

**VARIAÇÃO INTER-POPULACIONAL NO
COMPORTAMENTO REPRODUTOR DO BLENÍDEO**
Salaria pavo

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Dissertação de doutoramento em Ciências do Meio Aquático

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**VARIAÇÃO INTER-POPULACIONAL NO COMPORTAMENTO
REPRODUTOR DO BLENÍDEO *Salaria pavo***

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RESUMO

Neste trabalho foi investigada a variação inter-populacional no comportamento reprodutor do peixe blenídeo *Salaria pavo*, em particular a influência do ambiente ecológico na plasticidade desses comportamentos. Neste sentido, foram investigadas duas populações que habitam em locais com características ecológicas distintas: a Ria Formosa, um sistema lagunar de ilhas barreira localizado na costa Sul de Portugal, apresentando substrato de sedimento móvel, e o Golfo de Trieste, localizado no topo norte do mar Adriático, apresentando substrato rochoso.

Esta espécie apresenta um elevado dimorfismo sexual: os machos são maiores que as fêmeas e possuem caracteres sexuais secundários muito conspícuos, como uma crista cefálica e uma glândula anal que consiste numa modificação dos primeiros raios da barbatana anal. Estes caracteres desenvolvem-se principalmente na época de reprodução. O sistema de acasalamento é promíscuo e os cuidados parentais às posturas são prestados exclusivamente por parte do macho. Nas populações de substrato rochoso, os machos nidificam em cavidades na rocha e mantêm um território de corte em redor da entrada do ninho onde executam elaborados exibições de cortejamento, assumindo uma tática 'burguesa'. As fêmeas normalmente assumem um papel passivo na corte, respondendo com mudanças de cor e algumas exibições antes de entrarem no ninho para a desova.

A população da Ria Formosa apresenta no entanto consideráveis modificações ao padrão observado em costas rochosas. Nesta lagoa costeira, os substratos de nidificação são escassos e os únicos locais adequados para o estabelecimento de ninhos são tijolos que se encontram em recifes artificiais, construídos por viveiristas de bivalves para delimitar as suas áreas concessionadas. Durante a época de reprodução, os ninhos encontrados estão muitas vezes altamente agregados, parecendo impossibilitar a manutenção de territórios típicos dos machos bugueses desta espécie. Nesta população foi também observada uma reversão dos papéis sexuais, com as fêmeas a cortejarem intensamente os machos e a competirem entre si por acesso a ninhos. As fêmeas neste local apresentam um padrão de corte elaborado, enquanto que os machos bugueses assumem um papel quase passivo durante a corte. Na população da Ria Formosa existe também uma alta proporção de machos parasitas, que apresentam um tamanho menor que os que nidificam e que imitam tanto a morfologia como o comportamento de corte das fêmeas de modo a conseguirem aproximar-se do ninho e fertilizar as posturas durante episódios de desova. Estas táticas alternativas de

reprodução são sequenciais e os machos que apresentam uma tática parasita numa época de reprodução normalmente adquirem um ninho na época seguinte.

No presente estudo, foram colocadas algumas questões relativas ao aparecimento destas diferenças comportamentais entre populações. Na hipótese de trabalho considerou-se que as alterações ao sistema de acasalamento desta espécie se deveriam a uma elevada plasticidade comportamental sob influência do ambiente ecológico, nomeadamente a abundância e dispersão dos locais de nidificação. Concretamente, as principais questões estudadas foram:

- 1) Será que nesta espécie os papéis sexuais são dinâmicos e podem ser modulados através de uma manipulação experimental da agregação dos ninhos?
- 2) Como funciona o sistema de acasalamento em populações com abundância e dispersão de ninhos diferentes? Haverá diferenças genéticas entre as populações? Será que existem táticas alternativas de reprodução em populações com abundância de ninhos?
- 3) Haverá diferenças morfológicas nas fêmeas e nos diferentes fenótipos de machos entre populações? Quais os correlatos endócrinos dessas diferenças?
- 4) Quais as diferenças na regulação neuroendócrina do comportamento reprodutor entre as duas populações?

Os resultados apontam para uma elevada plasticidade comportamental nesta espécie, com uma forte influência da abundância e dispersão de ninhos na modulação do sistema de acasalamento e comportamento reprodutor. A agregação e escassez de locais de nidificação na Ria Formosa parecem promover uma forte competição para acesso a ninhos, que favorece os machos maiores e pode promover o aparecimento de caracteres sexuais secundários mais pronunciados como forma de sinalização intra e intersexual. Uma vez que apenas os maiores machos conseguem acesso a ninhos, o *sex-ratio* operacional (número de fêmeas maduras / número de machos em condição de reproduzir) vai ficar desviado para as fêmeas, limitando o seu potencial reprodutivo e causando a reversão dos papéis sexuais. Deste modo, na Ria Formosa as fêmeas assumem o papel principal na corte e competem entre si para terem acesso a machos. Por outro lado, uma grande percentagem de machos sexualmente maduros são impedidos de se reproduzir, sendo que os mais pequenos adoptam táticas alternativas de reprodução. Surpreendentemente, também foram encontrados machos parasitas na população de Trieste, embora numa proporção muito inferior. Estes machos, embora imitem a morfologia das fêmeas, não cortejam em exibições semelhantes às fêmeas,

permanecendo por descobrir qual a tática que adoptam para ter acesso às posturas das fêmeas. Uma vez que não existem diferenças genéticas significativas entre populações, estas diferenças no comportamento reprodutor são aparentemente devidas a plasticidade comportamental.

Embora os machos burgueses do Golfo de Trieste sejam menores e tenham caracteres sexuais secundários menos desenvolvidos, apresentam gonadas relativamente maiores. Uma vez que também os machos parasitas também apresentam gonadas mais desenvolvidas na população do Adriático, é provável que a competição de esperma seja mais acentuada no Golfo de Trieste. Isto pode ser explicado porque os machos burgueses passam mais tempo fora do ninho, havendo mais hipótese para invasões de machos rivais, e porque os machos parasitas não têm uma tática activa das fêmeas para imitar e portanto há muito maior imprevisibilidade nas oportunidades de reprodução.

A análise dos níveis circulantes de androgénios revelou maiores concentrações de 11-ceto-testosterona nos machos burgueses da Ria Formosa, sugerindo por um lado uma correspondência entre esta hormona e os caracteres sexuais secundários, e por outro que existe uma alta sensibilidade dos androgénios ao ambiente social, mais competitivo na população portuguesa. O estudo da enzima aromatase, que converte a testosterona em estradiol e que está envolvida na regulação de comportamentos de corte e agressividade, revela uma maior expressão cerebral desta enzima no cérebro dos machos burgueses da Ria Formosa. O estradiol tem sido relacionado com a inibição dos comportamentos agonísticos, e uma maior taxa de conversão local de testosterona em estradiol pode ser uma forma de reduzir a agressividade e os comportamentos de corte nestes machos, permitindo-lhes coabitar com vizinhos muito próximos.

Em conclusão, os dados presentes neste trabalho permitem uma compreensão integrada de alguns dos mecanismos que regulam a plasticidade comportamental em *S. pavo*, cuja expressão de comportamentos reprodutores parece estar intimamente relacionada com factores ecológicos.

ABSTRACT

The inter-population variation in the reproductive behaviour of the peacock blenny *Salaria pavo*, particularly the influence of the ecologic environment, was investigated in the present work. Two populations of this species inhabiting contrasting environments were studied: the Ria Formosa population, a coastal lagoon with sandy/muddy substrate located in the south of Portugal, and the Gulf of Trieste, an area presenting rocky substrate located in the northern Adriatic sea.

This species presents high sexual dimorphism: males are larger than females and exhibit conspicuous secondary sex characters, like a head crest and anal gland, that consists of modified rays of the anal fin. These characters develop mainly in the breeding season. The mating system is promiscuous, with exclusive male parental care. In rocky shore populations, males nest in crevices or holes in the rock and defend a courting territory around the entrance of the nest where they perform elaborate courtship displays, assuming a bourgeois reproductive tactic. Females usually have a passive role in courtship, responding with changes in colouration and a few displays before they enter the nest to spawn.

The Ria Formosa population, however, presents severe changes to this pattern. In this coastal lagoon, nesting substrates are scarce and the only adequate sites to establish nests are bricks located in artificial reefs, used by clam culturists to delimit their fields. During the breeding season, nests are highly aggregated, rendering the maintenance of the typical bourgeois territory impossible. Sex-role reversal has been described for this population, with females displaying intense and elaborate courtship and males assuming an almost passive role in courtship. Furthermore in the Ria Formosa there is also a high proportion of small parasitic males that mimic female morphology and courtship, and try to achieve parasitic fertilizations during spawning episodes. These alternative reproductive tactics are sequential and males that assumed a parasitic tactic in one breeding season usually acquire a nest in the next season.

In the present study, some questions regarding the emergence of these behavioural differences between populations were addressed. The main hypothesis was that the variations in the mating system should be due to high behavioural plasticity under the influence of the ecological environment, namely the abundance and dispersion of nesting sites. More precisely, the following questions were studied:

- 1- Are the sex-roles dynamic in this species and can they be modulated through experimental manipulation of nest-site aggregation?

- 2- How does the mating system work in populations inhabiting sites with different nest availability and dispersion? Are there genetic differences between populations? Are there alternative reproductive tactics in populations where nest-sites are abundant?
- 3- Are there morphological changes in females and different male morphs between populations? What are the endocrine correlates of such differences?
- 4- What are the differences in the neuroendocrine regulation of reproductive behaviour between the two populations?

The results point to a high behavioural plasticity in this species, with a strong influence of nest-site abundance and dispersal in the modulation of the mating system and reproductive behaviour. In fact, the aggregation and scarcity of nest sites in Ria Formosa apparently promote a strong competition for access to nests sites, favouring larger males and probably promoting the development of more pronounced secondary sex characters as intra and intersexual signalling. As only the largest males acquire nests, the operational sex-ratio (number of mature females / number of males qualified to mate) will be biased towards females, limiting their reproductive potential and causing sex-role reversal. This way, in Ria Formosa females assume the major role in courtship and compete for access to males. On the other hand, a large proportion of sexually mature males cannot breed and the smallest adopt alternative reproductive tactics. Surprisingly, we also found parasitic males in the Gulf of Trieste, although in a much lesser proportion. These males mimic female morphology but do not display female-like courtship behaviours. The tactic they use to achieve parasitic spawning remains unknown. Since there are no significant genetic differences between populations, the differences in reproductive behaviour are interpreted as being due to behavioural plasticity.

Although bourgeois males in the Gulf of Trieste are smaller and have less developed secondary sex characters than in Ria Formosa, they present relatively larger gonads. And since parasitic males also present larger gonads in the Adriatic population, it is probable that sperm competition is higher in the Gulf of Trieste. This can be explained by the longer periods outside of the nest that bourgeois males from this population spend, increasing the chance for nest-takeovers or stealing fertilizations by rivals; and as parasitic males do not have an active female courtship to mimic, there is much higher unpredictability in reproductive opportunities.

The analysis of circulating levels of androgens revealed higher concentrations of 11-keto-testosterone in bourgeois males from Ria Formosa, suggesting on one hand a correspondence between this hormone and the development of secondary sex

characters, and on the other that androgens are highly sensitive to the social environment, probably more competitive in this population. The study on the aromatase enzyme, that converts testosterone into estradiol and is involved in the regulation of courtship and aggressive behaviours, reveals a higher expression of this enzyme in the brains of bourgeois males from Ria Formosa. Estradiol has been described to be linked to the inhibition of agonistic behaviours, and a higher local testosterone-estradiol conversion rate may be down-regulating the aggressiveness and courtship in these males, allowing them to cohabitate with neighbours.

In conclusion, the data present in this work allow an integrated understanding of some of the mechanisms that regulate behavioural plasticity in *S. pavo*, whose expression of reproductive behaviours seem to be closely related to ecological factors.

RESUMÉ

La variation inter-populationnel dans le comportement reproducteur du poisson blenniide *Salaria pavo*, notamment l'influence de l'environnement écologique, a été examinée dans le travail présent. Deux populations de cette espèce habitant des environnements contrastés ont été étudiés : la population de Ria Formosa, une lagune côtière avec le substrat sableux/boueux localisé dans le sud de Portugal, et le Golfe de Trieste, un secteur présentant le substrat rocheux localisé aux nord de la mer Adriatique.

Cette espèce présente l'haut dimorphisme sexuel : les mâles sont plus grands que les femelles et expose les caractères de sexe secondaires apparents, comme une crête à la tête et la glande anale, cela consiste en des rayons modifiés de la nageoire anale. Ces caractères développent principalement dans la saison reproductive. Le système accouplant est promiscue, avec le soin parental exclusivement du mâle. Dans les populations des rives rocheuses, les mâles font le nid dans les fissures ou les trous dans la roche et défend un territoire courtisant autour de l'entrée du nid où ils exécutent les expositions de cour soignées, en une tactique reproductrice bourgeoise. Les femelles normalement un rôle passif dans la cour, répondant avec les changements dans la coloration et quelques expositions avant qu'ils entrent le nid pour frayer. La population de Formosa de Ria, cependant, présente des changements sévères à ce modèle. Dans cette lagune côtière, les substrats de nidification sont rares et les seuls sites suffisants pour établir des nids sont des briques, localisés dans les récifs artificiels, utilisés par culturistes de bivalves pour délimiter leurs champs. Pendant la saison élevant, les nids sont extrêmement agrégés, rendant l'entretien du territoire bourgeois typique impossible. Le renversement de rôle sexuelle a été décrit pour cette population, avec les femelles affichant la cour et les mâles intense et soignée supposant un rôle presque passif dans la cour. De plus dans le Ria Formosa il y a aussi une haute proportion de petits mâles parasites qui imitent la morphologie et la cour femelles, et essayent d'atteindre des fertilisations parasites pendant les épisodes de fraye. Ces tactiques reproductrices alternatives sont des séquentielles et mâles qui a supposé qu'une tactique parasite dans une saison élevant acquiert d'ordinaire un nid dans la prochaine saison.

Dans l'étude présente, quelques questions quant à l'apparition de ces différences du comportement entre les populations ont été adressées. L'hypothèse principale était que les variations dans le système accouplant devraient être en raison de la plasticité haut du comportement sous l'influence de l'environnement écologique, à savoir l'abondance et la dispersion de sites de nidification. Plus précisément, les questions suivantes ont été étudiées : 1- Est-ce que les rôles sexuelles sont dynamiques dans cette espèce et

peuvent-ils être modulés par la manipulation expérimentale d'agrégation de nids ? 2- Comment est-ce que le système accouplant fonctionne dans les populations habitant des sites avec la disponibilité de nids et la dispersion différente? Est-ce qu'il y a des différences génétiques entre les populations ? Est-ce qu'il y a des tactiques reproductrices alternatives dans les populations où les nids sont abondants ? 3- Est-ce qu'ils ont des différences morphologiques dans les femelles et les types de mâle différents entre les populations? Quels sont les correspondances endocrines de telles différences ? 4- Quels sont les différences dans le neuroendocrine règlement de comportement reproducteur entre les deux populations ?

