993). The individuals were sexed (Johannesson *et al.*, 1993) and the adult males were anaesthetised by immersion in a solution of Mg₂Cl 7.5 % during 30 minutes. The seminal vesicle of each individual was dissected, preserved in Chromacell's kit buffer (see appendix 1) and maintained at -80 °C until further analyses. All the individuals infected with parasites were discarded. For each individual (appendix 2 for sampling size) sperm cells were taken from the dissected seminal vesicle and treated according to the Chromacell's kit manufacturer's *instructions* and analysed according to López-Fernández *et al.* (2009). For each individual, 100 sperm cells were randomly chosen and the percentage of these cells with normal and fragmented DNA was determined by counting under fluorescence microscope (appendix 2; Johnston *et al.*, 2007; López-Fernández *et al.*, 2009).

Using PopTool (Hood, 2010), we conducted hierarchical G-tests (Sokal & Rohlf, 1995) to test for the existence of significant heterogeneity in SDF of individuals both within and between each of the three morphological classes of individuals. G-tests were chosen over chi-square tests, due to the small sample size of each morphological class (Hoey, 2009; McDonald, 2009). To test for the heterogeneity caused by joining distinct morphological class we have calculated the value of G-test for samples including the individuals from two morphological classes and subtracted to this value

the sum of the G-test values obtained for each of the two morphological classes. The same procedure was applied to estimate the degrees of freedom of this test.

Results

Chromacell's protocol greatly facilitated the distinction between healthy sperms and sperm cells with fragmented DNA (Fig. 1) under the fluorescence microscope. Healthy sperms present a tight halo while sperms with fragmented DNA present a

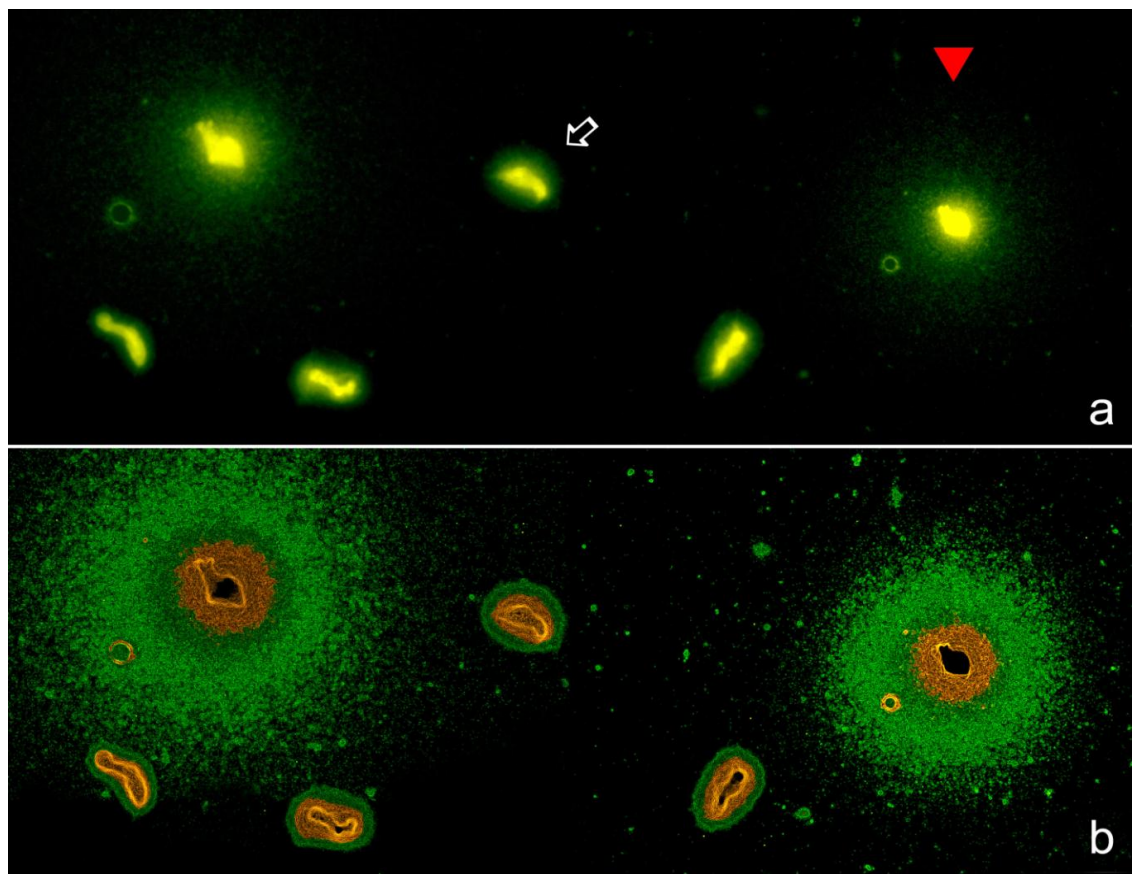


Figure 1: Sperm treated with Halomax Proto-Littorina, observed under Fluorescence microscope (a) and after informatic image correction (b). Red arrow points to sperm with DNA fragmentation and yellow arrow points to normal sperms.

larger and more diffuse halo (Fig. 1b). The percentage of normal and DNA damaged sperms observed for each individual is presented in appendix 2. The results, summarised per ecotype, are presented in Table 1.

According to the results of the G-test, SU individuals show no significant heterogeneity in the percentage of DNA fragmentation of its gametes, contrasting with RB and HY individuals (table 1). The same test reveals no significant differences in the

percentage of SDF between RB and SU individuals. However, G-tests reveal significantly higher levels of SDF in HYs when compared to both RB and SU. In fact, HY show, in average, more than twice the number of sperm cells with fragmented DNA than each of the two pure forms (HY: 7.95%, RB: 3.23%; SU: 2.00%).

Table 1: Results of G-tests conducted for testing the existence of significant differences in sperm DNA fragmentation (SDF) between pairs of morphological classes. Fragmentation: percentage of sperm cells with fragmented DNA observed in each morphological class; Variance: variance observed in SDF; G: value of G test; Df: degrees of freedom; P: probability of homogeneity within groups ($P < 0.05$ indicated in bold); N: number of males analysed; Sperm cells: Total number of sperms analysed; SU: smooth and unbanded ecotype; RB: ridged and banded ecotype; HY: hybrids.

	Among all	Between RB and SU	Between RB and HY	Between HY and SU	RB	HY	SU
Fragmentation					3.23	7.95	2.00
Variance					15.85	79.08	2.91
G	56.39	3.35	28.80	48.65	53.75	115.09	17.25
DF	2	1	1	1	12	12	11
Probability	5.70E-13	6.71E-02	8.01E-08	3.05E-12	3.03E-07	5.96E-19	1.01E-01
N					13	13	12
Sperm cells					1297	1316	1194

Discussion

Our results reveal significantly higher SDF rates in HY, when compared to both pure forms, suggesting the existence of an incipient post-zygotic isolation between these two ecotypes due to reduced HY fertility.

In several species (such as bull, ram, boar, rabbit, dog and humans), cryopreservation processes were shown to increase SDF (Bailey, Bilodeau, and Cormier 2000). However potential technical artefacts cannot explain the increased SDF rates detected in HY when compared to pure forms, as methodological artefacts would be expected to similarly impact all samples. Therefore we assume that our results

reflect real differences in SDF rates observed in sperm stored at the animals' seminal vesicles. It should be noticed that we have analysed sperm stored in seminal vesicle that had not yet been capacitated (Buckland-Nicks & Chia, 1977) nor ejaculated and the impact of these processes in the SDF rates of the *L. saxatilis* ecotypes and HY is unknown. However, for each individual the rate of SDF is expected to increase with sperms aging.

The degree of SDF observed in HY is still compatible with mating experiments conducted in laboratory that showed that HYs were fertile (Johannesson *et al.*, 2000). Indeed several studies showed that only males with > 30 % SDF are unfertile (Cohen 1973; Evenson & Wixon, 2006; Wallace, 1974; but see Agarwal & Allamaneni, 2004, for lower estimates of SDF cutoffs). However if SDF differences between HYs and pure forms observed in sperm stored in seminal vesicles reflect those observed at the ejaculation, hybrid males are expected to display reduced fertility when competing with pure forms. Competition between sperm from distinct males is expected to occur frequently in *L. saxatilis* as females are known to be highly promiscuous (Panova *et al.*, 2010) and to store sperm in *bursa copulatrix* for several months (Johannesson *et al.*, 1993; Panova *et al.*, 2010). The fertilisation of eggs by sperm with fragmented DNA increases the probability of abnormal development of the embryos, abortive apoptosis (Borini *et al.*, 2006) and decreases the viability of adult progeny (Fernández-Gonzalez *et al.*, 2008). Accordingly, females that are able to select sperm from males from the same ecotype are expected to leave more descendants, thus creating the chance for reinforcement to act. The occurrence of reinforcement in *L. saxatilis* has been debated (Butlin *et al.*, 2008; Johannesson *et al.*, 2000) but so far no evidence supporting reinforcement has been reported.

The heterogeneity in the SDF rates observed between HY individuals may be explained by different degrees of genomic admixture that are expected to occur among these individuals. Indeed the classification of males was done based on morphological

characters (Carballo *et al.*, 2005; Johannesson *et al.*, 1993) and not on genetic markers. Accordingly, individuals classified as hybrids may include F_1 , F_2 and several backcrosses. This fact may also explain why heterogeneity in SDF was observed between RB but not between SU individuals. Actually, as *L. saxatilis* males favour same size to slightly bigger females (Conde-Padín *et al.* 2007; R. Cruz, Carballo, *et al.* 2004; Quesada *et al.* 2007; Rolán-Álvarez *et al.* 1999; Rolán-Álvarez 2007) there is an increased chance for SU males to mate with RB or HY females than the reverse. Therefore, RB is expected to have more genetic introgression than SU. Moreover sampling was conducted in the hybrid zone, where the degree of introgression and genetic admixture is expected to be higher. Accordingly, the heterogeneity in SDF observed between RB individuals may be explained by the presence of genetically admixed individuals. To test this hypothesis further studies comparing the degree of SDF with hybrid indexes estimated from genetic data are necessary. An alternative explanation is that SDF rates are dependent of external environmental factors that may show strong differences across the intertidal area, even at micro-geographical scales. In fact, the present data does not allow us to determine if the increased SDF observed in hybrid males has an intrinsic or extrinsic origin. In other animal species, SDF rates can be affected by both intrinsic and extrinsic factors. These include: defective spermatogenesis and gamete maturation (reviewed in (Borini *et al.*, 2006); abortive apoptosis (Sakkas *et al.*, 1999); oxidative stress due to excessive production of reactive oxygen species (ROS; Aitken *et al.*, 1998); infections and exposure to drugs and pollutants (Evenson *et al.*, 1999; Evenson & Wixon, 2006) and male aging (Borini *et al.*, 2006; Wyrobek *et al.*, 2006). In order to investigate whether the increased SDF observed in hybrids has an intrinsic or extrinsic origin, further studies are required that compare SDF rates between individuals with similar genomic composition obtained from the wild and reared under laboratorial controlled conditions.

It would also be advisable to further extend the present study to other Galician locations where the two ecotypes are known to co-exist and mate. Such studies may provide additional information not only about the factors causing the reported incipient reproductive barrier but also on its importance across this entire region. In fact, the limited dispersal ability of this species and the specific environmental conditions of each location may allow for different reproductive barriers to have different impact on the on-going speciation process in areas separated by large geographic distances.

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Appendix

Appendix 1

Chromacell's kit buffer:

- ✓ Hepes 10 mM
- ✓ KCl 80mM
- ✓ NaCl 45mM
- ✓ C₂H₃NaO₂ 45 mM
- ✓ CaCl₂ 0.4mM
- ✓ MgCl₂ 0,2 mM

Fixation



Buffer 0.1M:

- ✓ Disodium phosphate
- ✓ Monosodium phosphate
- ✓ Fixator 37 %
- ✎ Paraformaldehyde 4 %
- ✎ Picric acid saturated 15 %

Appendix 2

Number of sperm cells with normal and fragmented DNA observed in individuals morphologically classified as rough and banded (RB), smooth and unbanded (SU), or hybrids (HY). A hundred sperm cells were analysed *per* individual. The cells were classified as normal cells or cells with fragmented DNA. Individuals for which no sperm cells with fragmented DNA were detected are highlighted in green. Individuals with more than 30% sperm cells with fragmented DNA are highlighted in red.

		Normal cells	Cells with fragmented DNA
Individuals RB	1	100	0
	2	100	0
	3	99	1
	4	99	1
	5	97	3
	6	98	2
	7	99	1
	8	93	7
	9	96	4
	10	100	0
	11	88	12
	12	96	1
	13	90	10
Individuals HY	1	82	18
	2	92	8
	3	96	4
	4	99	1
	5	93	7
	6	71	34
	7	100	11
	8	89	11
	9	96	4
	10	96	4
	11	100	0
	12	100	0
	13	96	4
Individual SU	1	94	0
	2	100	0
	3	98	2
	4	94	6
	5	98	2
	6	98	2
	7	96	4
	8	98	2
	9	98	2
	10	98	2
	11	100	0
	12	98	2

Chapter 3: Identification of reproductive proteins in *Littorina saxatilis*.

Abstract

The species *Littorina saxatilis* is a well-known example of non-allopatric speciation with ecotypes adapted to distinct shore levels evolving independently in several places across its distribution. The contribution of reproductive proteins (RPs) for these on-going speciation processes is however unknown. With the present work, we aim to identify RPs expressing in *L. saxatilis* through shotgun proteomics. This technique was applied to three male (testis, seminal vesicle and prostate) and two female (ovary and bursa) reproductive tissues (RTs) resulting in the detection and analyses of 1513 peptide fragments. To increase the rate of proteins identification, we further analysed 23 peptide fragments from 96 spots obtained by bi-dimensional electrophoresis of protein extracts from the seminal vesicle. From the peptides matching *L. saxatilis* transcriptomic databases, 7 were shown to express exclusively in male or female RTs both at proteomic and transcriptomic level, thus representing good candidates for reproductive proteins. Improvements on transcriptomic and genomic databases of this species are expected to increase the number of candidate reproductive proteins identified.