Les résultats indiquent à une haute plasticité du comportement dans cette espèce, avec une forte influence d'abondance de nids et de dispersion dans la modulation du système accouplant et de comportement reproducteur. En fait, l'agrégation et la rareté de sites de nid dans Ria Formosa promeuvent apparemment une forte compétition pour l'accès aux nids, favorisant les plus grands mâles et promouvant probablement le développement de caractères sexuelles secondaires plus prononcés comme signalisation intersexuél. Comme seulement les plus grands mâles acquièrent des nids, le *sex-ratio* opérationnelle (le nombre de femelles mûres/le nombre de mâles qualifiés pour accoupler) sera dévié vers les femelles, limitant leur potentiel reproducteurs et causant le renversement de rôle sexuelle. Dans les femelles de Ria Formosa suppose que le rôle majeur dans la cour et concourt pour l'accès aux mâles. D'autre part, une grande proportion de mâles sexuellement mûrs ne peut pas reproduire et le plus petit adopte des tactiques reproductrices alternatives. Étonnamment, nous avons trouvé aussi des mâles parasites dans le Golfe de Trieste, bien que dans une beaucoup de moindre proportion. Ces mâles imitent la morphologie femelle mais n'affichent pas les comportements de cour des femelles. La tactique qu'ils utilisent atteindre des frais parasites reste inconnus. Puisqu'il n'y a pas de différences génétiques significatives entre les populations, les différences dans le comportement reproducteur sont interprétées comme plasticité du comportement reproductif. Bien que les mâles bourgeois dans le Golfe de Trieste sont plus petits et moins a développé les caractères secondaires de sexe que dans Ria Formosa, ils présentent relativement plus grandes gonades. Et depuis les mâles parasites plus grandes gonades aussi présentes dans la population Adriatique, il est probable que la compétition de sperme est plus haute dans le Golfe de Trieste. Ceci peut être expliqué par plus périodes long du nid que les mâles bourgeois de cette population dépensent, augmentant le hasard pour les acquisitions du nid ou le vole les fertilisations par les rivaux ; et comme les mâles parasites n'ont pas une cour femelle active pour imiter, il y a beaucoup plus haute imprévisibilité dans les occasions reproductrices. L'analyse de niveaux circulants

d'androgènes a révélé plus hautes concentrations de de 11 keto-testostérone dans les mâles bourgeois de Ria Formosa, suggérant d'une part une correspondance entre cette hormone et le développement de caractères sexuelles secondaires, et sur l'autre que les androgènes sont extrêmement sensibles à l'environnement social, probablement plus compétitif dans cette population. L'étude sur l'enzyme d'aromatase, cela convertit la testostérone en estradiol et est impliqué dans le règlement de cour et les comportements agressifs, révèle une plus haute expression de cette enzyme dans les cerveaux de mâles bourgeois de Ria Formosa. Estradiol a été décrit être relié à l'inhibition des comportements agonistiques, et un plus haut taux de conversion local de testostérone en estradiol réglemente l'agressivité et la cour dans ces mâles, les permettant à cohabiter avec les voisins. En conclusion, les résultats présentent dans ce travail permet une compréhension intégrée de certains mécanismes qui réglementent la plasticité du comportement dans *S. pavo*, dont l'expression de comportements reproducteurs semble de près être relaté aux facteurs écologiques.

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GENERAL INTRODUCTION

INTRODUCTION

Fish reproduction presents an extraordinary diversity. In these animals, reproductive patterns range from gonochoristic species, sequential sex-changing species - male-to-female as well as female-to-male (Warner, 1988), serial sex-changing species - i.e. male-to-female-to-male (Sunobe and Nakazono, 1993), simultaneous hermaphrodites and even asexual reproduction (Schartl *et al.*, 1995). This astonishing variety is patent on virtually all aspects of reproductive activity: fertilization mode is mostly external but can also be internal in some species (Goodwin *et al.*, 2002), mating systems range from monogamous to promiscuous (Turner, 1993), parental care can vary from null to paternal, maternal or bi-parental (Sargent, 1997) and sex-roles can be conventional - where male competition for mates is strongest-, or reversed - where females competition for mates is strongest (e.g. Almada *et al.*, 1995). Sex-roles are dynamic and within the same species they may shift from conventional to reversed throughout the breeding season (Forsgren *et al.*, 2004; Shibata and Kohda, 2006).

In addition, males can adopt a wide variety of tactics (Taborsky, 1994; Gonçalves *et al.*, 1996; Taborsky, 2001; Oliveira *et al.*, 2005), which can differ greatly between species but also within the same species, i.e. between populations (Luyten and Liley, 1985; Magurran and Seghers, 1991; Almada *et al.*, 1995), within populations or between individuals (Taborsky, 1994; Taborsky, 2001; Oliveira *et al.*, 2005). This variability suggests that competition should be a strong factor in the evolution of fish mating systems, i.e., their mating behaviour and parental care (Reynolds, 1996). This is considered to be one of the driving forces of sexual selection: the process in which sexually dimorphic features evolve through direct competition, usually between individuals of the same sex (Darwin, 1871).

Ecological modulation of behaviour

Emphasizing the competitive aspect of sexual selection, Emlen & Oring (1977) provided a framework to better understand the ecological factors that influence the degree and form of polygamous mating systems. Accordingly, the evolution of such a system is dependent on two conditions. First, multiple mates, or resources sufficient to attract multiple mates, must be energetically defensible by individuals. Many environmental factors affect the potential for such control, most importantly spatial and temporal patterns of resource dispersion. In other words, this first condition refers to the economically monopolizability of several mates, directly related to the environmental potential for polygamy. These depend on the degree to which multiple mates, or resources critical to gaining multiple mates, are economically defensible. The second

condition refers to the ability of animals to capitalize on this potential. This comprises the time budget devoted either to defending critical resources or to other activities, including parental care. so the degree to which an animal can take advantage of the environmental potential for polygamy is largely dependent of the degree of parental care necessary to rear the young (Emlen and Oring, 1977).

The first condition assumes that a key factor for the monopolization of resources is their spatial distribution, especially in territorial species. Interestingly, some attention has been given to the spatial relationship between resource availability and aggression in a foraging context (e.g. Dunbrack *et al.*, 1996; Lahti *et al.*, 2002), but very few studies have examined the relationship between resource availability and aggression in a mating context (e.g. Kolhuru and Grether, 2004).

When important resources are highly clumped, a small percentage of the population may monopolize a large proportion of the available resources and the environment has a high polygamy potential (Weir and Grant, 2004). On such a system, a high variance in reproductive success and sexual selection should be expected. On the contrary, when resources are abundant and spatially dispersed there is little opportunity for resource monopolization and low variance in reproduction, as well as low sexual selection pressures should be expected (Emlen and Oring, 1977).

The distribution and abundance of resources also has an important impact on the ratio of sexually mature males to sexually receptive females (Operational Sex Ratio, OSR), which may provide an empirical measure of the degree of monopolization of mates (Emlen and Oring, 1977). Since Emlen (1976) introduced the concept of OSR, many studies have been carried out on this subject (e.g. Simmons and Bailey, 1990; Vincent, 1994; Kvarnemo and Ahnesjö, 1996; Kvarnemo and Simmons, 1999; Kokko and Johnstone, 2002; Kvarnemo and Merilaita, 2006). The OSR is primarily determined by the potential reproductive rate of the two sexes (Clutton-Brock and Parker, 1992) but this definition has to take into account species-specific qualitative details, such as having an essential nutrient (Simmons and Bailey, 1990) or owning an adequate nest site (Oliveira *et al.*, 1999; Ahnesjö *et al.*, 2001). Thus, the OSR is considered determinant for the opportunity of sexual selection (Kvarnemo and Ahnesjö, 1996).

Sex Roles

In general terms, anisogamy, or the difference in size of gametes from males and females, is ultimately responsible for the typical differences we observe in male and female sexual behaviour (see Kodric-Brown and Brown, 1987 for a review). Sperm is a fast producing, rapidly renewable resource produced in large quantities while eggs are energy and time consuming resources produced in low quantity. This results in a highly

asymmetric reproductive potential for both sexes: males can presumably fertilize hundreds or thousands of females with little effort or investment if given the chance, while most females must invest a lot of energy and time to each ovum before fertilization (Forsgren *et al.*, 2002).

The reproductive success of males is usually limited by access to fertilizable females, while females are not typically limited by access to males. This skewed equilibrium in reproductive potential leads to a male-biased OSR, so that competition for mates is traditionally more intense amongst males, while choosiness towards mates is usually higher in females. Nonetheless, the OSR is not a fixed parameter as it can be affected by various factors, *e.g.* differences between the sexes in age at maturity, longevity, migration schedules, resource availability, spatial distribution or mortality (Emlen and Oring, 1977; Clutton-Brock and Vincent, 1991; Kvarnemo and Ahnesjö, 1996). If the OSR varies within a species, spatially or temporally, the intensity of mating competition should vary accordingly and, if the variation is large enough, a change in sex roles is expected (Forsgren *et al.*, 2004). In fact, the sex roles in fish have proven to be highly dynamic, varying not only among populations of the same species (Almada *et al.*, 1995) but also from traditional to reversed in the same population throughout the season (Forsgren *et al.*, 2004) and in some cases, shifts from traditional to reversed and back during one breeding season (Shibata and Kohda, 2006). Altogether, sex roles reflect a response to social and ecological circumstances (Owens and Thompson, 1994), as these can override the effects of anisogamy and promote female intra-sexual competition for access to mates and male choosiness for better quality females.

Alternative reproductive tactics

In a typical competitive sexual context, males may either invest in ways to gain direct access to females or fertilizable eggs (bourgeois tactic), or attempt to circumvent direct competition with other males (parasitic tactic; Taborsky, 1997; Taborsky, 2001). Bourgeois males invest either in direct defence of mates, in monopolizing resources that are important for reproduction or for females, or in developing and displaying traits that signal male quality, such as courtship behaviour or secondary sex characters. On the other hand, parasitic males assume alternative reproductive tactics (ART) that exploit the reproductive investment of bourgeois males by behaving swiftly or inconspicuously, in order to break the monopolization of mates by bourgeois males (Gross, 1982; Taborsky, 1997; Taborsky, 2001). Parasitic males may use female mimicry, sneaking behaviour, or even cooperation with bourgeois males in order to get access to the spawning area and reproductive resources defended by the bourgeois male (Taborsky, 1994; 1997; 1998; Taborsky, 2001). In fact, “parasitic” males can cooperate with the

bourgeois males, for example in territory defence, and thereby gain access to the spawning scene and fertilizable eggs. Indeed, the investment of these males, e.g., in providing assistance in the bourgeois male defence or mate attraction behaviours can change the relationship between parasitic / bourgeois into a more cooperative association (Taborsky, 1994; Taborsky, 2001). Three main factors may explain why many species of fish are so prone to ART:

- 1-Indeterminate growth, resulting in large size differences among sexually mature males and allowing smaller competitors to be successful if they adopt a parasitic tactic, instead of directly competing with older, larger males;
- 2-External fertilization, allowing simultaneous parasitic spawning;
- 3-Male parental care, permitting parasitic males to spawn without the costs associated with parental care (Taborsky, 1999).

Among these factors, the pronounced size asymmetries among reproductive males seems to be the most important one, which has been suggested to result from the fact that ART evolved in systems presenting high male intra-sexual competition (Oliveira, 2006).

In general, these condition-dependent ART reflect behavioural and/or morphological characteristics whose expression depends either on environmental conditions or on the 'status' of the individuals in which they appear (Shuster, 2002). These individuals presumably assess their potential mating opportunities and make a behavioural or developmental 'decision' that positively influences their mating success (Shuster, 2002). This plasticity in behaviour is often based on a succession of ontogenic stages and the 'decision' for the optimal tactic generally depends on size. Thus, if monopolization of locations, resources or females provides prime access to mates, small individuals should succeed better if they adopt parasitic tactics until a size at which direct competition for resources might be feasible is reached (Taborsky, 1998). Nonetheless, the fertilization success of any male is ultimately dependent on sperm competition, and also on the development of many morphological structures that have evolved in order to gain advantage in the 'sperm wars'. These include traits useful in intra-sexual competition, signalling attributes used in inter-sexual communication and, most importantly, size and structure of testes that, in principle, determines sperm production (Billard, 1986). In this respect, the relative size of testes, usually expressed by the gonado-somatic index (GSI: gonad weight / body weight x 100), is a very good indicator of how strong sperm competition is in a given system (Stockley *et al.*, 1997).

Mechanisms of behavioural plasticity

Behavioural plasticity can be defined as the variation in the expression of a genotype

that accompanies fluctuations in the environment. In a reproductive context, these variations should occur in highly competitive scenarios, i.e. when sexual selection is strong (Shuster, 2002). Reproductive plasticity is usually studied at the intra-population level, mostly in cases where there is phenotypic variation in male ART (Gross, 1984; Bass, 1992; Gonçalves *et al.*, 1996; Oliveira *et al.*, 2001b; Oliveira *et al.*, in press). However, it is assumed that the mechanisms underlying behavioural plasticity at the inter-population level are similar, both at ultimate and proximate levels. Phenotypic plasticity is a life-history trait that is thought to have evolved in order to allow animals to shift resources from one life-history stage to another (e.g. from reproduction to growth or vice-versa; West-Eberhard (1989)). These shifts between life-history stages may be controlled by endocrine mechanisms: androgens are both involved in the animal's investment in current reproduction and in modulating different phenotypic traits (both morphological and behavioural), hence they are excellent candidates to mediate transitions between life-history stages (Oliveira, 2006). Secondary sex characters (SSC) are generally viewed as a product of sexual selection, either associated to female preference for exaggerated traits or to male advantage in bearing such traits (Andersson, 1994). The expression of SSC in teleost fish is known to be related to the circulating levels of androgens (Liley and Stacey, 1983; Borg, 1994; Oliveira *et al.*, 2001b). Indeed, testosterone (T) and 11-ketotestosterone (11KT) have been shown to be the main androgens involved in the expression of these SSC (Kime, 1993), while simultaneously mediating the expression of reproductive behaviours (Oliveira *et al.*, 1996; Oliveira *et al.*, 2001a; Oliveira *et al.*, 2001b; Oliveira *et al.*, 2001c; Oliveira, 2004; 2005). In species that present ART, males that follow a typical or 'bourgeois' tactic, i.e. investing in resources or competing for mates (Taborsky, 2001), present higher circulating levels of androgens than parasitic males ((Oliveira *et al.*, 2001b). In the peacock blenny for example, bourgeois males have higher circulating levels of 11KT than parasitic males, while T shows no clear pattern (Brantley *et al.*, 1993; Oliveira, 2006). In this species, as in other blenniids, bourgeois males present well developed testicular glands (de Jonge *et al.*, 1989) where 11KT is thought to be synthesized (Reinboth and Becker, 1986; Oliveira *et al.*, 2001a). Interestingly, administration of 11KT to female-mimicking parasitic males also promotes the appearance of SSC and inhibits the expression of female-like behaviours (Oliveira *et al.*, 2001d). Intriguingly, administration of estrogen to males has also been reported to promote male typical sexual displays in several taxa (e.g. Ball, 1937; Guhl, 1949). In order to explain this apparent paradox, Naftolin *et al.* (1975) proposed an hypothesis suggesting that the masculinizing effects of T on reproductive behaviour are partially dependent on its aromatization into estradiol by the enzyme aromatase in the brain. Several studies have

experimentally confirmed this hypothesis in a number of taxa (see Baum (2003), for a review), although in fish, this hypothesis is still yet to be demonstrated. Recently, Gonçalves *et al.* (2007), have tried to establish a link between steroid levels and the mechanism promoting the tactic switch in the peacock blenny *Salaria pavo*, a fish with sequential ART (males may either begin their reproductive activity as sneakers in the first season, then switch into bourgeois in their second year). Nonetheless, the results left unclear what endocrine mechanism is responsible for promoting the tactic switch, since administration of T, 11-KT and estradiol failed to induce male-like displays in sneakers. A model proposed by Oliveira *et al.* (2005) integrates set of proximate mechanisms acting in synergy to promote the differentiation of tactic-specific characters: while 11KT is responsible for the development of secondary sex characters, the expression of sexual behaviours may be dependent on the neuropeptide arginine-vasotocin, and the development of gonads and spermatogenesis may be dependent on a 11KT/T ratio. Therefore, it is possible that a constellation of traits that make up a reproductive phenotype may not all depend on a single agent (Oliveira *et al.*, 2005).

Study species

In this thesis, the reproductive behaviour of the peacock blenny *Salaria pavo* (Risso) was investigated. The peacock blenny is a small intertidal fish occurring in the Mediterranean and adjacent Atlantic coasts (Zander, 1986). The species presents a strong sexual dimorphism with males being larger than females and presenting several conspicuous secondary sexual characters such as a head crest and a sex pheromone-producing anal gland in the first two rays of the anal fin (Fishelson, 1963; Papaconstantinou, 1979; Patzner *et al.*, 1986). Males defend nests in rock crevices or holes and take care of the eggs until they hatch (Patzner *et al.*, 1986). There is evidence for ecological modulation of sex roles in this species. While in Mediterranean rocky shore populations parental males defend nesting territories and actively court females (Fishelson 1963; Patzner *et al.* 1986), in a natural population of Ria Formosa coastal lagoon (Algarve, Southern Portugal) nest sites are very scarce, courtship is almost entirely initiated by females, and both male and female intra-sexual competition occurs (Almada *et al.*, 1995).

In addition, male alternative reproductive tactics have been described in lagoon populations (Gulf of Lion, France (Ruchon *et al.*, 1995) and in Ria Formosa, Portugal, (Gonçalves *et al.*, 1996)) where the shortage and aggregation of nest sites seem to promote a strong male-male competition for nests, with small males being unable to acquire nests and adopting an ART. In the Ria Formosa population, the only adequate nesting sites are found in artificial reefs that delimit clam culture fields in muddy intertidal

flats. These reefs are made of bricks, stones, tiles and other debris, and nesting males use brick holes as nests (Almada *et al.*, 1994). In this population small parasite “sneaker” males mimic the females’ courtship behaviour and morphology, in order to approach nesting males and sneak fertilizations of eggs during spawning events (Almada *et al.*, 1994; Gonçalves *et al.*, 1996). The female-mimicry seems to be efficient as nesting males court and attack small sneakers and females with equal frequency (Gonçalves *et al.* 2005). Sneaker males are younger than nesting males, have higher gonadosomatic indexes (Gonçalves *et al.*, 1996) and switch into bourgeois males from one breeding season to the next (Fagundes, *et al.*, unpublished data). Since some males do not seem to breed in their first year, these data suggest a condition-dependent tactic for small males that can either reproduce as sneakers or post-pone reproduction to subsequent breeding seasons (Oliveira *et al.*, 2005).

Interestingly, the existing data on the Ria Formosa population is generally coincident with the description of a closely related but rather rare species, the zebra blenny *Salaria basilisca* (Valenciennes, 1836). This blenny is described to inhabit the *Posidonia oceanica* seagrass plains throughout the Mediterranean, sharing many morphological characteristics with the portuguese population of *S. pavo* (Heymer, 1985a). Furthermore, many behavioural features from *Salaria basilisca* coincide with those of the *S. pavo* in Portugal (see Heymer, 1985b; Heymer, 1987; also Almada *et al.*, 1995), differing from that described in other populations (Patzner *et al.*, 1986). In addition, since the female-to-male sex change in *S. basilisca* described by Heymer (1980; 1985a) was based solely on behavioural observations and histological studies were involved, different authors have suggested that this tactic switch is most likely a female-mimicking sneaker into a parental male existing in *S. pavo* (Gonçalves *et al.*, 1996; Gonçalves *et al.*, 2003). Thus, these similarities raised questions regarding the phylogenetic identity of the Ria Formosa population of *S. pavo*.

Objectives of this study

In this study we compared two populations of the peacock blenny: the Ria Formosa (RF) (Portugal) population, with low availability of nests sites and occurrence of ART and sex-role reversal (Almada *et al.*, 1994; Almada *et al.*, 1995; Gonçalves *et al.*, 1996), and a population in the Gulf of Trieste (GT) (Italy), with high availability of nest sites, no description of ART and typical sex-roles (Patzner *et al.*, 1986).

Specifically, we addressed the following questions:

- Are the sex roles dynamic in this species? Can the sex roles be modulated by experimentally manipulating the aggregation of nest sites?

- How does the mating system function in populations with contrasting nest site availability? Do these populations differ genetically? Do ART exist in populations with nest site abundance?
- Are the sex roles and reproductive tactics flexible in both populations? What are the fine differences in behaviour when individuals from both populations are tested under standardized conditions?
- What are the morphological differences in females and different male phenotypes between populations? What are the endocrine correlates of those differences?
- What is the expression of brain aromatase in different male morphs as well as in females in both populations? Does it correlate with observed behavioural differences?

A multi-disciplinary approach was followed, integrating different levels of analysis. At a zoogeographical scale, we used population genetics to assess the phylogenetic proximity of the populations studied; at a habitat scale, we characterized the ecological setting of both populations, namely the type of substrate as well as the abundance and dispersal of nest sites; at a populational scale, we observed the reproductive behaviour in the field; at an individual scale, we studied the reproductive behaviour in the lab; at a physiological scale, we examined the morphology and the endocrine correlates of the behavioural differences; and finally at a molecular scale, we investigated the brain aromatase expression in these animals. The integration of observational and experimental approaches and the analysis of results both at ultimate (adaptive) and proximate (physiological) levels, in an integrative approach, will hopefully contribute to a better understanding of the mechanisms underlying behavioural plasticity and ecological modulation.

The information gathered was organized in five chapters:

- I. The effect of nest aggregation on the reproductive behaviour of the peacock blenny;
- II. Inter-populational variation of the mating system in the peacock blenny;
- III. Plasticity of reproductive behaviour in two populations of the peacock blenny;
- IV. Environmental modulation of secondary sex characters and androgen levels in two populations of the peacock blenny *Salaria pavo*;
- V. Brain aromatase mRNA expression in two populations of the peacock blenny *Salaria pavo* with divergent mating systems.

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CHAPTER I

The effect of nest aggregation on the reproductive behaviour of the peacock blenny *Salaria pavo*

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ABSTRACT

The effect of nest aggregation in courtship behaviour was tested experimentally in an ecologically constrained, sex-role reversed population of the peacock blenny *Salarias pavo*. Mixed sex groups of 8 males and 8 females were tested in experimental tanks, containing eight potential nests either aggregated or dispersed. In the aggregated treatment, males spent more time inside their nests and monopolized other potential nests, causing a female biased operational sex ratio(OSR). In the aggregated treatment, females also expressed more courtship behaviour. The results in general support the prediction that the aggregation of nests promotes male monopolization of potential nests, resulting in fewer nest-holding males and therefore a female biased OSR that leads to the reversal of sex roles.

Keywords: resource monopolization; sex-role reversal; operational sex ratio.

INTRODUCTION

Emlen & Oring (1977) provided an ecological framework for the evolution and expression of polygamous mating systems. In this seminal paper it was proposed that the differences in the intensity and direction of sexual selection found in different species, and frequently between populations within the same species, are a consequence of the ability of a portion of the population to control the access of others to potential mates. This can be achieved either directly through the physical exclusion of competitors or indirectly through the monopolization of critical resources for reproduction.

In general, males have a higher potential reproductive rate than females, resulting in a male-biased operational sex ratio (OSR, i.e. the ratio of ready-to-mate males to ready-to-mate females; (Emlen and Oring, 1977; Kvarnemo and Ahnesjö, 1996). Under these circumstances, males are the courting sex, there is high level of male competition to access mates, and females are choosy for potential mates. However, sexual selection operating both through intra-sexual competition for breeding opportunities and through male mating preferences is common in females (Clutton-Brock, 2007). For example, in some cases male parental care may result in a lower potential reproductive rate in males than in females (Ahnesjö *et al.*, 2001). This may result in the reversal of sex roles, i.e. females compete for access to mates and/or are the courting sex (Almada *et al.*, 1995). Furthermore, sex-role reversal can occur within a population throughout the breeding season. In the two-spotted goby *Gobiusculus flavescens* (Fabricius), sex-role reversal occurs while the OSR becomes increasingly female-biased towards the end of the breeding season due to a decrease in available nest-holder males in the population (Forsgren *et al.*, 2004). Also, in the blenniid *Petroscirtes breviceps* (Valenciennes), a nest brooder with exclusive paternal care, sex-role reversal was found only in the middle of the breeding season when the availability of nesting sites is lower, therefore making the OSR female-biased for a period of time (Shibata and Kohda, 2006). These studies point to a special importance of resources (such as nesting sites) to the definition of a mating system. For example, the spatial aggregation of resources may limit the number of individuals who can actually breed, by exclusion of less able competitors (Emlen and Oring, 1977; Hastings, 1988; Oliveira *et al.*, 1999; Ahnesjö *et al.*, 2001; Clutton-Brock, 2007). In fish this might be particularly relevant for cavity spawners with paternal care, in which both the ratio of sexually mature males and females and the availability of spawning sites may affect the OSR. In fact, the spatial aggregation of nest sites may lead to monopolization by a small number of males. This impedes

the remaining males from accessing a nest and alters the number of individuals qualified to mate (Ahnesjö *et al.*, 2001). In the common goby *Pomatochistus microps* (Krøyer), an experimental decrease in nest availability lead to a female biased OSR and a shift in the sex roles, with females actively courting and competing for partners (Borg *et al.*, 2002).

In the present study, the effect of nest aggregation on the reproductive behaviour of *Salaria pavo* (Risso) was tested. This species presents a promiscuous mating system in which both sexes have active courtship displays. This particularity allowed testing the effects of resource spatial distribution not only on males' territoriality and resource monopolization but also on the courtship of both sexes.

S. pavo inhabits the rocky shore of the Mediterranean and adjacent Atlantic coast (Zander, 1986). In general, males are the courting sex and actively attract females into a hole or crevice in the rock that is used as a nest. In a territory around the nest, males display courtship that includes bright coloration, body jerking, 8-figure swimming and trying to chase females into the nest; females are typically more passive than males in their behaviour, sometimes responding to male initiatives by turning on a conspicuous nuptial coloration that consists on a pattern of brown and white bars, and by displaying fast opercular movements, and/or body jerks (Patzner *et al.*, 1986). However, in Ria Formosa (Algarve, Southern Portugal), a costal lagoon where adequate nesting substrate is scarce and highly aggregated, the sex roles are reversed and females lead the courtship (Almada *et al.*, 1994; 1995). In this population, males nest in artificial reefs built by clam culturists in the muddy intertidal flats to delimit clam culture fields. These reefs are made of materials like bricks, tiles, stones and other debris. Brick holes are often used as nests and eventually adjacent holes are occupied by nest-holders, so territories can be virtually absent. Since there is a high scarceness of nesting sites, many sexually mature males are unable to establish nests throughout the breeding season, and this seems to promote high levels of male competition for access to nesting sites (Almada *et al.*, 1994). These ecological constraints seem to be responsible for a female-biased OSR since the low number of nest-holder males seems to limit the female reproductive potential as nests become filled with eggs (Almada *et al.*, 1995; Oliveira *et al.*, 1999). These factors seem to explain the sex-role reversal in this population of *S. pavo*, in which females are the courting sex and compete for access to mates. In the current study, the effect of nesting site aggregation on the reproductive behaviour of male and female *Salaria pavo* was tested experimentally. More specifically, it is predicted that the aggregation of nest sites will promote the monopolization of nests by males, resulting in a lower number of breeding males. Therefore, the OSR will become

female biased with a concomitant increase in female courtship and intra-sexual aggression, paralleled by an increase in male intra- and inter-sexual aggression and choosiness when nests are aggregated.

MATERIALS AND METHODS

The experiment was carried out in outdoor flow-through tanks (120 x 100 x 60 cm) at the Ramalhete Experimental Station (University of Algarve) in the Ria Formosa, Portugal (36°59' N, 7°51' W). Seawater was delivered to the tanks from the station reservoirs and the outflow into the drainage pools. Two tanks, containing sand on the bottom and abundant sheltering provided by stones and shells, were used to maintain separately male and female stocks; two other tanks of similar size were used for the experiment. An eight-hole brick (25 x 19.5 x 10 cm) was placed in the centre of one of the tanks providing eight aggregated nesting sites (nearest nest distance = 0.5 cm), hereafter designated Aggregated Treatment (AT). In the other tank, eight brick-made individual nesting sites (25 x 4.5 x 4.5 cm) were uniformly distributed (nearest nest distance = 20 cm) along the wall of the tank and with the entrances facing the centre of the tank. This treatment is hereafter called Dispersed Treatment (DT) (Fig. 1). All potential nests had a 3.5 X 3.5 cm entrance. The end of each brick hole was sealed with cement. A removable glass drawer was inserted inside each cavity so that the identity of the occupant of each nest and the number of eggs in the nest could be easily determined. These drawers were hermetic and had their entrance partially obstructed, since both males and females prefer narrow entrances (Oliveira *et al.*, 1999; Ruchon *et al.*, 1999). Between replicates, the sand on the bottom was renewed and treatments were switched between the two tanks to avoid tank effects. New glass drawers were used in each replicate. Replicates were carried out with eight sexually mature fish of each sex.

S.pavo were captured during the breeding season in Ria Formosa and the experiment was carried out from May to July 2001 and in the same period in 2002. The fish were kept for at least one week in the stock tanks and were fed with sliced mussel whilst in the stock tanks and during the experiment. On the day before the experiment, males and females were anaesthetized with 1:12000 MS 222 (ethyl 3-aminobenzoate, SIGMA, St. Louis, USA), had their Ls measured and were tagged with two coloured beads (ca. 3 mm) attached with a thread to the dorsal body muscle, immediately before the first ray of the dorsal fin (Patzner, 1986). Fish were left to recover in small aquaria with abundant aeration. The tags were very conspicuous both by direct observation and on video, allowing discrimination of

individuals in a tank. Males and females were placed simultaneously in the experimental tanks 24 hours prior to the start of the behavioural observations. Each replicate lasted for four days. Seven replicates were conducted for each treatment, three in 2001 and four in 2002. The average standard length and head crest size of fish between treatments did not differ (Wilcoxon W unpaired test for Ls of males: AT=9.81±0.95, DT=9.90±0.20, N= 7, $P>0.05$. Wilcoxon W unpaired test for head height of males including crest: AT=2.59 ± 0.27, DT=2.62 ± 0.63, N=7, $P>0.05$; Wilcoxon W unpaired test for Ls of females: AT=6.90±0.20, DT=6.94±0.25, N= 7, $P>0.05$). Only females presenting an evident abdominal swell (an indicator of ripeness) were used. The proportion of females in each treatment that released eggs upon abdominal pressure did not differ (AT=0.16±0.05, DT=0.17±0.05, Wilcoxon W unpaired test, N= 7, $P>0.05$). All the fish were released in good condition in the same place as their capture.

This study was carried out in conformity with ASAB guidelines and the Portuguese law on animal experimentation (1005/92, Project 'Hormones and Life History Trade-Offs and Plasticity').

Video recordings were carried out twice a day at 7.30am and 5.30pm, during the four days of the experiment. These sampling times are within the periods of higher spawning activity (Patzner *et al.*, 1986). To control for order effects, the recording sessions started in a different treatment and in a different nest each day.

In the DT, the video image was centred at the nest entrance, covering an area of about 30 cm². Each focal sampling lasted for 10 min, and the first 5 min were discarded as habituation time to the observer and camera. At the end of each focal sampling the camera was moved to the next nest and so on, providing a total of 40 min of observation per session. In the AT, the focal field covering the front of the brick and adjacent 30 cm² of tank bottom was recorded continuously for 40 min. As analyzing nests individually in this treatment was virtually impossible due to the high proximity of the entrances (0.5 cm), the whole period of 40 min was analyzed for all nests. This resulted in an asymmetry of nest focal time between treatments and so each fish individual focal time was estimated as the actual time that specific individual was visible in the film.

The behaviours selected for analyses were: female courtship, female intra-sexual aggression, male intra- and inter-sexual aggression, time spent inside the nest and male courtship (see Patzner *et al.* (1986) for a detailed description of these behaviours). The focal observations were registered with *The Observer*® v.3 (Noldus, the Netherlands).

Nest occupation was recorded twice a day after the video recordings took place. Removing the glass drawer and identifying the occupant assessed the occupation of the nests. The number of eggs was calculated by visual estimation of the area covered by the egg clutch (a priori observations revealed approximately 80 eggs per cm²).

Variables that did not follow the parametric assumptions were analysed by non-parametric statistical methods. All tests were two-tailed and $\alpha=0.05$.

The behaviours were expressed as frequency per time of observation (i.e. time during which the fish was visible in the video). Data are presented as means per replicate except when stated otherwise. The statistical analysis was performed using the freeware statistical analysis software 'R' (R Development Core Team, 2005). In this software, the two-sample non-parametric test corresponding to Mann-Whitney U test in other software packages is the Wilcoxon W unpaired test (R Development Core Team, 2005).

RESULTS

A higher percentage of females courted the males (mean $\pm$ S.E.: AT = 53.6 ± 23.3 %, DT = 5.3 ± 6.0 %, Wilcoxon W unpaired test N=7, $P<0.01$) and with higher frequency (Fig. 2) in the AT. No significant difference was found in female intra-sexual aggression corrected for the exploratory behaviour (agonistic interactions between females x female nest approaches⁻¹) (Fig. 3). Females visited more nests in the DT than in the AT (AT = 0.391 ± 0.131 , DT = 2.059 ± 0.820 , Wilcoxon W unpaired test N=7, $P<0.01$).

Male intra- or inter-sexual aggression did not differ between treatments (Fig. 3).

Males spent a significantly larger proportion of time inside a nest in the AT than in DT (Fig. 4a) and the variability (SD) among males in the proportion of time spent inside a nest was also higher in AT (AT = 23.1 ± 4.9 %, DT = 3.0 ± 1.0 %, Wilcoxon W unpaired test N=7, $P<0.01$). The maximum proportion of time inside the nest was higher in AT than in DT (AT = 72.0 ± 12.7 %, DT = 12.3 ± 1.4 %, Wilcoxon W unpaired test N=7, $P<0.01$) but no significant differences were found in the minimum time between the two treatments (AT = 9.2 ± 7.7 %, DT = 4.1 ± 2.7 %, Wilcoxon W unpaired test N=7, $P>0.05$). Also, higher proportion of nesting sites were occupied by males in DT than in AT (Fig. 4b).