Introduction

Speciation in the face of gene flow is expected to occur much less frequently than allopatric speciation (Coyne & Orr, 2004) and few widely accepted examples of such process are currently known. The evolution of the ecotypes of the marine snail *Littorina saxatilis* (Olivi, 1792) is one of the best well studied examples of non-allopatric incipient ecological speciation. *L. saxatilis* is a dioecious and polygamous species with internal

fertilization (Cruz *et al.*, 2004a; 2004b). In this species, two ecotypes can be found in some highly exposed shores, each one adapted to different shore levels and habitats (Johannesson *et al.*, 1995; Rolán-Alvarez *et al.*, 2004). In Spain the two ecotypes are called the ridged and banded ecotype (RB) and the smooth and unbanded ecotype (SU). RB lives in the upper shore associated with *Chtamalus stellatus* and *C. montagui* (Rolán-Alvarez *et al.*, 1997; Rolán-Alvarez, 2007; Butlin *et al.*, 2008). RB individuals possess a thick shell that protects them from predation. In these individuals shell aperture is smaller when scaled to its body size diminishing desiccation that results from long exposure to air and sun (Johannesson *et al.*, 1993; Conde-Padín *et al.*, 2007). The smooth and unbanded ecotype (SU) lives in the lower shore associated with *Mytilus galloprovincialis* (Rolán-Alvarez *et al.*, 1997; Conde-Padín *et al.*, 2007). The small size (about half of RB size) and the strong muscular foot of this ecotype increase its resistance to dislodgement by the strong wave strength, typical of its habitat (Johannesson *et al.*, 1993; Rolán-Álvarez *et al.*, 1997). At the mid-shore, the two ecotypes meet and occasionally mate producing fertile hybrids (Johannesson *et al.*, 2000). The morphological, behavioural and physiological differences observed between ecotypes have been shown to be, at least partially, heritable (Rolán-Alvarez *et al.*, 2004; Martínez-Fernández *et al.*, 2010).

The ecological displacement of the two ecotypes (Rolán-Álvarez, 2007) results in a low probability of hybridisation (Cruz *et al.* 2004a; Fernández *et al.*, 2005). However, it has been demonstrated that, even in the face of strong disruptive selection, speciation can only be achieved in the presence of mechanisms that cause assortative mating (reviewed by Kirkpatrick & Ravigné 2002). In *L. saxatilis* assortative mating has been shown to occur due to male preferences based on body size (Cruz *et al.*, 2004a; Conde-Padín *et al.*, 2008). But assortative mating may also be promoted by factors acting after copulation (Birkhead & Pizzari, 2002; Bretman *et al.*, 2004). Notwithstanding most of the studies aiming to understand factors driving mate choice in

internal fertilizers (including those performed in *L. saxatilis*) focused on pre-copula mechanisms and little is known regarding the role of post-copula mechanisms on individuals' reproductive success and on the evolution of these characters. This is particularly worrying as in most of the studied cases, proteins expressing in reproductive tissues have been shown to evolve rapidly and under positive selection across several taxa (Swanson & Vacquier 1998; Swanson, Clark, *et al.*, 2001; Landry *et al.*, 2003; Aagaard *et al.*, 2006; Swanson *et al.*, 2003; Berlin *et al.*, 2008) and to be able to cause assortative mating within a population (Palumbi, 2008; Geyer & Palumbi, 2003)

With the present study, we aim to identify candidate proteins involved in reproductive functions (RPs) in *L. saxatilis*. With this purpose, we have performed shotgun proteomic analyses in male and female reproductive tissues (RTs) of *L. saxatilis*. Shotgun analyses allow to identify the most abundant proteins in protein extracts, even those with extreme pI or molecular mass (< 15 or > 200 kDa; Old *et al.*, 2005). To complement these results, we have also analysed protein spots obtained from bi-dimensional electrophoresis (2DE) of seminal vesicle protein extracts. The 2DE is a reproducible technique that allows the separation of proteins according to their isoelectric point (pI; first dimension) and relative molecular mass (second dimension; Görg *et al.*, 2009). The Mass spectra (MS) obtained from these analyses were compared to public databases as well as with tissue specific transcriptomic databases available for this species. Candidate RPs identified in the present study can then be studied in *L. saxatilis* ecotypes and on other species from this genus.

Material and Methods

All the individuals, morphologically identified as RB ecotype, were collected simultaneously at the upper rocky shore of *Cabo Silleiro*, Spain (42°6'N, 8°53'W) in

September 2010. The individuals were kept in aquariums for three days to reduce potential environmental effects, and then dissected. The RTs of 20 males (testis, seminal vesicle and prostate) and 20 females (bursa and ovary, see figure 1) were collected and preserved at -80°C.

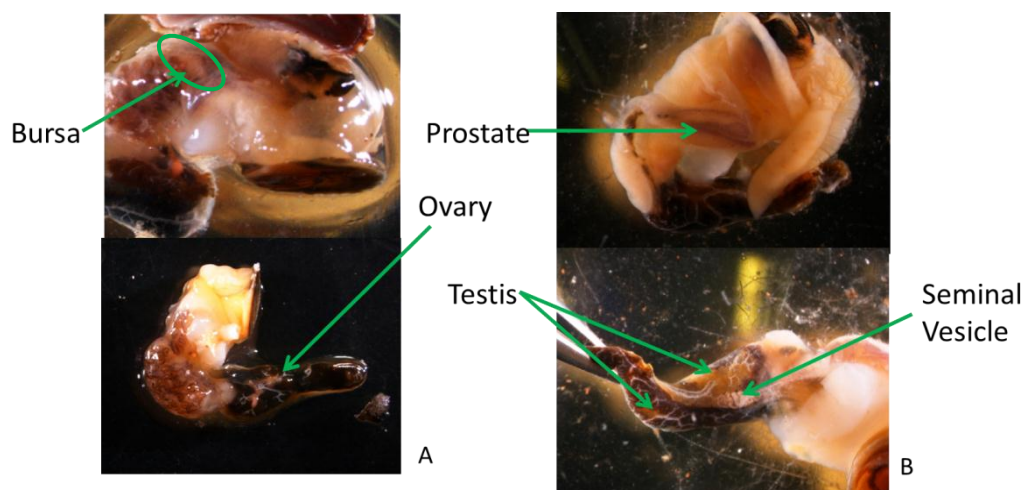


Figure 1: Female (left) and male (right) individuals of *Littorina saxatilis* (RB ecotype) after removing the shell. Arrows indicate the reproductive tissues dissected and analysed in the present work.