No significant differences were found between treatments for the frequency of male courtship (Fig. 2) and proportion of courting males (AT = 3.6 ± 5.8 %, DT = 7.1 ± 4.1

%, Wilcoxon W unpaired test $N=7$, $P>0.05$). However, females in the AT courted significantly more than males, whereas the courtship frequency did not differ between sexes in DT (Fig. 2).

No significant difference was found in the proportion of males with eggs (AT: 17.9 ± 12.4 %, DT: 19.6 ± 10.7 %, Wilcoxon W unpaired test $N=7$, $P>0.05$) or in the number of eggs between treatments (AT: 20.2 ± 12.9 , DT: 15.9 ± 9.6 , Wilcoxon W unpaired test $N=7$, $P>0.05$)

DISCUSSION

Nest aggregation seems to have promoted resource monopolization by some of the males, with a consequential female-biased OSR that resulted in higher frequencies of female courtship and female intra-sexual competition. The results indicate that fewer males in the AT were able to occupy nesting sites than in DT and that the few nest holders in the AT spent more time inside the nest than the higher number of nest holders in DT. This suggests an effort to monopolize several nests as valuable resources in the AT, which become economically defendable because of their spatial aggregation. In fact, the aggregation of nests altered the number of males qualified to mate, excluding those who did not gain access to a nest (Ahnesjö *et al.*, 2001). Grant (1993) reports a parallel situation in food competition in several fish species and other vertebrates, where the spatial aggregation of food patches also promotes resource monopolization.

Since females spawn multiple clutches and males can receive clutches from several females, it could be argued that the female biased OSR due to nest aggregation would not be as high as expected from theory. Nevertheless, a response in female behaviour to the treatments was detected. In fact, the low percentage of occupied nests in the AT resulted in about one nest-holder male to two mature females. In this female-biased OSR, females responded by courting more in the AT than the DT, where the OSR was more even. This paradox can be explained by two non-exclusive mechanisms: 1) although there is enough nest surface to receive clutches from different females, they would have to take turns to spawn; apparently *S. pavo* females do not adopt this queuing behaviour and the resulting overlap in spawning attempts by different females promotes female competition near the nest sites; 2) Although on average there is only one more breeding male in the DT than in the AT, females in the AT have to aggregate in front of a single brick. This may lead to a perceived higher density by the females resulting in an increased rate of courtship.

Similar results were found by Forsgren et al. (2004) in the gobiid *Gobiusculus flavescens* and emphasize the importance of factors that directly affect the OSR, especially in cavity spawners which seem to be heavily influenced by the availability of nesting sites (Almada et al., 1995; Oliveira et al., 1999; Ahnesjö et al., 2001). Also Shibata & Kohda (2006) found dynamic sex roles in the blenniid *Petroscirtes breviceps*, where the number of available nests showed only a small but significant fluctuation across the breeding season. Furthermore, the spatial aggregation of resources may also lead to increasing female intra-sexual competition for breeding opportunities in other animal groups, such as mammals (Clutton-Brock et al., 2006).

Despite the fact that variability among males in the time spent in the nests was higher in the AT, the aggregation of nests did not result in higher male intra-sexual aggression. The explanation can lie in the fact that an increase in population density might be accompanied by mechanisms to avoid aggression and males might be using other mechanism of male-male competition. In the population of Ria Formosa, breeding aggregations of *S. pavo* are so dense that territories are virtually absent due to the high-risk of nest take-overs (Almada et al., 1994). Nest-holder males restrain from leaving their nests and most behavioural activities, including courting females or feeding are restricted to the nest itself (Gonçalves and Almada, 1998). It has also been observed that aggression among males does not seem to rise with increasing density of nest-holding males (Oliveira et al., 2001). As expected, in the AT males spent a larger proportion of time inside the nests suggesting that endurance instead of overt aggression is being used in male-male competition in this species. Furthermore, Borg et al. (2002) did not find differences regarding male-male competition in the goby *Pomatochistus microps* in a study where nest availability was manipulated, suggesting that sexual selection is still acting in males even when the OSR is female biased and females lead the courtship.

Males did not present differences in courtship between treatments. This result is supported by a field study that compared two populations of *S. pavo* living under different nest-site availability conditions that also found higher differences in female rather than in male behaviour (Saraiva et al., in prep.). A decrease in nest availability should increase the sexual selection pressure on females and elicit a higher response in direct competition. In males, the effect should be noted mainly on competition for access to nests rather than courtship, because although males were spatially separated in the DT, they were all in visual contact. This perception of competitors may constrain their courtship behaviour, due to the risk of nest take-over (Almada et al., 1995). In populations of *S. pavo* living in rocky shores with no

limitation of nest sites, the nests are hardly ever in visual contact (Patzner *et al.*, 1986; Saraiva *et al.*, in prep). This would also help to explain why males also did not present differences in aggression between treatments, since the perception of competition would still be present in the DT (although to a lesser degree).

Differences in male aggression towards females were expected to occur only when and if nest space available for spawning would become a limiting factor so that no more females can spawn. In fact, since there was no difference found in number of eggs or number of males with eggs, and these values were overall low, there was no apparent reason for males to reject females.

In conclusion, this study shows that aggregation of nests may bias the OSR towards females and decreases the number of males qualified to mate, thereby creating a competitive mating situation for females. This apparently leads to predominant female courtship and intra-sexual competition. The results found in this work suggest that nest aggregation has a clear impact in the dynamic of sex-roles.

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FIGURE LEGENDS

Figure 1 – Disposition of nest in the Aggregated Treatment (AT) and Dispersed Treatment (DT). Not to scale. Distance between nest entrances in the dispersed treatment: 20cm.

Figure 2 – Courtship behaviours per minute of males and females in both treatments. FAT: Females in the AT; MAT: Males in the AT; FDT: Females in the DT; MDT: Males in the DT. Female courting: Wilcoxon W unpaired test $N=7$, $P<0.05$; Male courting: Wilcoxon W unpaired test $N=7$, $P>0.05$. FAT Vs MAT courting: Wilcoxon V paired test, $N=7$, $V=0$, $P<0.05$. FDT Vs MDT courting: Wilcoxon V paired test, $N=7$, $P>0.05$. Error bars represent S.E.. * indicates significant differences at $P<0.05$.

Figure 3 – Agonistic Interactions between individuals: Male-male: Wilcoxon W unpaired test, $N=7$, $P>0.05$; Female-Female: Wilcoxon W unpaired test, $N=7$, $P>0.05$; Male-Female: Wilcoxon W unpaired test, $N=7$, $P>0.05$. Error bars represent S.E.. NS: non-significant. * indicates significant differences at $P<0.05$.

Figure 4 – A) Proportion of time spent inside the nest by males: Wilcoxon W unpaired test, $N=7$, $P<0.01$. B) Proportion of nest occupation: Wilcoxon W unpaired test, $N=7$, $P<0.05$. Error bars represent S.E.. * indicates significant differences at $P<0.05$.

FIGURES

Figure 1

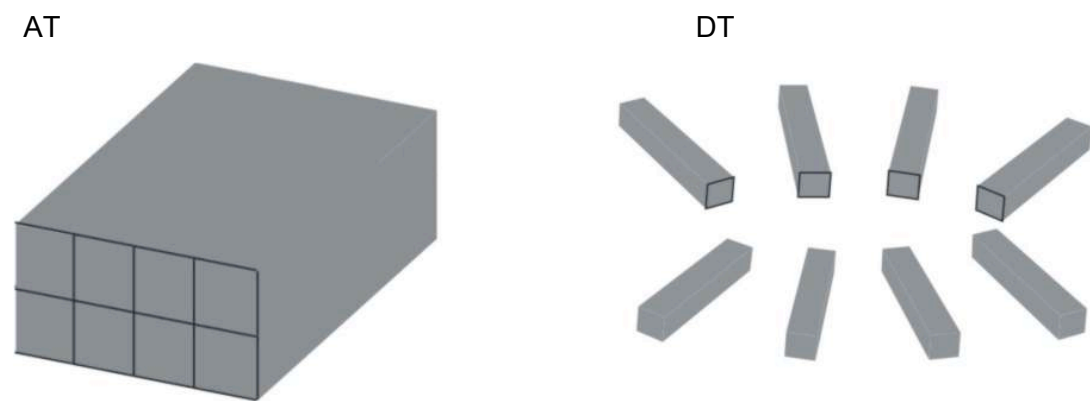


Figure 2

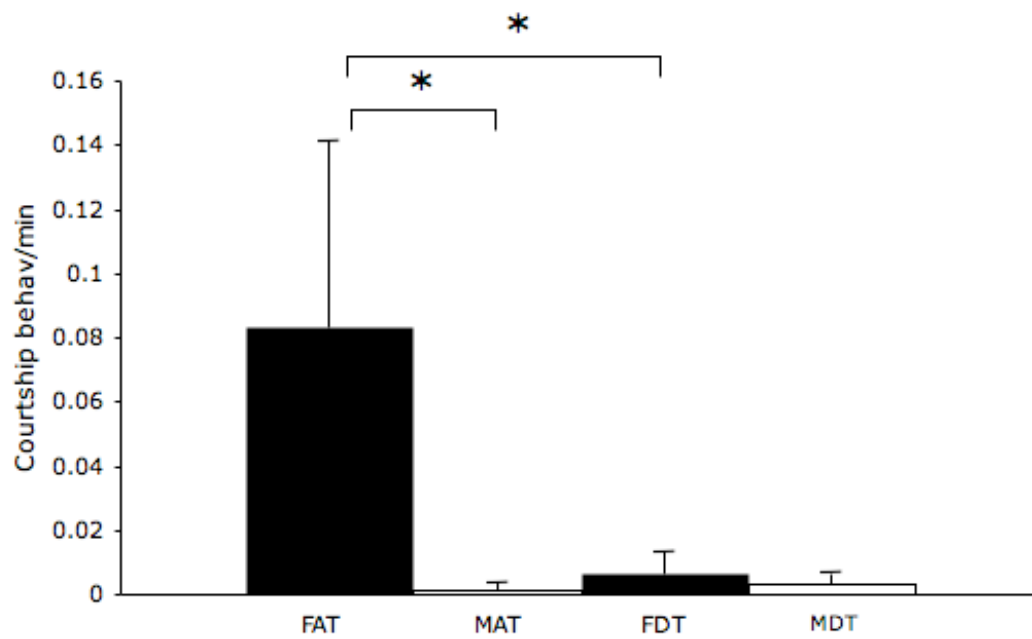


Figure 3

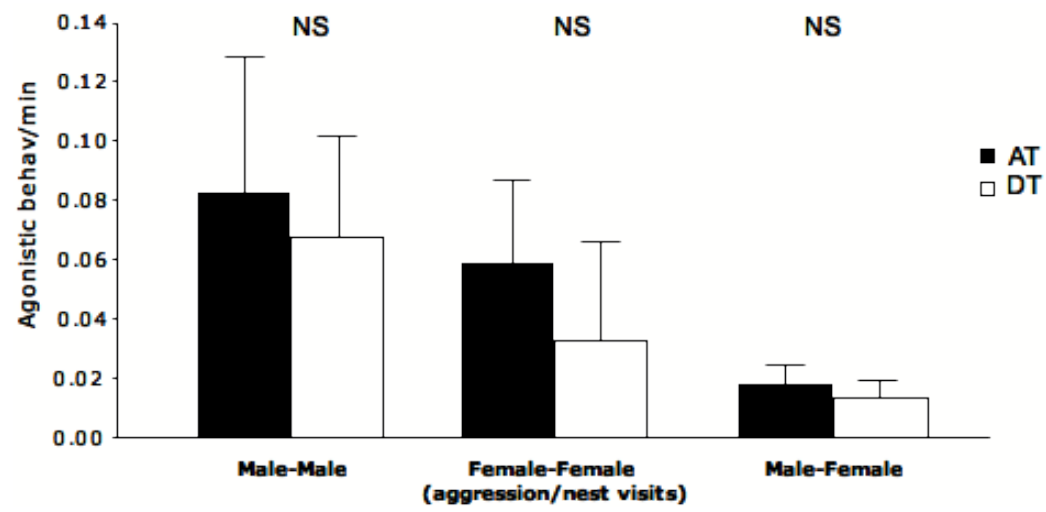
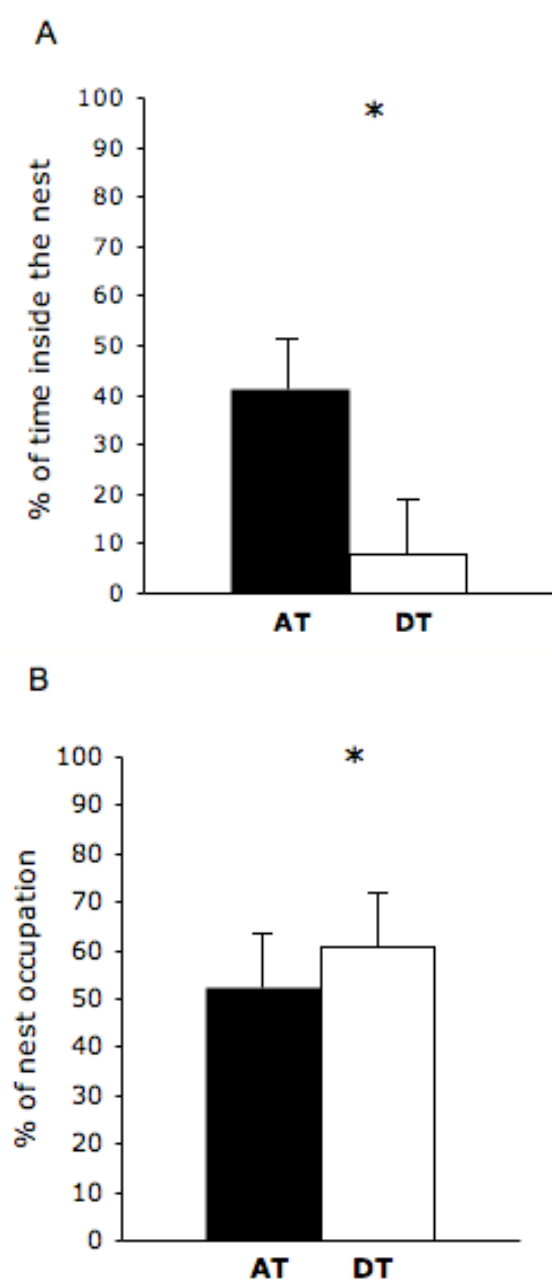


Figure 4



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CHAPTER II

Inter-populational variation of the mating system in the peacock blenny

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ABSTRACT

The peacock blenny *Salaria pavo* is a small benthic fish that can be found throughout the Mediterranean and adjacent Atlantic areas. Typically, males establish nests in rock crevices from where they attract females. Inter-population differences in the mating system of *S. pavo* in relation to ecological variations have been suggested. The available data suggests that in rocky shores males take the main role in courtship while in populations found in coastal lagoons, where nesting substrate is scarce, sex-roles are reversed. The presence of sneaker males that reproduce parasitically (a male alternative reproductive tactic) has only been reported so far for coastal lagoons populations. To better characterize the possible differences in the species mating system in relation to ecological and genetic factors one rocky shore (Gulf of Trieste, Italy, GT) and one lagoon population (Ria Formosa, Portugal, RF) that had been reported to differ in sexual behaviour were studied. Nest availability was higher and nest aggregation was lower at GT. As expected, GT males courted more and in higher proportion than RF males. Reversely, RF females directed more courtship displays towards nest-holders than GT females. The frequency of parasitic males was lower in the GT population than at RF. A genetic analysis using mitochondrial (12S, 16S and control region) and nuclear (S7) genes showed no major differences between these two populations. Thus the inter-population differences observed in the mating system of *S. pavo* most probably reflect a plastic behavioural response to varying ecological conditions and the occurrence of ART seems to be condition dependent.

INTRODUCTION

In a seminal paper published over 30 years ago Emlen & Oring (1977) proposed that the ecological settings influence the mating system present in any given species. They hypothesized that the spatial and temporal dispersion of mates, or the resources required to attract them, is the key component of the environmental potential for polygamy (i.e. resource monopolization). Several studies over the last decades have extensively confirmed this hypothesis and showed how the ecological modulation of behaviour can have severe impacts on mating strategies (e.g. Kolhuru & Grether, 2004; Weir & Grant, 2004).

One important effect of ecological modulation of reproductive behaviour is the reversal of sex roles (Almada *et al.*, 1995; Borg *et al.*, 2002). Generally, in teleost fishes sex role reversal occurs as a result of a female biased operational sex ratio (OSR: the ratio of sexually active males to fertilisable females). This may happen because males have a limited capacity to care for the offspring, so comparing male and female potential reproductive rates should predict the sex with higher levels of intrasexual competition (Ahnesjö *et al.*, 2001). Current parental investment theory asserts that regardless of whether males or females provide parental care, the sex with the higher potential reproductive rate will compete more strongly for mates (Clutton-Brock and Vincent, 1991).

In cavity spawners with exclusive male parental care, the number of nesting sites can limit the fecundity of females by limiting the number of nest-holder males at a given point in time (Kvarnemo and Ahnesjö, 1996). Consequently, the OSR of species who are cavity spawners must take into account the availability of spawning sites as well as the present mature male / female ratio (Hastings, 1988; Oliveira *et al.*, 1999). Forsgren *et al.* (2004), showed that along the spawning season of a Baltic population of the two spotted goby *Gobiusculus flavescens*, the OSR shifts towards females and fish progressively shift sex-roles. Similar results have been found by Shibata & Kohda (2006) in the blenny *Petroscirtes breviceps*. In this species, males start the breeding season actively courting females but the sex roles change from typical to reversed as the breeding season advances. Towards the end of the season, the sex roles change again so males return to be the courting sex. Together with the sex roles, so does the intra-sexual competition shifts from male-male to female-female and back. These changes seem to be explained by a decrease in available nests as the season moves forward, reaching a critical point at the peak of the breeding season, and by a gradual increase in the number of available nests towards the end (Shibata and Kohda, 2006).

These studies reveal a high degree of plasticity in these species, showing how the sex roles reverse along the spawning season as the number of available males decreases. They demonstrate that there are factors underlying the definition of sex roles that remain poorly understood, and these can have important implications in sexual selection processes (Forsgren *et al.*, 2004).

Another consequence of high male intra-sexual competition for limited reproductive resources is the occurrence of male alternative reproductive tactics (ART; Taborsky *et al.*, 2008). When resources are limiting and defensible, some males invest in territorial defence and female attraction (bourgeois males, *sensu* Taborsky, 1997) while others parasitically exploit reproductive resources (parasitic males *sensu* Taborsky, 1997). Bourgeois males invest in resources to attract mates, such as the differentiation of morphological ornaments, the expression of courtship signals, and/or the defense of a breeding territory (Taborsky, 1994; 1997; Taborsky, 2001). Parasitic males use female mimicry, sneaking behaviour, or cooperation with bourgeois males in order to get access to the spawning area and reproductive resources defended by the bourgeois male (Taborsky, 1994; 1997; 1998; Taborsky, 2001).

Here we test the hypothesis that variation in nest-site availability leads to different OSR regimes that will impact the mating system, namely which sex takes the initiative in courtship and the occurrence of alternative reproductive phenotypes. For this purpose, we compared the reproductive behaviour of the peacock blenny *Salaria pavo* (Risso) in two populations that vary in nest site availability.

The peacock blenny is a small intertidal fish occurring in the Mediterranean and adjacent Atlantic coasts (Zander, 1986). The species presents a strong sexual dimorphism with males being larger than females and presenting several conspicuous secondary sexual characters such as a head crest and a sex pheromone-producing anal gland in the first two rays of the anal fin (Fishelson, 1963; Papaconstantinou, 1979; Patzner *et al.*, 1986). Males defend nests in rock crevices or holes and take care of the eggs until they hatch (Patzner *et al.*, 1986). Differences in the reproductive behaviour of this species have been described in the literature. In Mediterranean rocky shore populations of this species parental males defend nesting territories and actively court females (Fishelson 1963; Patzner *et al.* 1986), whereas in a natural population of *S. pavo* living in the Ria Formosa coastal lagoon (Algarve, Southern Portugal) courtship is almost entirely initiated by females, and both male and female intra-sexual competition occurs (Almada *et al.*, 1995). The aggregation of nests in this population seems to have a clear impact on the dynamics of the sex roles (Saraiva *et al.*, in press). In addition, alternative reproductive male phenotypes

(sneaker males) have been described in two lagoon populations (Gulf of Lion, France, (Ruchon *et al.*, 1995) and Ria Formosa, Portugal, Gonçalves *et al.*, 1996) where the shortage and aggregation of hard-substrates that provide nest sites seem to promote a strong male-male competition for nests, with small males being unable to acquire nests and adopting an alternative reproductive tactic (ART). In the Ria Formosa population small parasite “sneaker” males mimic the females’ courtship behaviour and morphology, in order to approach nesting males and sneak fertilizations of eggs during spawning events (Almada *et al.*, 1994; Gonçalves *et al.*, 1996). The female-mimicry seems to be efficient as nesting males court and attack small sneakers and females with equal frequency (Gonçalves *et al.* 2005). Sneaker males are younger than nesting males, have higher gonadosomatic indexes (Gonçalves *et al.*, 1996) and switch into bourgeois males from one breeding season to the next (T. Fagundes, D. Gonçalves, J. L. Saraiva and R.F. Oliveira, unpublished data). Since some males do not seem to breed in their first year, these data suggest a condition-dependent tactic for small males, that can either reproduce as sneakers or post-pone reproduction to subsequent breeding seasons (Oliveira *et al.*, 2005). Sneaker males have not been described for the Mediterranean populations where nest-sites are widely available (Fishelson 1963; Patzner *et al.* 1986).

In this study we compare two populations of the peacock blenny: the Ria Formosa (RF) (Portugal) population, with low availability of nests sites and a population in the Gulf of Trieste (GT) (Italy), with high availability of nest sites. We characterized the ecological conditions, conducted field observations on reproductive behaviour to assess the mating system and the occurrence of ART and performed genetic analyses to assess the genetic divergence between the two populations.

MATERIALS AND METHODS

Breeding ecology

In order to characterize the ecological setting of the *S. pavo* population at GT (45°40' N, 13°35' E), transects were carried out in the rocky shore between May and August of 2004 and 2005. Each transect covered 25m² of substrate, with quadrats of 1m² placed alternately left and right of a 25m line parallel to the shoreline, at approximately 1.5m deep. The number of nests occupied and potentially available was registered in each quadrat. A total of 18 transects were performed, covering 450 m² of substrate along the GT.

At RF (36°59' N, 7°51' W), the only adequate nesting sites are found in artificial reefs that delimit clam culture fields in muddy intertidal flats. These reefs are made of bricks, stones, tiles and other debris, and nesting males use brick holes as nests

(Almada *et al.*, 1994). We have inspected artificial reefs made of 25 bricks, laid in 3 separate groups of 5, 14 and 6 bricks were inspected. Bricks were located at regular intervals of approximately 50cm in the intertidal zone. Individuals that sheltered or nested inside the brick holes were inspected during low tide. The fish were carefully removed from their holes, and morphometry was performed *in situ*. The following measures were taken using a calliper: standard length (SL), head height (HH), body height (BH) and crest height (CH). The fish were then returned to the brick hole they were in. Every unobstructed brick hole was considered a potentially available nest. The relationship between male and nest morphometry was also determined. At GF nest holder males were attracted out of the nest with food (usually an open mussel) inside a transparent plastic bag, which was opened around the nest entrance. Once the male was out of the nest and inside the bag, the opening was sealed. Morphometry was performed *in situ* with the male inside the bag, and the following measures were taken using a calliper: standard length (SL), head height (HH), body height (BH) and crest height (CH). While the male was still in the plastic bag the following nest measurements were taken: nest height (NH), nest width (NW) and nest depth (ND). NH was the maximum height of the nest entrance in which the nest holder would fit. NW was the maximum width of the nest entrance, perpendicular to NH, in which the nest holder would fit. ND was the longest straight distance to the inside of the nest, measured from the plane defined by NH and NW. All measures were performed to the nearest 0.1 mm. In cases where ND exceeded the size of the calliper, a value of 121mm was assigned (corresponding to the maximum size of the calliper + 1). At the end of these measurements, the male was returned to the nest. No case of nest rejection by the handled males was registered.

The operational criteria to classify cavities as potential nests was that their NH, NW and ND would be within the limits defined by mean $\pm$ standard deviation of the NH, NW and ND (in this case only a minimum depth was defined) of 46 occupied nests measured *a priori*.

Behavioural Observations

Behavioural observations in GT were carried out on the breeding seasons (May to July) of 2004 and 2005. Focal observations were performed on 31 occupied nests. Each observation lasted for 20min and observations for each nest were repeated on 3 different days, except in cases where the male had meanwhile abandoned the nest. Observations were performed while snorkelling and behaviours were registered in Plexiglas tables using continuous recording. After the last observation period and

whenever possible, both the nest and the nest-holder male were measured following the procedure described above.

Field observations at RF were performed in the breeding season (June-July) of 1996 over artificial reefs made of 25 bricks, laid in 3 separate groups of 5, 14 and 6 bricks. In each group, bricks were separated by approximately 20cm. Observations were performed while snorkelling and the frequency of the different behaviours was registered in Plexiglas tables. The following behaviours were registered in both populations: frequency of exits from the nest, frequency of male courtship, frequency of male intra- and inter-sexual aggression, frequency of visits to the nest by other males, frequency of female visits to the nest, frequency of female courtship, frequency of female entrances in the nest, frequency of sneaker visits to the nest and sneaker courtship, and visits to the nest by other species.

Genetic analysis

We used four markers: fragments of the 12S and 16S ribosomal mitochondrial DNA, which tend to evolve at a slower rate in blenniids (Almada *et al.*, 2005), the mitochondrial control region (considered a fragment with a very high rate of evolution (Bowen *et al.*, 2006)) and the first intron of the nuclear S7 ribosomal protein gene.

1. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from fin or muscle samples preserved in 96% ethanol with the REDExtract-N-Amp kit (Sigma-Aldrich) following the manufacturers instructions. Voucher specimens are deposited in ISPA collections (ethanol preserved tissues). PCR amplification of mitochondrial fragments and the first intron of the nuclear S7 ribosomal protein gene, were performed with the following pairs of primers: 12s rDNA — 12S For and 12SRev (Almada *et al.*, 2005); 16s rDNA — 16SFor and 16SRev (Almada *et al.*, 2005); control region — L-Pro1 and H-DL1 (Ostellari *et al.*, 1996) and the first intron of the S7 ribosomal protein gene — S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998). Further details on primers and PCR conditions are summarized in supplementary material. For all genes, each sample was sequenced in both directions using the same PCR primers. Sequencing reactions were performed by Stabvida (Oeiras, Portugal; <http://www.stabvida.com>). All sequences have been deposited in GenBank (available at www.ncbi.nlm.nih.gov/).

2. DNA analysis

Sequences were edited with CodonCode Aligner v. 2.0 (<http://www.codoncode.com/>) and aligned with Clustal X (Thompson *et al.*, 1997). For each fragment, Maximum Parsimony (MP) and Minimum Evolution (Neighbor-joining, NJ) were performed separately. MP and NJ analyses were performed with PAUP* 4.0 (Swofford, 2003). Maximum parsimony-based (MP) phylogenetic relationships were estimated with 100 heuristic searches using random additions of sequences and implementing the TBR algorithm. Support values for individual nodes was assessed using 100 bootstrap resamplings (Felsenstein, 1985). We conducted phylogenetic analysis on each fragment separately. Minimum-evolution (ME; Saitou and Nei, 1987) phylogenetic trees were inferred, in PAUP*, based on uncorrected patristic distances because previous studies (Robalo *et al.*, unpublished data) indicated that there was little divergence among populations, so saturation was unlikely. *Salaria fasciatus* and *Salaria fluviatilis* were used as outgroups in all analysis.

For the control region dataset ARLEQUIN software package version 3.01 (Schneider *et al.*, 2001) was used to access population differentiation (AMOVA - Excoffier *et al.*, 1992) and to determine the number of haplotypes. Inter-population distances were corrected by subtracting the mean intraspecific pairwise distances as implemented in Arlequin. Relationships among haplotypes were analysed with a parsimony network estimated by the software TCS version 1.18 (Clement *et al.*, 2000)

Occurrence of ART's

In order to assess the relative frequency of the two male morphotypes in both populations, two types of samplings were performed in June 2004:

In the GT, a food trap as described in 'Nest Characterization' was used, placed in an open area and left open so that several animals could enter. Once one or more animals entered, the trap was closed and the fish were placed in an opaque container. The trap would then be placed again successively until there were no more fish in sight.

In the same month, the population from RF was sampled, using the technique described in Gonçalves *et al.* (2003a): during low tide, a transect of 80 bricks was inspected, and animals that sheltered or nested in the brick holes were sexed.

Males are easily distinguished from females upon inspection of the genital papillae. Furthermore nest-holder males express secondary sex characters (head crest and anal gland). Males that do not express secondary sex characters can be either immature males or sneakers. Since sneakers have mature gonads and release sperm we have used sperm release as a criteria for distinguishing between these two male types. Thus, males that did not express secondary sex characters were tested

by gently pressing the ventral lateral surface of the abdomen after which sneakers visibly release sperm and immature males did not.

Statistical analyses

The statistical analyses were performed using the software SPSS 13.0 for Mac OSX. All tests were two tailed and $\alpha=0.05$. When assumptions for normality were not met, non-parametric tests were used.

RESULTS

Breeding ecology

The distance to the nearest neighbour at RF was found to be significantly shorter than at GT (RF=32.57 $\pm$ 12.34 cm, GT=148.17 $\pm$ 44.88 cm; $N_{RF}=36$, $N_{GT}=41$ Mann-Whitney U-test $P<0.001$). Males occupied a significantly higher proportion of available potential nests at RF than at GT (RF=36 out of 96, GT=66 out of 838; χ^2 test $P<0.001$). At GT, there was a positive correlation in all three dimensions between male and nest measures (HH vs. NH: Pearson correlation, $r=0.426$, $df=27$, $p=0.022$; HW vs. NW: $r=0.604$, $df=22$, $p=0.002$; SL vs. ND: $r=0.694$, $df=27$, $P<0.001$, Fig 1).

Sex-roles

Males courted tenfold more at GT than at RF, while females courted tenfold more at RF than at GT (Male courtship: RF= 0.157 $\pm$ 0.089, GT= 1.785 $\pm$ 0.367 acts/20min, $N_{RF}=36$, $N_{GT}=31$, Mann-Whitney U test $U=329$, $P<0.001$; Female courtships: RF= 0.843 $\pm$ 0.221, GT= 0.086 $\pm$ 0.049 acts/20min, $N_{RF}=36$, $N_{GT}=31$, Mann-Whitney U test $U=232$, $P<0.001$) (Fig. 2). At GT, males played the leading role in courtship, while females did so in RF (Wilcoxon signed ranks test: GT: $Z=-4.032$, $P<0.001$; RF: $Z=-2.619$, $P<0.01$) (Fig. 2). There was a higher frequency of both male and female visits to the nests in RF (Male visits: RF= 2.426 $\pm$ 0.401, GT= 0.849 $\pm$ 0.172 visits/20min, $N_{RF}=36$, $N_{GT}=31$, Mann-Whitney U test $U=318$, $P<0.05$; Female visits: RF= 4.102 $\pm$ 0.277, GT= 1.441 $\pm$ 0.243 visits/20min, $N_{RF}=36$, $N_{GT}=31$, Mann-Whitney U test $U=112.5$, $P<0.001$) (Fig. 3a and 3b). Nest holders from GT attacked females more often, but there was no difference between the two populations in male-male attacks (Male-female attacks: RF= 0.093 $\pm$ 0.038, GT= 0.296 $\pm$ 0.080 acts/20min, $N_{RF}=36$, $N_{GT}=31$, Mann-Whitney U test $U=415$, $P<0.05$; male-male attacks: RF= 0.139 $\pm$ 0.048, GT= 0.183 $\pm$ 0.055 acts/20min, ANOVA $F_{1,66}=0.365$, $P>0.05$). Nest-holders exited the nest less often at RF (RF= 0.424 $\pm$ 0.186, GT= 1.871 $\pm$ 0.372 exits/min, $N_{RF}=35$, $N_{GT}=31$ Mann-Whitney U test $U=226.5$, $P<0.001$), but in this

group there were positive correlations found between the distance to the closest neighbour and overall male activity (distance to nearest neighbour Vs nest exits: Pearson correlation, $r = 0.527$, $N = 36$, $P < 0.05$; distance to nearest neighbour Vs male courts: Pearson correlation, $r = 0.602$, $N = 36$, $P < 0.001$) (Fig 4). No such correlations were found for the GT population (distance to neighbour Vs nest exits: Pearson correlation, $r = -0.11$, $N = 27$, $P > 0.05$; distance to neighbour Vs male courts: Pearson correlation, $r = -0.09$, $N = 27$, $P > 0.05$).