Sterile water was added to RT's samples and proteins were extracted through sonication and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies). For each tissue 20µg of protein were dried using a Speed-Vac and 0.5 µg trypsin were added for an overnight digestion (37° C). The reaction was stopped by adding 1µL of a formic acid solution (20%). The samples were cleaned with the Ziptip procedure (C18 Ziptip; Millipore) using acetonitrile 50%-0.1% formic acid. After elution, 10 µL were used for high-pressure liquid chromatography combined to MS/MS analysis.

In-gel tryptic digests were analyzed by LC-MS/MS using an Apex-Qe 7T FT-ICR (Bruker Daltonics, Billerica, MA, USA). LC separations were performed using an Ultimate 3000 HPLC system (Dionex, Amsterdam, Netherland), operated at a flow rate of 250 nL/min onto a 75 mm x 15 cm, 3 micron particle size C18 reversed phase column (Acclaim PepMap 100 from Dionex). Peptides were eluted using a linear

gradient starting at 98% A (0.1% formic acid in water) and ending at 60% B (0.1% formic acid in ACN) for 100 minutes. Mass measurements were taken from m/z (mass-to-charge ratio) 200 to 2000 and data-dependent MS/MS was performed on multiple charged precursors. A cell fill time of 0.5 s was used for MS measurements and 1 s for MS/MS. Data was acquired using the ApexControl 1.1 software (Bruker Daltonics, Bremen, Germany). Data files were processed using the software Data Analysis 4.0 SP 2 (Bruker Daltonics, Bremen, Germany) generating a Mascot generic file (mgf) that was submitted for database searching using MASCOT 2.2.04 search engine (Matrix science, MA, USA). The mgf files were compared to proteins from the NCBI nr 20070216 (4626804 sequences; 1596079197 residues) database and to proteins predicted from the public available *Littorina saxatilis* database (LSD; Canbäck *et al.*, 2012) with MASCOT 2.2.04 search engine (Matrix science, MA, USA). The mgf files were also compared with proteins predicted from unpublished tissue specific transcriptomic databases (Sá-Pinto *et al.*, unpublished data) generated for *L. saxatilis* and *L. obtusata* by 454 sequencing of mRNA extracted from the RTs here analysed as well as from two non RTs (foot and head). These databases included: the translation of all the reads obtained across all the tissues (AIIIMIDsprouts); the translation of the contigs generated after the assembly of the reads per tissue (HCRsprouts); the translation of the assembly performed across tissues (PRNASprouts). Search parameters were set as follows: taxonomy all entries, enzyme trypsin; allowance of one missed cleavage site; carbamidomethyl of cysteine as fixed modification; oxidation of methionine as variable modification; monoisotopic mass values; 10 ppm of mass tolerance for precursor ions; 0.03 Da of mass tolerance for fragment ions and protein mass unrestricted.

Some of the most interesting RPs are expressed in sperm cells (see examples in Swanson & Vacquier, 1998). Therefore and in order to increase the number of sperm proteins identified, we have additionally analysed protein spots from the seminal

vesicle separated after a 2DE analysis. For that analysis, proteins were extracted from the RTs of 50 individuals with a lysis buffer (7M urea, 2M thiourea and 4% CHAPS) to prevent oxidation and protein aggregation and to solubilise hydrophobic proteins and neutralize proteases (López, 2005). The extraction was done through a 90 seconds sonication, with pulses of 5 seconds interrupted by 10 seconds pauses to chill the samples, with oscillation parameters of 10%. Sonication was performed with the samples immersed on ice to avoid protein degradation by overheating. The resulting protein extract was incubated at 25°C for 60 minutes with gentle shaking 200g to facilitate protein solubilisation. Samples were centrifuged 15 minutes at 14,000g at room temperature and supernatant stored at -80° C. Proteins in each sample were quantified using the Bradford's method (Bradford, 1976) adapted to 2DE (Ramagli & Rodriguez, 1985).

Even though of that other proteomic studies have been already performed in *L. saxatilis* (Martínez-Fernández *et al.*, 2008; Martínez-Fernández *et al.*, 2010), this is the first time that 2DE technique was applied exclusively to reproductive tissues. Due to this, a first map was obtained using immobilised pH gradient (IPG) strips (ReadyStrip™ from Bio-Rad®, 17 cm) with a wide pH range from 3 to 10 in the first dimension. As most of the proteins detected ranged from pH 5 to 8 (data not shown), subsequent runs were carried out using this narrower pH range in order to ensure a better resolution of the protein map.

In this first dimension, 100 µg of protein were charged with a rehydration buffer (0.3% dithiothreitol [DTT], 0.5% Bio-Lytes, 3/10 ampholytes and lysis buffer up to 350 µL) (Martínez-Fernández *et al.*, 2008). The IEF ran a linear rehydration with a constant voltage (50 V) for 12 h at 20 °C (Görg *et al.*, 1991). Rehydrated gels were focused up to 60 000 Vh with current restricted to 50 µA per gel.

Then, IPG were equilibrated by incubation with gentle shaking during 20 minutes in a buffer (tris 50mM, urea 6M, glycerol 30% and SDS 2%) supplied with DTT 1% to reduce electroendosmotic effects and to improve protein transfer (Görg *et al.*, 2004). IPG were then incubated during 20 minutes with gentle shaking, in the same buffer, but supplied with iodoacetamide 2.5% and Bromophenol blue 0.02% instead of DTT (Görg *et al.*, 2004). The equilibrated strips were placed in a 12% denaturalizing polyacrylamide gel (acrylamide: 30%; bisacrylamide 0.8%) (Läemmli, 1970). Electrophoresis was performed at 20 mA per gel during 15 minutes and then at 40 mA until the bromophenol reached the bottom of the gel (Martínez-Fernández *et al.*, 2008). The gels were silver stained (Heukeshoven & Dernick 1985). Silver staining is not the only staining technique of polyacrylamide gels, but it allows to stain spots with protein amounts as low as 0.1 ng (Görg *et al.*, 2004; Heukeshoven & Dernick 1985).

A total of 96 spots were excised from the 2DE gel and digested overnight (37° C) with trypsin 1:40. Mass measurements were taken from m/z 700 to 4000 and data-dependent MS/MS was performed on multiple charged precursors as previously described for shotgun analyses. The mgf files were submitted for database searching with the databases previously mentioned and the SwissProt 56.6 database (405506 sequences; 146166984 residues). Search parameters were the same as those used for the identification of proteins from shotgun analyses, except the mass tolerances. 100 ppm of mass tolerance for precursor ions; 0.5 Da of mass tolerance for fragment ions and protein mass unrestricted.

To identify putative function of the peptides matched only to *L. saxatilis* databases, we carried out a standard protein BLAST with the BlastP algorithm (Altschul *et al.*, 1997) and compared the aminoacidic sequence inferred for each of these peptides with the nr GenBank database (GenBank CDS translations + PDB + SwissProt + PIR + PRF, excluding those in environmental samples from WGSprojects),

using default search parameters. The results with scores higher than 40 were retained and summarised in table 1 in appendix.

RPs are all the proteins involved in the reproductive function from gametogenesis to polyspermy avoidance system (typically associated with prezygotic-postmating reproductive barriers; Swanson & Vacquier, 2002), as well as those involved in gestation (Bernstein *et al.*, 1988) and, in mammals, in lactation (Anderson *et al.*, 2007; Burdon *et al.*, 1991). Although some RPs have been found to express also in non reproductive tissues (Wong *et al.*, 1994; Kirszbaum *et al.*, 1989) we will only consider here RPs that express exclusively in RTs. In order to identify these proteins we compared the proteomic expression profile of each peptide with its transcriptomic expression profile, obtained from tissue specific transcriptomic analyses. We classified as candidate female RPs, those that are only found to express in female RTs both at proteomic and transcriptomic levels and as male RPs those expressing exclusively in male RTs

Results

Shotgun analyses returned MS/MS results for 1513 peptides, from which 387 were detected in the bursa, 402 in the ovary, 395 in the testis, 139 in the seminal vesicle and 190 in the prostate. From these, only 46 distinct peptides were significantly matched to genes from at least one of the databases used for MASCOT analyses (see Table 1 in appendix). According to the results from Swissprot comparison (table 1 in appendix) some of these peptides were similar to proteins belonging to the actin, tubulin and tropomyosin gene families, heat shock proteins, Glycoprotein 93 CG5520-PA and a putative sporulation protein SOJ from *Tropheryma whipplei*. The results from the blastp searches allowed to identify putative homologous proteins for the peptides only matched to *L. saxatilis* databases (see table 1 in appendix).

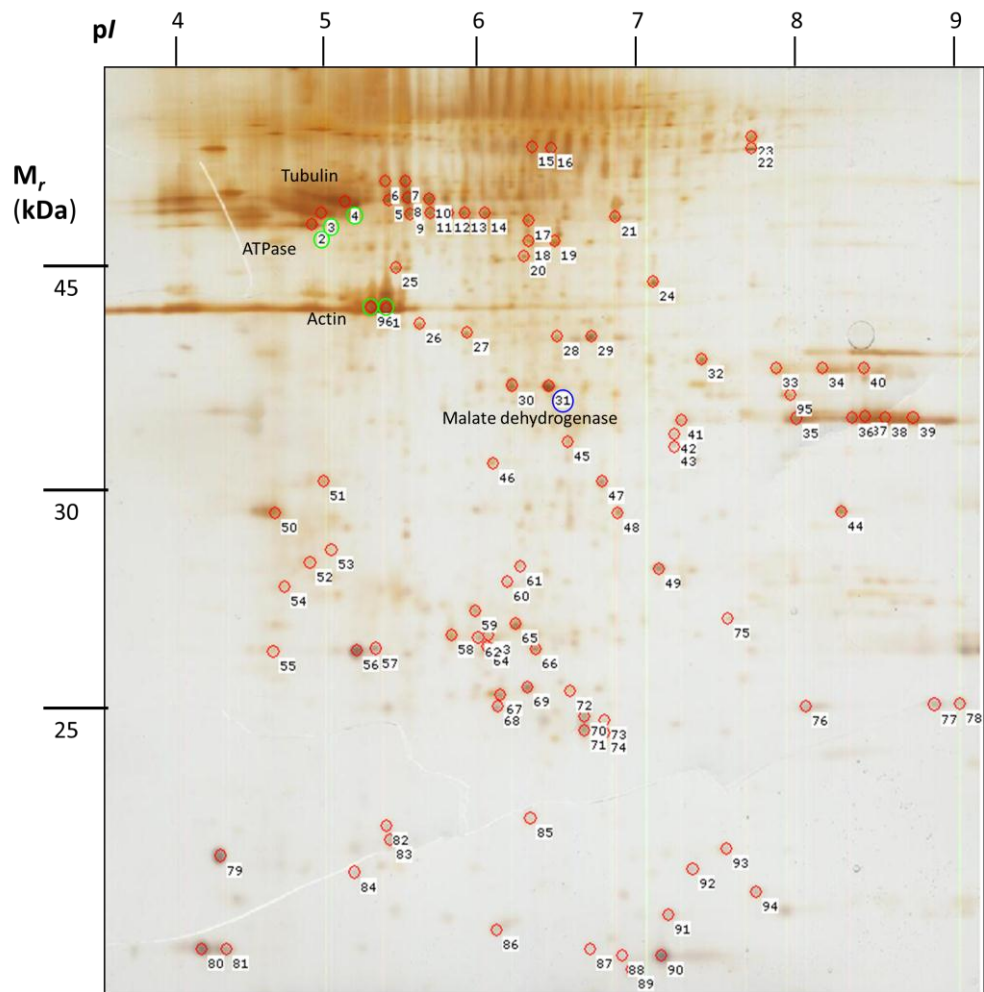


Figure 2: 2DE map of the seminal vesicle of RB ecotype. All the spots analysed are shown with their number and with their putative protein family (when identified cf. table 2 in appendix for more details). Spots analysed with MS/MS analyses are circled in blue and spots analysed with MS analyses are circled in red. Spots analysed with both MS and MS/MS are circled in green. *pI*: isoelectric point *Mr*: molecular weight in kilodaltons (kDa).

From the 96 spots excised from the map obtained for the seminal vesicle (Figure 2), 6 proteins were significantly matched to genes from one of the databases (spots: 1, 2, 3, 4, 31 and 96; see table 2 in appendix). According to the results obtained from the Swissprot comparison (table 2 in appendix), 2 of these proteins are similar to proteins from the actin family, 2 are similar to proteins from the tubulin family, one is similar to ATPase dehydrogenase and another one to malate dehydrogenase. The comparison with tissue specific transcriptomic databases allowed us to identify candidate RPs. From all the peptides here analysed, only 2 and 5 were found to express exclusively in female and male RTs, respectively, at both proteomic and transcriptomic level, and are probably part of candidate RPs (see table 1 in appendix).

Discussion

During the present work, we have detected and analysed 1513 peptide fragments through shotgun proteomics of 3 male and 2 female RTs and analysed 96 spots from 2DE of protein extract obtained from the seminal vesicle. With this study, we have been able to identify 2 peptides belonging to candidate female RPs and 5 peptides belonging to candidate male RPs.

According to the results of the MASCOT search in NCBI, 1 of the 5 candidate male reproductive peptides shows significant homology to proteins from the tubulin family, including β -tubulins of *Drosophila melanogaster* and β -tubulins of Gastropods from the genera *Patella* and *Haliotis*. This peptide was exclusively found in the testis and seminal vesicle and its corresponding mRNA was exclusively detected in these tissues. Proteins from the tubulin family are ubiquitous and known to be part of cytoskeleton, but Kemphues *et al.* (1979; 1983) demonstrated the existence of a testis-specific subunit of the Tubulin (β_2 -tubulin) in *Drosophila melanogaster* with strong effects on sperm fertility (Kemphues *et al.*, 1983) further supporting this protein as a candidate RP. The blastp searches performed in GenBank allowed us to identify putative homologous sequences for another candidate RP that was found to express exclusively in the testis. This peptide displays strong sequence similarity to proteins belonging to the H2A histone family from several plant and animal species, including molluscs and mammals (max score from 59.3 to 57.0). In mammals, proteins belonging to H2A histone family have been shown to be involved in spermiogenesis and like other sperm specific histones, have been shown to evolve extremely fast (Lieber *et al.*, 1986; Carlos *et al.*, 1993; González-Romero *et al.*, 2010; Ishibashi *et al.*, 2010). These results further support this protein as a candidate male RP. For the remaining candidate male RPs, no matches with scores higher than 40 were obtained in blastp searches.