Alternative reproductive tactics

At RF, 16 out of the 40 males sampled (40%) were identified as sneakers, while in the GT only 4 sneaker males out of the 30 males sampled (13.3%) were detected. Therefore, the incidence of the alternative tactic is significantly higher at RF ($\chi^2 P < 0.05$), but it is also present at GT. It should be noted that the expression of the tactics is not exactly the same in the two populations. Sneaker-like males at GT were also of smaller size than nest-holders, exhibited no or only vestigial secondary sex characters and released sperm when pressured in the abdominal area). However, during the all period of behavioural observations at GT sneaking attempts have never been observed. That is, smaller males lacking SSC have never been observed neither to adopt the female nuptial coloration nor to court males. These behaviours are frequently expressed by sneaker males at RF (0.630 ± 0.232 acts/min). On the other hand spawning interferences from other nest-holder males have been observed during spawning events at GT but smaller males have never been observed to try to enter nests during these spawning events. Together, this evidence suggests that sneaker males at GT do not adopt female-mimicking behaviour and that sneaking attempts are very rare events at GT.

Genetic analysis

For the 12S rDNA(400 bp), 6 fish were sequenced (2 from Trieste, 2 from Chioggia and 2 from Ria Formosa) and only 1 haplotype was obtained thus no further analysis was performed on this marker. The same individuals were sequenced for the 16S rDNA (613 bp) and 4 haplotypes were obtained. For the control region (341 bp), 45 fish were sequenced (27 from Trieste, 5 from Chioggia and 13 from Ria Formosa) and 21 haplotypes were obtained. For the S7 gene (630 bp), 57 fishes were sequenced (29 from Trieste, 5 from Chioggia and 23 from Ria Formosa) and 14 haplotypes were obtained. Both parsimony and minimum evolution trees for 16S rDNA failed to recover the fish from Portugal and Italy in separate groups (trees not shown). Both inference methods also failed to display any geographic pattern.

Considering the control region, the result of the AMOVA considering two groups (RF in Portugal vs Italian populations) was significant ($P < 0.001$), although the percentage of variation between populations was 32.33 and the corresponding value within populations was 67.67 ($F_{ST}=0.32$, $P < 0.001$). The corrected average pair-wise difference between both populations was 4.45 (which for a fragment of 341bp corresponds to 1,3%). The average number of pair-wise differences within groups of samples was 10.86 (3,2%) and 3.17 (0,9%) for the Italian and Portuguese groups of samples respectively.

The haplotype network obtained with TCS for the mitochondrial control region failed to connect all haplotypes at a 95% confidence limit. Interestingly two distinct networks were obtained, one including most fish from GT, Chioggia and RF, with a subset of haplotypes from GT grouped in another network. The same pattern was recovered by both phylogenetic inference methods where no structure was observed except for the presence of a distinct clade representing a subset of fish from GT (data not shown).

DISCUSSION

The results presented here strongly support Emlen & Oring's classic hypothesis of the influence of spatial distribution of mates and resources on the mating system of a given species. This hypothesis is widely accepted and seems to be valid not only to explain inter-specific differences but also intra-specific variation in mating systems. As mentioned in the introduction, a growing body of evidence shows that temporal variation in operational sex roles within the same breeding season may induce changes in sex roles within the same population (Almada et al. 1995; Borg et al. 2002; Forsgren et al. 2004; Shibata & Kohda 2006). In the current study we show that also geographic differences in resource (i.e. nest site) availability, that result in biased operational sex ratios, induce differences in the mating system between populations of the same species. The scarcity and high density of nest sites at RF bias the OSR towards females, leading to sex-role reversal in courtship behaviour and to a higher incidence of males adopting a sneaker tactic to reproduce. At GT nest dimensions seem to be selected according to male dimensions, and therefore even small males can efficiently defend a nest and breed as nest-holders.

Choosing a tightly fitting nest may prevent intrusion, while still enabling females to enter because they are smaller than males (Kotrschal, 1988). At RF, however, nearly 100% of nesting males nest in bricks (Almada *et al.*, 1994). Due to the particular standardized shape of brick holes, the choice of an adequate nest is a difficult task in this population. Not all brick holes are selected, with a preference for

those with one of the openings obstructed and the other usually narrowed (Almada *et al.*, 1994; pers. obs.). In this low nest availability, and therefore highly competitive, scenario, only the largest and more competitive males get to occupy a nest (Almada *et al.*, 1994). Those males who do acquire a nest face competition from floater males in search for nests, and nest-holders apparently restrain from leaving the nest to court females or attack other males. Staying inside the nest in high-density situations may work as a mechanism to prevent nest take-overs (Kotrschal, 1988). In fact, our data show that at RF, the denser are distributed the nests, the less active are the nest-holders. Since they receive more visits from possible intruders than those from GT, but there are no differences in the expression of agonistic behaviours, this differential inter-male aggression is evidence pointing to behavioural plasticity in contrasting ecological situations, as suggested by Almada *et al.* (1994). As the number of males qualified to mate (Kvarnemo *et al.*, 2001) is reduced at RF, the OSR shifts towards females who take on the leading role in courtship. In this population, females visit nests and court nest-holders more frequently than at GT, which suggests high competition among females for mating opportunities. Due to the high aggregation of nest sites at RF, many females can be found simultaneously visiting nests in the same patch. In such high densities females may increase their courtship rate as a competitive mechanism (Saraiva *et al.*, in press).

Interestingly, there are more male-female chases at GT, where the OSR is more balanced, while theory would predict the reverse situation (e.g. Andersson, 1994; Clutton-Brock, 2007). A possible explanation may be the higher overall activity of males from GT, who are not constrained by environmental or social factors and are free to interact outside of the nest. A previous study on the reproductive behaviour of the peacock blenny at the Gulf of Trieste also found high expression of male-female aggressive behaviour, with no obvious reason (Patzner *et al.*, 1986).

The genetic results do not support the hypothesis of a genetic base for the behavioural differences observed between the two populations. The more conservative 12S rDNA yielded a single haplotype in both Portugal and Italy. This mitochondrial gene tends to evolve at a slow pace in blenniids (Almada *et al.*, 2005) but finding the same haplotype in such a geographic distance strongly suggests that the two populations must have shared a common ancestor in recent times. If we turn to the opposite end of the spectrum of rates of evolution, we have the control region, which in fish may evolve at rates that have been estimated to reach 10 % or higher per million years (Bowen *et al.*, 2006). These estimates, together with a corrected inter-population net average distance of 1.3 % (see results) indicate that the divergence between Italian and Portuguese populations is likely younger than

130,000 years. Such a low value, although yielding significant results in AMOVA, is easily explained by the geographical separation of the two groups of samples and fails to support the view that we are dealing with distinct taxonomic entities in Trieste and in Ria Formosa. Typically, sister species of blenniids diverge by values of several percent units (Almada *et al.*, 2005). The failure of the phylogenetic trees and the haplotype network to recover the Italian and Portuguese samples as two distinct and cohesive groups is further evidence on the very recent nature of their divergence. It could be argued that behavioural traits evolved at such a fast rate that even a period of less than 100 000 years was sufficient for the evolution of distinct breeding patterns. In this respect it is interesting to note that both for the control region and S7 genes the fish from Chioggia (where typical lagoon conditions prevailed) share haplotypes with the fish from GT, which is to be expected due to their geographic proximity. Apart from the fish from RF the only other lagoon population studied so far (Ruchon *et al.*, 1995) apparently contains both parental males and sneakers. If the fish from Chioggia prove to be similar behaviourally to those of the Ria Formosa the hypothesis of a genetic basis for the differences in life-history among populations can be ruled out with a high level of confidence. Further studies on the reproductive biology and behaviour in lagoons like Chioggia (which are close to Trieste) and on rocky substrata on Algarve, close to the Ria Formosa, will provide a definitive test of this hypothesis. As no genetic differences were detected between both populations, there is no evidence for genetic differentiation as the cause for the behavioural variation. More likely, differences in behaviour reflect a very high degree of behavioural plasticity of the species and condition-dependent strategies.

The confirmation that both populations (RF and GT) belong to the same phylogenetic unit is particularly important since the RF population shares some behavioural features with those described for a closely related, but rather rare, species, the zebra-blenny *Salaria basilisca* (Valenciennes, 1836) (see Heymer, 1985b; Heymer, 1987; also Almada *et al.*, 1995). Like *S. pavo* at RF also *S. basilisca* inhabits seagrass (*Posidonia oceanica*) plains throughout the Mediterranean (Heymer, 1985a). Female-to-male sex change in *S. basilisca* have been described based solely on behavioural observations (Heymer (1980; 1985a), at a time when ART had not yet been detected in blenniids. Thus, this putative sex-change can be interpreted instead as a tactic switch from female-mimicking sneaker to parental male behaviour as described for *S. pavo* (Gonçalves *et al.*, 1996; Gonçalves *et al.*, 2003a). These similarities raised questions about the phylogenetic status of the *S. pavo* RF population.

The available data at this point suggests that the mating system functions in different ways in different ecological conditions. At RF, the mating system seems to have two distinct phases: at the onset of the breeding season, there is high male intra-sexual competition for access to nest sites. Once the larger males establish nests and the smaller males are excluded, the intra-sexual competition decreases in males and increases in females as the sex-roles become reversed. This should be the point where female-mimicking male ART also begins to be expressed in sneakers. Experimental evidence suggests that sneakers not only mimic female behaviour and morphology, but also copy females when it comes to choose which nest-holders to court (Gonçalves *et al.*, 2003b). The sneaking tactic probably appears in high frequencies at RF because only the largest males can acquire and secure nests. While floaters (i.e. slightly smaller males with fully developed SSC that could not secure a nest) swim around ready to take over vacant nests (Almada *et al.*, 1994; Almada *et al.*, 1995), the only option for lower size classes is to compromise the growth of SSC and invest in growing large testis that allow them to spawn parasitically (Taborsky, 1998). The severe nest scarcity in this population should maintain and enhance this pattern throughout the rest of the season, as the nests become more and more filled with eggs.

At GT, there is virtually an excess of nesting sites for every mature male during the whole breeding season. This reduces male intra-sexual competition for nests and allows small mature males to acquire a nest. Apparently only a very reduced number of males 'choose' to follow an ART. Given the availability of potential nests, it is surprising that these parasitic males even exist at all. One hypothesis is that these males mature too late in the season to grow SSC and adopt a bourgeois tactic, and, as those from RF, invest in large testis that still allow them to spawn parasitically. However, in this case it should be not an effect of size, since even very small males are capable of finding a nest (Saraiva *et al.*, unpublished data). Instead, this may represent an extreme example of a birthdate effect in a condition-dependent ART (Taborsky, 1998), with only a very small proportion of males being caught in this ontogenic trap.

In this system with traditional sex-roles, theory predicts that female choice should be higher (Andersson, 1994), but an accurate assessment of this prediction should only be possible through focal observations to females in studies such as those performed by Fagundes *et al.* (2007).

An experimental approach using either long-term common garden experiments (i.e. allowing individuals from the two populations to grow in the same environment) or translocation of individuals between populations (i.e. growing RF

juveniles in GT conditions and vice-versa) should help to clarify these issues. Nevertheless, these populations represent a rare case where the assumptions for ecological modulation of behavioural plasticity can be tested at a large scale.

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FIGURE LEGENDS

Fig 1 – NH: nest height; HH: head height; ND: nest depth; HW: head width; NW: nest width

FIG 2 – Male and female courtship displays in both populations (M: males; F: females; RF: Ria Formosa; GT: Gulf of Trieste; * signals significant differences at $P<0.05$; ** signals significant differences at $P<0.001$)

FIG 3A male visits to the nest; 3B female visits to the nest. * signals significant differences at $P<0.05$

FIG 4A: relationship between distance to closest neighbour and frequency of exits from nest at RF; 4B: relationship between distance to closest neighbour and frequency of courtship displays