The blastp searches performed in GenBank also allowed us to identify putative homologous sequences for a candidate female RP for which expression was only

detected in the bursa (both at proteomic and transcriptomic levels). According to this result, this peptide is similar to paramyosin, a muscle protein found in several invertebrate species (Winkelman, 1976) but up to our knowledge no studies suggested this protein as a putative RP. This result suggests that additional sequencing of tissue specific transcriptome is required to confirm the expression patterns of this and other putative candidate RPs.

When planning the present study, we were aware of the difficulties we would face to identify *L. saxatilis* RPs in classical databases, given the lack of available information on non-model organisms. Notwithstanding the majority of peptides detected and analysed during the present work did not match to any protein in any of the databases. This is particularly strange because the peptides were compared to *L. saxatilis* transcriptomic databases, including tissue specific databases. The most probable explanation for the low peptide identification rate is the low coverage of current transcriptomic databases for reproductive genes. In fact, although LSD (Canbäck *et al.*, 2012) contains approximately 26 500 contigs, it does not contain information for male RTs and transcripts from female RPs are expected to be poorly represented as these are expected to constitute a small fraction of the entire individual transcriptome (Galindo *et al.*, 2010; Canbäck *et al.*, 2012). Also, the coverage of the tissue specific transcriptomic database, that is currently being constructed (Sá-Pinto *et al.*, unpublished data), is still low for many genes, limiting the number of RP transcripts identified. The 454 technology used to construct this database, allows faster and less expensive results than the classical Sanger sequencing but the sequence fragments obtained are usually shorter (≤ 450 bases) and with higher error rates, thus requiring high coverage. Insertion or deletion errors (Kircher & Kelso, 2010) may be retained after the assembly process and, in this case, false stop codons and/or frame shifts may be introduced preventing the correct translation from the DNA/RNA sequence to the protein sequence. Although several algorithms are nowadays available, that correct errors

introduced by sequencing methods and are able to infer the most probable reading frame (Schiex *et al.* 2003; Min *et al.* 2005; Rho *et al.* 2010), these were not used during the translation of transcriptomic databases to aminoacid sequences.

The observed discrepancies can also be partially explained by the fact that we are comparing data corresponding to two different molecular levels, each with its corresponding experimental errors, and where each level has its particular regulation mode and speed. However, the increase in the coverage of transcriptomic and genomic databases currently in progress together with the use of algorithms to infer the most probable protein sequence from each contig are expected to increase the rate of peptides matching, the accuracy of mRNA expression tissue profiles and the number of candidate RPs identified.

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Appendix

Table 1: List of peptides expressed in *Littorina saxatilis* reproductive tissues, detected with shotgun analyses and matched to genes and/or gene fragments contained in available genomic and/or proteomic databases, its most probable aminoacidic sequence, the tissues where these express at proteomic and transcriptomic level, databases that include proteins with significant similarity to the peptide and its putative identity inferred from significantly similar proteins with known function contained in NCBI. Putative male reproductive proteins are highlighted in blue and putative female reproductive proteins are highlighted in pink. Legend: m/z- mass-to-charge ratio; SV – seminal vesicle; L:sax- *Littorina saxatilis*; L:obt: *Littorina obtusata*

m/z	Most probable aminoacidic sequence	Protein expression	mRNA expression	Database used for Identification	Putative Identity
1151,58	K.TEGFLPFWR.G	SV	SV, Bursa, Head	AIIMIDsprts, LSD	
1211,75	R.GLALLLETVRK.I	Prostate		NCBIhr	putative sporulation protein Soj (Tropheryma whipplei)
1284,71	K.NELVNNLGTIAK.S	Testis		NCBIhr	Heat Shock protein 90 or Glycoprotein 93 CG5520-PA
1352,76	-VYNLLNYSIR.Y	Testis	SV	HCRsprts	
1376,68	K.NQAEISFASPWK.K	Ovary	Prostate	AIIMIDsprts	
1391,64	R.LEESEISSTLR.S	Ovary	Bursa	AIIMIDsprts	
1442,86	K.VAFLLGINAGDLLK.A	Bursa		LSD, NCBIhr	Myosin
1456,87	R.LIGQVSSITASLR.F	Prostate, Ovary	Prostate	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, NCBIhr	Tubulin
1474,81	R.SIFDQLVDIAVK.D	Ovary		LSD	
1514,74	K.IWHHTFYNELR.V	SV, Prostate	SV, Prostate, Bursa, Ovary, Foot	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, NCBIhr	Actin
1514,75	K.IWHHTFYNELR.V	Prostate		NCBIhr	Actin
1587,80	R.DDQGLLFYYVTR.C	SV	Testis	AIIMIDsprts, HCRsprts	
1607,80	K.EFVDSFQLNPSAVR.V	Prostate		LSD	
1653,86	K.FGFLPNAGLALYDTR.S	SV	Bursa	AIIMIDsprts	
1672,83	K.LNEEELINIAGDTSR.V	SV, Prostate, Bursa, Ovary	Testis, Bursa, Ovary, Head	AIIMIDsprts, LSD	
1695,83	K.NSSYFVEWIPNNVK.T	SV, Testis	SV, Testis	AIIMIDsprts, HCRsprts, LSD, NCBIhr	Beta-tubulin
1695,84	K.NSSYFVEWIPNNVK.T	Testis		NCBIhr	Beta-tubulin
1700,91	R.AVFVDLEPTVIDEVR.T	Testis		LSD, NCBIhr	Tubulin
1701,87	K.SGFLSFDEVFNIISK.N	Bursa		LSD	
1720,91	R.FEVSSLTLEQVSNLR.N	Prostate	Ovary, Bursa	AIIMIDsprts	
1780,85	R.NGFGDLEGNFWLGLDK.L	SV	SV, Testis, Ovary	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, NCBIhr	
1784,82	K.NISDELDTFAELAGY.-	Bursa	Prostate, Bursa, Foot	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, NCBIhr	Tropomyosin
1788,89	K.SYELPDGQVITIGNER.F	All Tissues	All Tissues	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, NCBIhr	Actin

Table 1: continuation

1789,90	K.SYELPDGQVITIGNER.F	Ovary			NCBInr	Actin
1806,93	R.IDEVSGLTADLVDSVFK.A	Bursa			LSD	
1838,93	R.LDVDDDKVYSVTTEIK.V	Ovary	Prostate		AIIMIDsprts	
1844,00	-RLVVLFFQTFHHYK.V	Testis	Testis (L.sax+L.obt)		PRNAfrags	
1846,90	K.SYWLPDGGQVITIGNER.X	Bursa			NCBInr	Actin
1846,91	K.SYWLPDGGQVITIGNER.X	SV, Prostate, Bursa			NCBInr	Actin
1846,92	K.SYWLPDGGQVITIGNER.X	Ovary, Testis			NCBInr	Actin
1849,91	K.DLELSNVSEFAAEASLR.K	Bursa	Bursa		AIIMIDsprts, NCBInr	Paramyosin
2001,07	K.QTLVWELDGLSGQLDSVTK.A	Prostate, Bursa	Bursa		AIIMIDsprts	
2008,06	K.NVASLSETEVLSQLSYLR.A	Prostate	Bursa		AIIMIDsprts	
2008,07	K.NVASLSETEVLSQLSYLR.A	Ovary, Prostate	Bursa		AIIMIDsprts	
2010,08	R.VAPEWHPVLLTEAPLNPK.A	Prostate, Bursa			NCBInr	Actin
2010,09	R.VAPEWHPVLLTEAPLNPK.A	SV, Prostate, Ovary, Testis			NCBInr	Actin
2015,00	K.GHYTEGAERIDSVLDVVR.K	SV			NCBInr	Beta-tubulin
2215,17	R.IQSVPSGGAAPAAAAAPAAEAK.K	Testis	Ovary		AIIMIDsprts	Cytochrome C
2486,17	K.TLAIQNDQASQREDSYEETIR.D	Prostate	Prostate, Foot		AIIMIDsprts	Tropomyosin
2498,14	K.SLEISEQEASQREDSYEETIR.D	Bursa, SV	Prostate, Bursa, Ovary, Foot		AIIMIDsprts, HCRsprts, LSD, NCBInr	Tropomyosin
2498,15	K.SLEISEQEASQREDSYEETIR.D	Bursa			NCBInr	Tropomyosin
2517,34	R.EAWVTLATNDAYALGALVGNLSR.R	Testis	Bursa		AIIMIDsprts	Glycogenin
2598,29	K.VGIPYWDWTEAFTDLPALFTEK.E	Testis	Bursa		PRNAsprts	Hemocyanin
2951,57	-RGATAAVVSAAILLYTAEVLELAGNASK.D	Testis	Testis		AIIMIDsprts, HCRsprts	Histone
3164,66	R.TTGIVLDSGDGVTHTVPIYEGYALPHAILR.L	Prostate, Bursa			LSD, NCBInr	Actin
3164,67	R.TTGIVLDSGDGVTHTVPIYEGYALPHAILR.L	Prostate			NCBInr	

Table 2: Summary of the results of Mass Spectrometry analyses of 2DE Seminal vesicle gel. For each spot it is presented the peptides which aminoacidic sequence was identified, the tissues where these express at transcriptomic level, databases that include proteins with significant similarity to the peptide and its putative identity inferred from significantly similar proteins with known function contained in NCBI. Legend: SV – seminal vesicle.

spots	Sequence	Tissue mRNA Expressed	Database used for Identification	Putative Identity
1	K.AGAGDDAPR.A	All Tissues	HCRsprts, LSD, PRNAsprts, SwissProt	Actin
	R.GYSFTTTAER.E	All Tissues	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, SwissProt	
	R.AVFPSIVGRPR.H	All Tissues	HCRsprts, LSD, PRNAsprts, SwissProt	
	K.SYELPDGQVITIGNER.F	All Tissues	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, SwissProt	
	R.TTGIVLDSDGDGVTHTVPIYEGYALPHAILR.L		SwissProt	
2	K.AHGGYSVFAGVGER.T		LSD, SwissProt	ATP synthase subunit beta
3	R.FPGQLNADLR.K	Bursa	AIIMIDsprts, LSD, SwissProt	β -Tubulin
	K.LAVNMVFPFR.L	Bursa	AIIMIDsprts, LSD, SwissProt	
	R.INVYYNEATGGK.Y	SV, Head	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, SwissProt	
	R.LHFFMPGFAPLTSR.G	Bursa	AIIMIDsprts, LSD, SwissProt	
	R.INVYYNEATGGKYVPR.A	Head	AIIMIDsprts, LSD, SwissProt	
	R.SGPFQGIERPDPNFVFGQSGAGNNWAK.G	SV, Head	AIIMIDsprts, HCRsprts, LSD, PRNAsprts, SwissProt	
4	K.EIVDLVLDRI		LSD, SwissProt	α -Tubulin
	-YMACCMILYR.G	SV, Ovary	LSD, PRNAsprts, SwissProt	
	R.AVFVDLEPTVIDEVR.T		LSD, SwissProt	
	R.QLFHPEQLITGKEDAAANNYAR.G		SwissProt	
31	R.VFGVTTLDVVR.S		LSD, SwissProt	Malato dehydrogenase
	R.DDLFNTNAGIVR.D			
	R.QCLEGAQVVIPAGVPR.K			
	R.GYSFTTTAER.E			
96	R.AVFPSIVGRPR.H		SwissProt	Actin
	K.QEYDESGPSIVHR.K			
	K.SYELPDGQVITIGNER.F			

Discussion

More than 200 years elapsed since Darwin (1859) presented his theory of evolution and speciation by natural selection. Despite the research conducted on the field of speciation, several questions remain poorly understood (Coyne & Orr, 2004) and some hypotheses hotly debated. As speciation is characterised by the emergence of reproductive isolation barriers, a profusion of studies were conducted over the last decades on the evolution of such barriers (Rice & Hostert, 1993).

Non allopatric speciation is one of the most hotly debated hypotheses. It was only recently that scientific community acknowledged that reproductive barriers could arise on the face of gene flow (Via, 2001). Accordingly, knowledge on the evolution of reproductive barriers in the face of gene flow is still scarce (see reviews by Schluter, 2009; Smadja & Butlin, 2011). To better understand the evolution of reproductive isolation mechanisms in the context of non-allopatric speciation, we have studied the Galician ecotypes of *Littorina saxatilis* (Quesada *et al*, 2007; Rolán-Álvarez, 2007). In the next sections we report our findings and discuss their implications and importance to the knowledge on this model system.

Sperm's DNA Fragmentation

Up to our knowledge, this work is the first to study sperm DNA fragmentation (SDF) in the context of speciation research. In our opinion, analyses of SDF rates and dynamics can greatly contribute to speciation research, as SDF was shown to be strongly associated not only with infertility and miscarriage rates (Benchabib, 2003), but also with progeny viability (Fernández-Gonzalez *et al.*, 2008). Both fertility and progeny viability are important parameters to account when studying post-zygotic isolation.