FIGURES

Figure 1

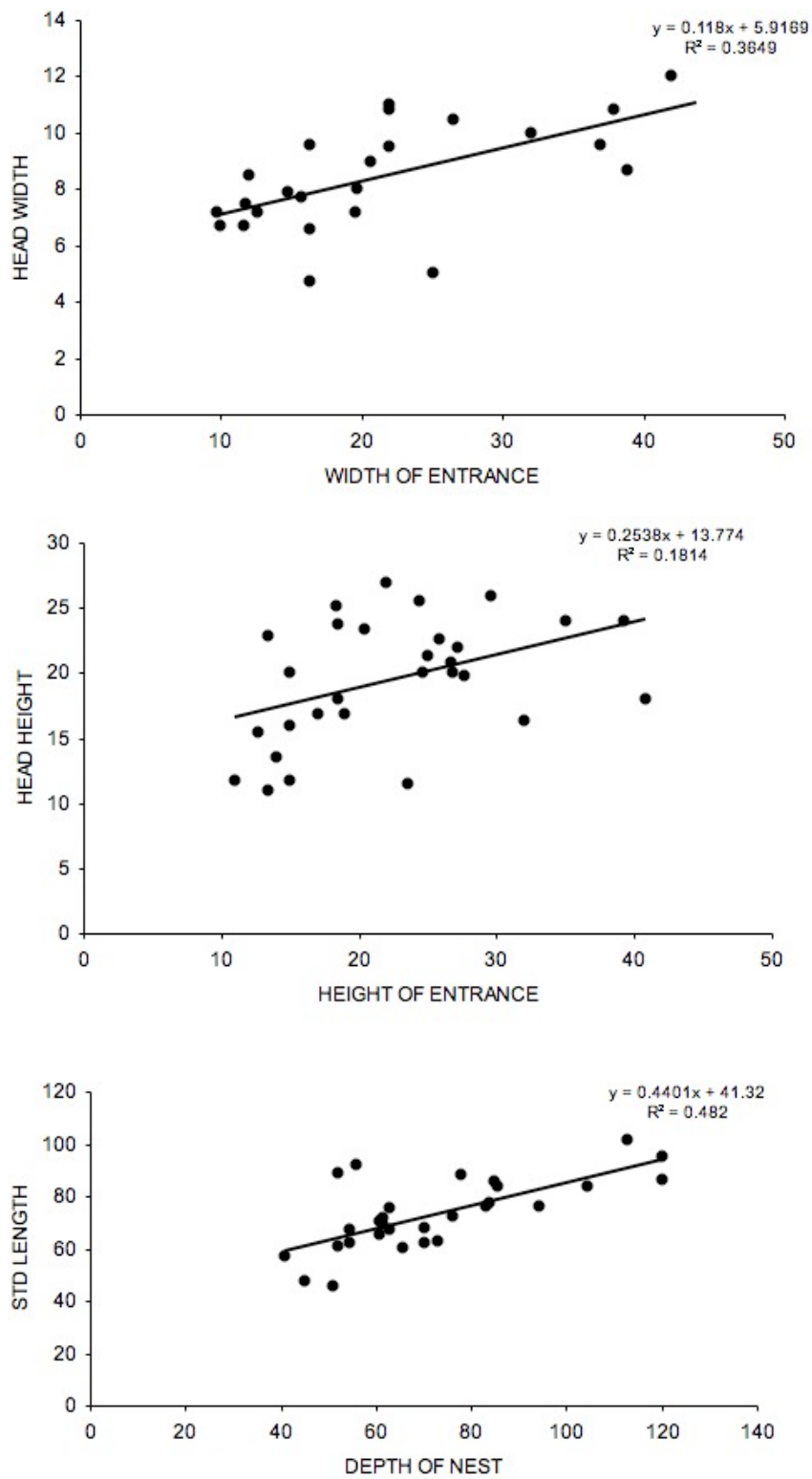


Figure 2

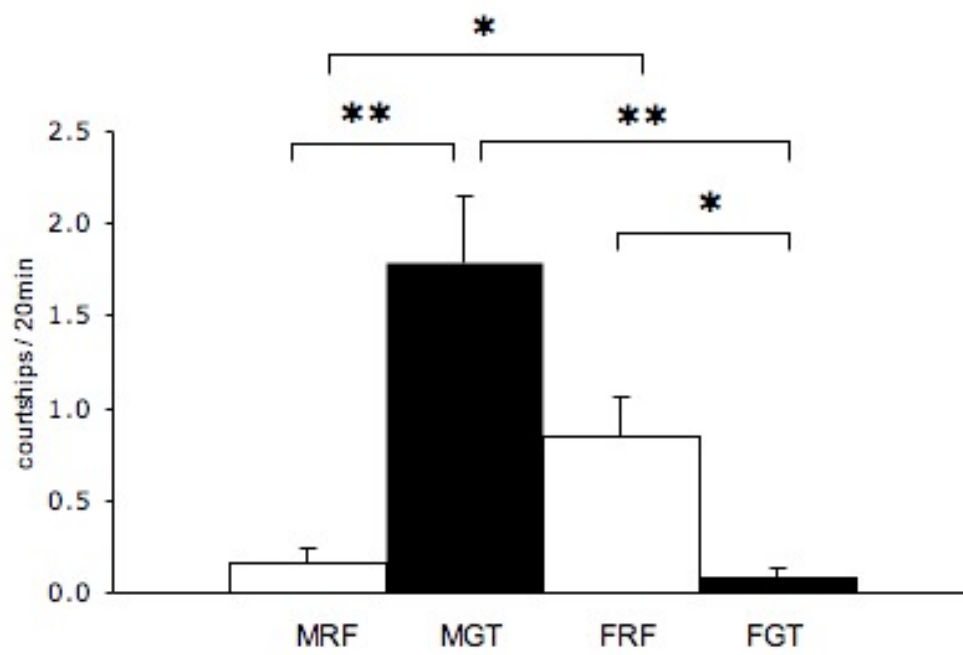


Figure 3A

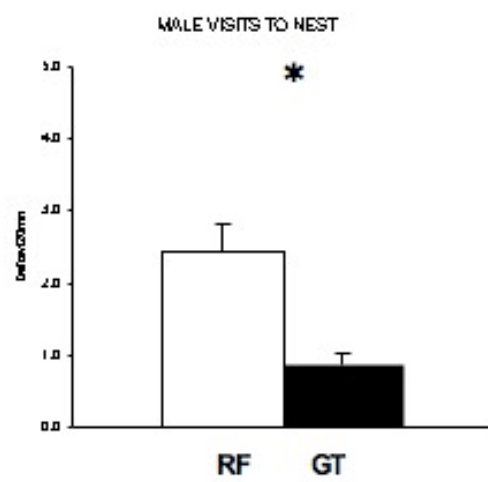


Figure 3B

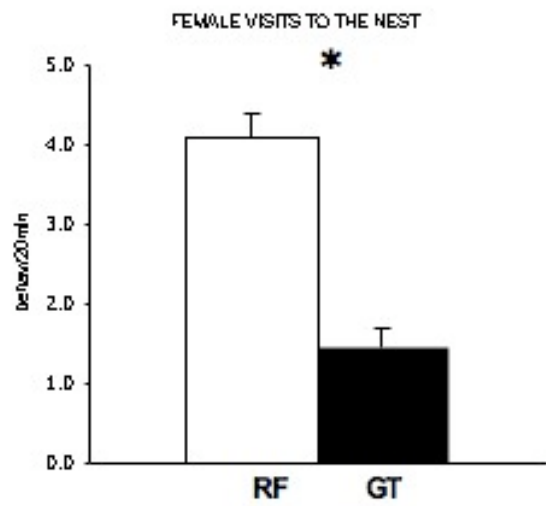


Figure 4A

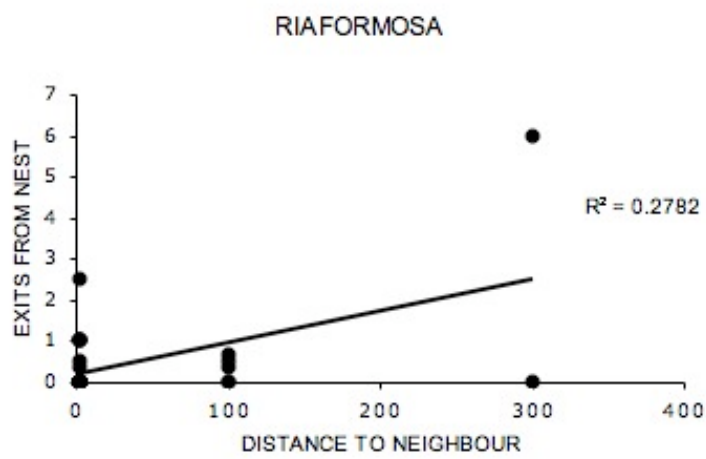
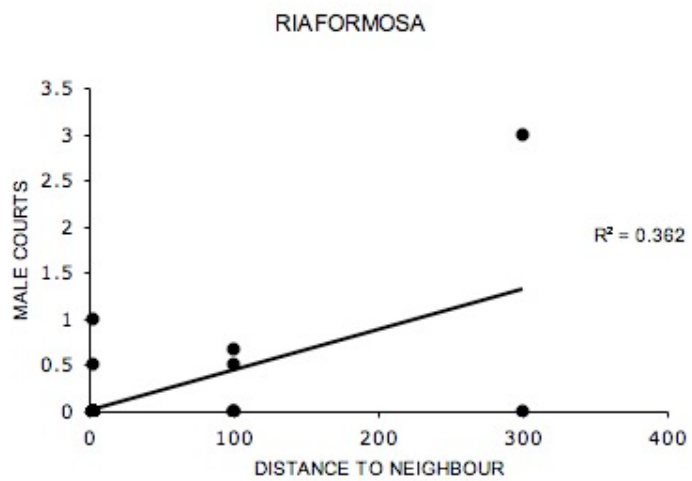


Figure 4B



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CHAPTER III

Plasticity in reproductive behaviour in two populations of the peacock blenny

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ABSTRACT

Sexual plasticity is ubiquitous among teleost fish. Temporal and inter-population intra-specific variations in reproductive mode have been described involving sex-change, changes in sex-roles and the expression of alternative reproductive tactics. In the peacock blenny (*Salaria pavo*) the reproductive behaviour varies between two populations that differ in nest availability. At the Gulf of Trieste (Italy), where nest availability is high, conventional sex roles are present and alternative reproductive phenotypes are rare. On the other hand at Ria Formosa (Portugal), where nest site availability is low, females are the courting sex and a high percentage of males adopting an alternative reproductive tactic to reproduce is present. Laboratory experiments were used to study the degree of flexibility of the behavioural tactics used by females and two male morphs in both populations. Nesting males were sequentially presented with a female and a putative female-like parasitic male. A second experiment was performed to assess the tactic choice in parasitic males: either occupying an empty nest to follow a nest-holder tactic or mimic female courtship behaviour and follow a sneaker tactic. In the lab experiments, females from Ria Formosa took the initiative in courtship and displayed courtship behaviour more frequently than males. In contrast, at Gulf of Trieste, males had the initiative in courtship and displayed more often courtship behaviour than females. When comparing both populations, females from Ria Formosa were overall more active in courtship than females from Trieste. Parasitic males from Ria Formosa displayed female-like courtship behaviour towards the nesting male, while no parasitic male from Trieste exhibited female-like behaviour. No parasitic male from both populations chose a bourgeois tactic. These results indicate clear differences in the mating system of the two populations, and a variable degree of behavioural flexibility across sex morphotypes once a reproductive tactic is assumed during the breeding season, with males being more plastic than females in the expression of sex specific courtship behaviour. GT parasitic males were unable to express female-like courtship behaviour suggesting that a ART not based on female-mimicry occurs in the Gulf of Trieste.

INTRODUCTION

Teleost fish are the champions of sexual diversity and plasticity among vertebrates. At the inter-specific level reproductive patterns range from gonochorism, to sequential hermaphroditism, either male-to-female as well as female-to-male sex-change (Warner 1988), to reversible hermaphroditism (e.g. male-to-female-to-male, (Sunobe & Nakazono 1993), to simultaneous hermaphroditism and even to asexual reproduction (Schartl et al. 1995). The diversity in teleost modes of reproduction is also present on virtually all aspects of reproductive activity: fertilization mode is mostly external but can also be internal in some species (Goodwin et al. 2002); mating systems range from monogamous to promiscuous (Turner 1993); parental care varies from null to paternal, maternal or bi-parental (Sargent 1997); sex-roles can be conventional - where male competition for mates is strongest-, or reversed - where females competition for mates is strongest (e.g. Almada et al. 1995); and within the same species individuals may use alternative reproductive tactics to reproduce (Taborsky et al., 2008).

Together with this wide variety of reproductive modes, teleosts also exhibit a high degree of flexibility in their reproductive patterns. Sex-roles are dynamic and within the same species they may shift from conventional to reversed throughout the breeding season depending on the temporal variation of male and female reproductive rates (Forsgren et al. 2004; Shibata & Kohda 2006). The expression of alternative reproductive tactics can differ greatly within the same species between populations (Almada et al. 1995; Luyten & Liley 1985; Magurran & Seghers 1991), within populations or between individuals (Oliveira et al. 2005; Taborsky 1994; Taborsky 2001).

In the peacock blenny *Salaria pavo* (Risso) inter-populational variation in sex roles and the expression of alternative reproductive tactics has been described (Almada et al. 1995; Almada et al. 1994; Saraiva et al. in press; Saraiva et al. in prep). This species is usually found in rocky shores of the Mediterranean and adjacent Atlantic areas, where males build nests in holes or crevices in the rock and defend courting territories around the entrance of the nest (Fishelson 1963; Patzner et al. 1986; Zander 1986). In these populations, nesting males are territorial, actively courting females with conspicuous displays (coloration changes, 'figure-8 swimming'), performed outside of the nest in the courting territory, while females respond to courting with changes in coloration and ultimately entering the nest (Patzner et al. 1986). In a population found in a coastal lagoon (Ria Formosa, southern Portugal) the reproductive behaviour is strikingly different, with females taking the leading role in courtship and males assuming a more passive role and hardly leaving the nest

(Almada et al. 1995; Almada et al. 1994). These differences were hypothesized to be due to ecological constraints. At Ria Formosa nesting substrate is very scarce and adequate sites are only found in artificial reefs built by clam culturists to delimit their fields (Almada et al. 1994). The scarcity and aggregation of nest sites has apparently promoted high competition for access to nests, thus excluding less competitive males from nesting. This resulted in a female biased operational sex-ratio (OSR), with a reversal in the sex-roles in this population (Almada et al. 1995; Saraiva et al. in press; Saraiva et al. in prep). Another consequence of the limitation of nesting sites in populations found in lagoon environments is the occurrence of a high proportion of alternative reproductive phenotypes among males (sneakers). These are small, less competitive males that could not acquire a nest and assume an alternative reproductive tactic (ART), mimicking female behaviour and morphology in order to enter nests and steal fertilizations from nest-holders (Gonçalves et al. 2003a; Gonçalves et al. 1996; Ruchon et al. 1995; Shuster 2002; Taborsky 2001). Inter-sexual copying by sneakers from Ria Formosa is not limited to behaviour and morphology but involves also copying female mate choice (Gonçalves et al. 2003b), suggesting a strong social component for this behavioural tactic in sneakers from the Ria Formosa population. A low proportion of males assuming a parasitic tactic has also been found in a population inhabiting a rocky shore environment with abundance of nest sites in the Gulf of Trieste (Saraiva et al. in prep). However, field observations suggested that these parasitic males do not follow a similar sneaking tactic to those of Ria Formosa.

In order to understand the proximate and ultimate mechanisms underlying sexual plasticity a detailed characterization of the phenomena is needed. In the peacock blenny system it is important to understand the degree of flexibility of the reproductive patterns expressed in the field in the two populations and the hidden potential of individuals from one population to express the behaviour present in the other population. For this purpose, we performed a behavioural screening of the reproductive behaviour of nest-holder males, females and sneaker males of *S. pavo* in a controlled laboratory environment. In particular the following questions were addressed:

- 1- Are the sex-roles context dependent, affected by the individuals' perception of local conditions, or are there inbuilt phenotypical differences between the two populations? More precisely, are males from Ria Formosa (RF) capable of actively courting females, once the ecological constraints are not present? And are females from Gulf of Trieste (GT) capable of taking the initiative in courtship?

- 2- Do parasitic males from GT have the ability to express female-like courtship?
- 3- If parasitic males are given the opportunity will they be able to express nest-holder behaviour?

MATERIALS AND METHODS

The experiments were carried out in the Marine Biology Laboratory of the University of Trieste, located in the GT in the Northern Adriatic Sea (Italy) and at Culatra Island, located in RF in Algarve (Portugal). The RF population occurs in a mudflat area where natural substrate appropriate for nesting is absent. Fish reproduce in artificial materials (usually bricks) used by clam culturists to delimit clam culture fields and the density of potential nesting sites is very low. In Trieste, the substrate is mainly constituted by limestone rocks and boulders that create an intricate labyrinth of crevices, holes and galleries of many shapes and sizes, thus providing appropriate nesting sites in abundance. Fish were captured with a food trap while snorkelling (GT) or during low tide in bricks (RF) and brought to the laboratory in containers with abundant aeration. Nest-holder males, females and small males that did not exhibit secondary sex characters and from which sperm release could be elicited after gently pressing the abdominal cavity (herein referred to as putative parasitic males) were kept separate in large tanks with abundant sheltering. The experimental room was illuminated by natural light (GT) or by artificial light (RF) and in this case photoperiod was adjusted according to natural conditions. All fish were fed daily with common cockles either fresh (GT) or frozen (RF).

Parental males were left to acclimate in 50L individual tanks either for 48 hours (GT) or for two hours (RF) prior to the onset of the experiment. These individual tanks had the bottom covered with sand and contained one potential nest placed in the centre of the tank. In the GT experiments the nest was a PVC tube measuring 15 X 3 cm diameter (106 cm^3) while in RF experiments the nest was an opaque glass box of 21 X 5 X 5.5 cm (577 cm^3). The nests had one of the extremities closed with an opaque piece of plastic. All males adopted the nest typically during the first minutes of the habituation period.

Each bourgeois male was sequentially presented with a ripe female (female presentation test, FPT) and a putative parasitic male (parasitic male presentation test, PPT) in the same day. The order of the FPT and PPT was balanced to control for order effects. Observations lasted 15 min (RF) or 20 min (GT).

In the RF experiments observations were video recorded and the behaviours exhibited by the nesting male and by the sneaker or female quantified using the Observer PC software V3.0 (Noldus Information Technology, Wageningen, The

Netherlands). In Trieste the behavioural variables were directly recorded through check sheets. The behavioural variables recorded for putative parasitic males, females and bourgeois males were the frequency and latency of courtship displays and, for bourgeois males, also the frequency of agonistic attacks towards sneakers or females (see Patzner et al. (1986) for details on these behaviours). Aggressiveness from bourgeois males towards females and putative parasitic males was expressed in relation to the total number of aggressive and sexual displays exhibited by the bourgeois male according to the following index: frequency of bourgeois male attacks/(frequency of bourgeois male attacks + frequency of bourgeois male courts). This index is used to assess the overall direction of the interactions of nesting males towards females and parasitic males (reproductive vs agonistic).

In a second experimental set-up, a tactic choice test (TCT) was presented to parasitic males from both populations. Inside 100L tanks, similar nests to those used in the previous set-up were placed in opposite walls. One of the nests had an established nest-holder with eggs and the other was empty. Two mature females and a parasitic male were inserted in the tank, and the proportion of sneakers that courted the male within 20 min was registered.

Methodological differences between populations in the FPT and PPT tests are assumed to be minor and results comparable. They were due to particular details of *a-priori* observations on sensitivity to the presence of human observers, habituation to captivity and nest size in natural conditions on both populations. Fish from Trieste were found to be more sensitive to captivity conditions, hence the longer habituation time. Nest internal volume in the Gulf of Trieste was found to range from 5 to 150 cm³ in nature (Saraiva et al. in prep), while at RF *S. pavo* males usually nest in bricks with holes of similar dimensions to those used in this study (Almada et al. 1994; Oliveira et al. 1999). The size of female and parasitic males in the FPT and PPT tests did not differ between populations (mean $\pm$ SE: Females RF= 7.06 $\pm$ 0.12 cm, Trieste= 7.36 $\pm$ 0.14 cm, ANOVA planned comparison: $t = -1.111$, $P = 0.271$, $N_{\text{Trieste}}=8$, $N_{\text{RF}}=11$; parasitic males RF= 5.75 $\pm$ 0.09 cm, Trieste= 5.53 $\pm$ 0.23 cm, ANOVA planned comparison: $t = 1.166$, $P = 0.248$, $N_{\text{Trieste}}=12$, $N_{\text{RF}}=36$). The frequency of behaviours per minute was used.