In chapter 2 we compared the fertility of the males, morphologically identified as pure RB, pure SU and HY, by determining the degree of SDF. Our results show that hybrids have SDF rates significantly higher than any of the parental ecotypes. The SDF

rate of $\approx 8\%$ observed in HY is far from the threshold of 30%, usually required for individuals to be considered unfertile (Agarwal & Allamaneni, 2004; Erenpreiss, *et al.*, 2006; Evenson & Wixon, 2006; Wyrobek *et al.*, 2006). However HY' s SDF rates are at least twice those observed in pure ecotypes suggesting that HYs could be less fertile than any of the parental forms, especially when competing. At the mid-shore pure RB and SUs are found together with HYs. In this area, it is highly likely that sperm from HYs competes directly with sperm from pure forms as females are highly promiscuous (Mäkinen *et al.*, 2007; Panova *et al.*, 2010) and are able to store sperm for several months (Buckland-Nicks *et al.*, 1999; Panova *et al.*, 2010) and from different males (Mäkinen *et al.*, 2007). In this context, the higher SDF rates observed in HYs may reduce their ability to fertilise eggs, a reproductive barrier whose effect adds to others already reported (Conde-Padín *et al.*, 2008; Cruz *et al.*, 2004; Nosil *et al.*, 2005; Rolán-Álvarez *et al.*, 1999) further contributing to reduce introgression between forms.

Our study also detected significant heterogeneity in SDF rates among HY as well as among RBs. These results may be explained by the existence of individuals with different degrees of genetic admixture within the same morphological class. In fact, although we classified our sampled individuals as pure RB, pure SU or HY we expect these classes to include genomically distinct individuals ranging from F1, to backcrosses with different introgression levels. The lower (and not significant) heterogeneity observed in SU when compared to RB and HY can be explained by the lower introgression expected in this form due to the preference of all males for same sized (Cruz *et al.*, 2004; Hollander *et al.*, 2005) to slightly larger females. In order to test the impact of genetic admixture in SDF rates, additional studies are required to compare SDF with individuals' hybrid index inferred from diagnostic genetic markers.

Our results do not allow to determine whether the reported barrier has an intrinsic or extrinsic origin. For that purpose, studies comparing SDF rates of the three morphological classes maintained under controlled laboratorial conditions are required.

Identification of reproductive proteins

Incipient post-zygotic barriers like the one reported in chapter 2 create the required conditions for reinforcement to act, favouring genetic and behavioural variants that enhance pre-zygotic isolation between the two ecotypes. Several studies have been conducted to test for pre-mating reproductive isolation between the Spanish ecotypes of *L. saxatilis* (Cruz *et al.*, 2001; Rolán-Álvarez *et al.*, 1999), but post-mating pre-zygotic isolation in this species has received much less attention, even though several features of this intertidal mollusc might favour the evolution of such mechanisms. In fact, *L. saxatilis* can reach high densities (Carballo *et al.*, 2005) and its females are highly promiscuous and able to store sperm for several months (Buckland-Nicks *et al.*, 1999; Panova *et al.*, 2010).

Reproductive proteins (RPs) are likely targets for natural selection to act, by increasing reproductive isolation through reinforcement. These proteins were shown to evolve faster than other proteins and under positive selection (Panhuis *et al.*, 2006; Swanson & Vacquier, 2002a; Turner & Hoekstra, 2008) and to be able to cause assortative mating and reinforcement (Geyer & Palumbi, 2003; Palumbi, 2009; Slaughter *et al.*, 2008). The work reported in chapter 3 represents the first attempt to identify RPs in *Littorina*, the required step for studying the role of such proteins in the on-going speciation process.

During this work we have analysed 1513 peptide fragments resulting from shotgun analyses, from which, only 46 distinct peptides exhibited a significant match to proteins inferred from *L. saxatilis* transcriptomic databases and/or available in public databases. Additionally, 6 proteins were identified from the 96 spots obtained from 2DE analyses. When compared to tissue specific transcriptomic databases obtained for this species and for the closely related *L. obtusata*, 5 peptide fragments were found to match genes expressed only in male reproductive tissues and 2 in female reproductive tissues (see tables in appendix, chapter 3), suggesting these genes as putative RPs. For most of these genes no information is available on their function, and their

expression profiles require further confirmation. Two of the putative male RPs were identified as probable members of the β -tubulin and H2A histone gene families. Both these gene families include genes that were shown to express exclusively in testis of other species and to affect male fertility (Kemphues *et al.*, 1979,1983; Carlos *et al.*, 1993; González-Romero *et al.*, 2010; Ishibashi *et al.*, 2010; Lieber, *et al.*, 1986), thus supporting these proteins as candidate male RPs. One of the candidate female RP shows significant similarity to paramyosin, a muscle protein found in several invertebrate species in both reproductive and non reproductive organs (Winkelman, 1976), highlighting the necessity of additional sequencing effort on tissue specific transcriptome to confirm the expression patterns of this and other putative candidate RPs.

The disparity between the number of peptides analysed and the number of proteins identified is probably explained by the scarcity of protein sequences available in public databases for non-model organisms (Martínez-Fernández *et al.*, 2008), but also by the quantity and quality of transcriptomic data available for this species. Although we did compare our results to the LSD (Canbäck *et al.*, 2012) which is constituted exclusively of *L. saxatilis* sequences, this database does not include males RT and female RPs are expected to be poorly represented (Galindo *et al.*, 2010; Canbäck *et al.*, 2012). The tissue specific transcriptomic databases that are currently under construction and some genes still have a low coverage and a high error rate. The 454 technology used to construct these databases, allows faster and cheaper results than classical Sanger sequencing but the sequence fragments obtained are usually shorter (≤ 450 bases) and with higher error rates (Morozova *et al.*, 2009), thus requiring high coverage (25-30 folds). The insertion or deletion errors (Kircher & Kelso, 2010) that are retained after the assembly process introduce false stop codons and/or frame shifts preventing the correct translation to proteins. Although some algorithms were developed to correct the errors introduced by sequencing methods and to infer the most probable reading frame (see review by Pop & Salzberg, 2008), these were not

used in the present work during the translation of both LSD and the tissue specific transcriptomic databases into protein sequences.

The observed discrepancies can also result in part from the fact that we are comparing data corresponding to two different molecular level with its corresponding experimental errors associated, and where each level has its particular regulation mode and speed.

The additional sequencing of *L. saxatilis* genome and transcriptome that is currently being done is expected to provide additional data and to improve the quality of available databases and therefore, to allow the identification of the peptides that were not matched to any gene increasing the number of candidate RPs.

Main conclusions and future directions

With this work we demonstrated the existence of a postzygotic reproductive isolation barrier which is expected to reduce fertility of hybrid males in competition with pure ecotypes thus creating a favourable framework for reinforcement to appear. However, the causes for this reduced fertility cannot be inferred from the present data. Studies that compare the degree of introgression of each individual with their SDF rate in several independent sampling locations are required to better understand the causes for the reduced fertility of hybrid males here reported.

We also identified seven candidate RPs which role on the on-going speciation process can now be studied. The increase of genomic and transcriptomic data available for this species is expected to increase both the number of peptides matching genes from this species and the number of candidate RPs identified from the present proteomic dataset. Further work is also required to know the function of the proteins identified during this study.

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