The statistical analysis was performed using the software Statsoft Statistica 8.0 for Windows. In this software package, the test between proportions does not return a test value. Means $\pm$ S.E. are presented throughout.

RESULTS

Female presentation test

In the RF FPT test, females courted more frequently than males (Females= 0.364 ± 0.058 , Males= 0.048 ± 0.022 behav/min, $N=11$, $F_{1,20}= 25.900$, $P < 0.001$), initiated courtship more often than males (Females= 91%, Males= 9%, $N=11$, Statistica test of proportions, $P < 0.001$) and the latency to the first courtship was lower in females than in males (Females= 69.1 ± 49.1 sec, Males= 341.8 ± 26.0 sec, $N=11$, Mann-Whitney U test, $U= 2$, $P < 0.05$). All females courted the nesting male while only 9% of the males courted females ($N=11$, Statistica test of proportions, $P < 0.001$). Contrarily, in the GT FPT test, males and females did not present differences in the frequency of courtship displays (Females= 0.169 ± 0.105 , Males= 0.169 ± 0.123 behav/min, $N=8$, $F_{1,14}=4.780$, $P > 0.05$, Fig. 1), probability of initiating courtship (Females= 25% of 8 trials, Males= 25% of 8 trials), latency to initiate courtship (Males= 256.0 ± 113.8 sec, Females= 193 ± 111.7 , $N= 8$, Mann-Whitney U test, $U=4.5$, $P > 0.05$) or in the proportion of animals exhibiting courtship displays (38% of each sex, $N=8$).

When comparing the FPT results between populations, a higher percentage of females from RF courted males (Fig. 2) and with a higher frequency (ANOVA planned comparison $t= 2.00$, $N_{GT}= 8$, $N_{RF}=11$, $P < 0.05$, Fig. 1), and with a lower latency (RF= 69.090 ± 25.999 , GT= 658.500 ± 123.587 sec, ANOVA planned comparison: $t= -10.4$, $N_{GT}= 8$, $N_{RF}=11$, $P < 0.001$). There was no difference between populations in the frequency of male courtship displays (ANOVA planned comparison $t= -1.652$, $N_{GT}= 8$, $N_{RF}=11$, $P > 0.05$ Fig. 1), neither in the percentage of males that courted females, (Fig. 2), nor in the latency of male courtship displays (RF= 697.027 ± 89.064 , GT= 360 ± 127.586 sec, ANOVA planned comparison $t= 0.466$, $N_{GT}=12$, $N_{RF}=11$, $P > 0.05$). Males also did not differ in aggressiveness towards females (RF= 0.769 ± 0.100 , GT= 0.422 ± 0.149 , ANOVA planned comparison $t= 0.832$, $N_{GT}=8$, $N_{RF}=11$, $P > 0.05$).

Parasitic male presentation test

In the PPT, there were no differences between populations regarding the proportion of nest-holders courting parasitic males (17% Vs 19%, $P= 0.832$, $N_{GT}=12$, $N_{RF}=36$, Statistica test of proportions, $P > 0.05$ Fig. 3). However, while in GT there was no courting from parasitic males towards nest-holders, in RF 83% of the parasitic males courted the nest-holder (Statistica test of proportions, $N_{GT}=12$, $N_{RF}=36$, $P < 0.001$, Fig 3). There was no difference between RF and GT in the overall aggressiveness of

nest-holders towards parasitic males (RF= 0.770 ± 0.066 , GT= 0.546 ± 0.142 , ANOVA planned comparison: $t = 1.634$, $N_{GT}=12$, $N_{RF}=36$, $P > 0.05$,).

Nest-holder males from both from RF and GT were equally aggressive towards females and parasitic males (Aggressiveness index: Wilcoxon matched pairs test, RF: $T = 5.5$, $N_{females} = 11$, $N_{p. males} = 36$, $P > 0.05$; GT: $T = 3$, $N_{females} = 8$, $N_{p. males} = 12$, $P > 0.05$).

Tactic choice test

In the tactic choice test, a much higher proportion of parasitic males courted the nest-holder with female-like behaviours (RF= 87.5%, GT= 7.7%, $N_{RF}=8$, $N_{GT}=13$, Statistica test of proportions $P < 0.001$). However, none of the parasitic males studied from either population occupied the empty nest and assumed a nest-holder tactic.

DISCUSSION

Overall our results suggest that, although there is substantial inter-population plasticity in the expression of reproductive behaviour, the individual ability to adjust its behavioural tactic is somewhat reduced. In general, animals continued to express in the laboratory tests the behaviours of their original population, suggesting some resilience in the mechanism controlling mating behaviours. The decision to follow a reproductive tactic in sexually mature *S. pavo* individuals is probably assumed at the onset of the breeding season, and although it is plastic, the changes may only occur gradually. In the two-spotted goby *Gobiusculus flavescens*, the sex roles gradually change from conventional to reversed over the course of breeding season that lasts for three months (Forsgren et al. 2004). The blennioid fish *Petroscirtes breviceps* shifts from typical sex-roles to reversed and back during an 8-month breeding season (Shibata & Kohda 2006). Changes in reproductive behaviour should imply gathering information from the environment and adjustments of the animals' internal state through hormonal processes that may produce a lag in the animals response (Forsgren et al. 2004). As the animals in our experiments only stayed in the lab for a short period, there was not enough time to adjust the behavioural response and therefore they adopted the standard pattern of courtship for their population. Thus, our results support the view that changes in reproductive behaviour are slow and long-lasting probably involving a re-organization of the physiological mechanisms underlying reproduction.

A more detailed analysis of our results reveals that nest-holder males are the morphotype with more flexible behavioural responses (i.e. courtship behaviour),

followed by females and sneaker males. Nest-holder males from both populations were equally active in courtship and aggressiveness towards females, indicating that RF males can express courtship behaviour at similar rates to GT nest-holder males in lab conditions. This can be due to the fact that nest-holder males did not have eggs in the nest at the beginning of the experiment. Therefore, both RF and GT males had to court in order to attract females and get the first spawn. For example Shibata & Khoda (2006) report a decrease in male courtship as the nest fills with eggs, and again an increase when it becomes empty in the blenny *Petroscirtes breviceps*. A nest without eggs is probably the reason why both the percentage and frequency of male courtship did not differ between populations in this laboratory set up.

On the other hand, female courtship behaviour in the lab reflected the differences observed in the field. This can be explained by one of two processes. Female courtship behaviour is only expressed in response to courtship behaviour initiated by the males and therefore taking the initiative to court is less plastic than male behaviour and changes on its expression require a longer period. Alternatively, in the FPT females were unaware of the presence or absence of eggs in the nests. Following the information they had available (i.e. from their home populations) females from RF assumed that the nest holder is a high-quality male with eggs in the nest (Oliveira et al. 1999; Saraiva et al. in prep), and thus courted more in order to be accepted; females from GT probably assumed that nests were empty of eggs and waited to be courted into spawning. This would explain why male courtship did not differ between the two populations but female courtship did.

The possible answer to the first question of our study is that the assumption of a sex-role in *S. pavo* seems to be context-dependent and plastic, but changing the behavioural profile should imply changes that cannot occur in a short period.

Regarding the second question, more than 80% of the RF parasitic males tested courted the nesting-male with female-like displays but none of the GT putative parasitic males courted the nesting-male or tried to sneak into the nest, suggesting that smaller males of the peacock blenny at GT do not use female-courtship as an ART and probably assume a novel technique. At RF the most distinctive feature of courtship events are the conspicuous female courtship displays towards nesting-males (Almada et al. 1995). Parasitic males in this population effectively imitate these displays in order to approach nests and steal fertilizations (Gonçalves et al. 2003b). At GT the most distinctive feature of courtship episodes is the male 'S courtship' display that occurs outside the nest (Patzner et al. 1986). Thus, at GT parasitic males presumably will rely on their female-like appearance either to be

courted by males or to remain inconspicuous in order to approach nests and steal fertilizations. This explains why parasitic males did not court the nesting-male. Adopting an active female-mimicking courting tactic should not pay off for these putative parasites, since females at GT do not court as actively as at RF.

The tactic choice test was used to answer the question of how flexible ART are and would help to unravel the spawning tactic used by the parasitic males from GT. Given the opportunity, would parasitic males 1) choose to try to steal fertilizations or would they 2) adopt the empty nest and court females as nest-holder males? While at RF the answer was clearly the first option, this test failed to elicit any spawning behaviour from parasitic males at GT. As none of the tested individuals adopted nest-holding behaviour, it is safe to conclude that *S. pavo* parasitic males cannot switch into the bourgeois tactic within a short period in both populations, thus confirming previous data on RF sneakers (Oliveira et al. 2001). In parasitic males, the transformations necessary for a tactic switch are even greater than in bourgeois males and probably cannot even occur during a single breeding season. This may be because the constellation of traits that make up a reproductive phenotype may not all depend on the same agent, but instead in several different mediators acting at different levels (Oliveira et al. 2005). For example, the development of secondary sex characters (typical in bourgeois but absent in parasitic males of *S. pavo*, such as a conspicuous head crest and an anal gland) is related to an increase in circulating levels of the androgen 11-keto-testosterone, that also acts on the inhibition of female-like behaviours of sneakers (Oliveira 2006; Oliveira et al. 2001). However, courting behaviour seems to be controlled independently by the neuropeptide arginine vasotocin (Carneiro et al. 2003). Thus, it may not be feasible for parasitic males to induce changes in both their endocrine system and neural circuits within a short time frame. In fact, in over 10 years of sampling in the RF population of *S. pavo* there is not a single documented case of a tactic switch within the duration of one breeding season; tactic switches only occur from one season to the next (T. Fagundes, DM Gonçalves, J Saraiva, RF Oliveira, unpublished data).

In conclusion, these experiments present experimental evidence that the degree of behavioural plasticity observed between two populations of the peacock blenny under different ecological environments varies across the different sex morphotypes. Male courtship behaviour can vary rapidly, whereas variation in female courtship although present is more resilient. Moreover, parasitic males from GT do not mimic female behaviour like as it happens with the same morph from RF, suggesting that they may adopt a novel, unknown tactic to achieve fertilizations

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FIGURE LEGENDS:

Figure 1 – Frequency of courtship displays of males and females in the Female Presentation Test (FPT) (see text for details). * indicates significant differences at $P<0.05$. NS: non-significant.

Figure 2 – Percentage of individuals that displayed courtship behaviours in the Female Presentation Test (FPT) (see text for details). * indicates significant differences at $P<0.05$. NS: non-significant.

Figure 3 – Percentage of individuals that displayed courtship behaviours in the Parasitic male Presentation Test (PPT) (see text for details). * indicates significant differences at $P<0.05$. NS: non-significant.

Figure 1

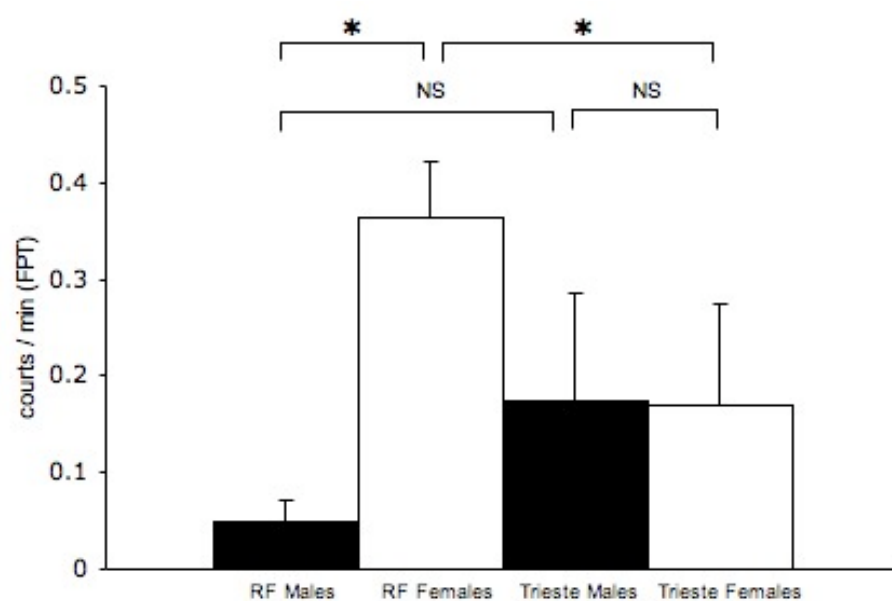


Figure 2

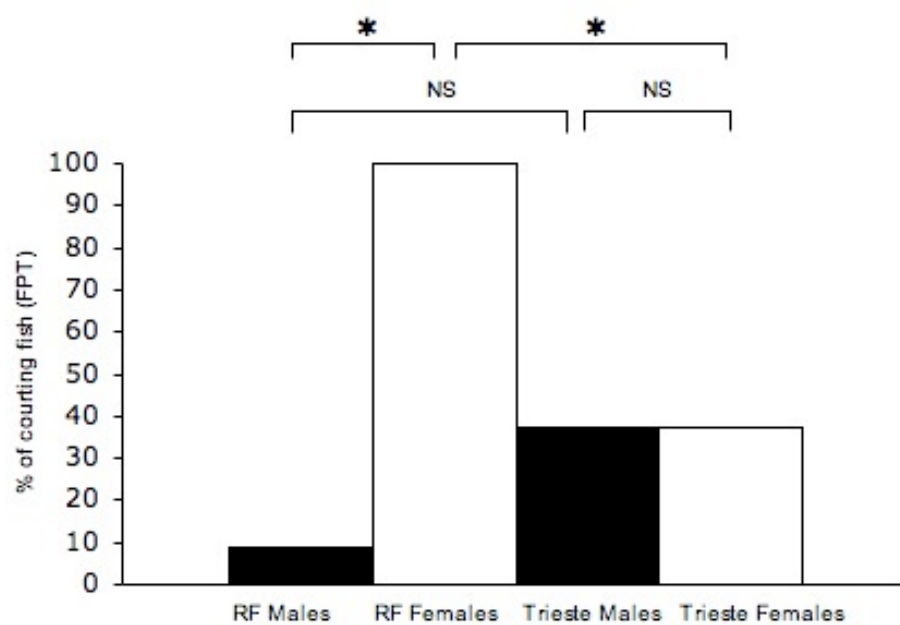
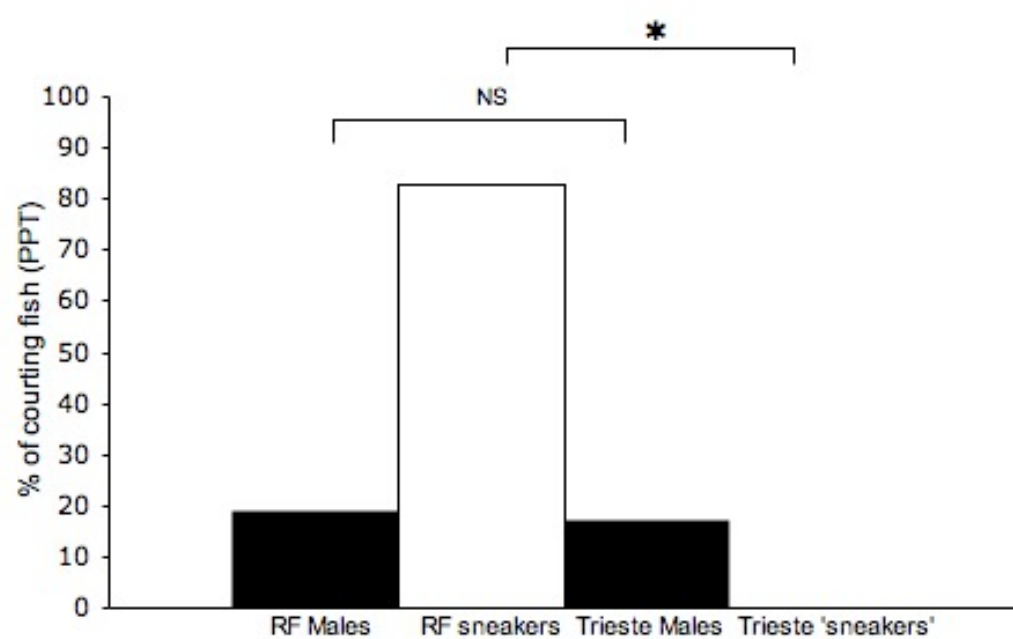


Figure 3



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CHAPTER IV

Environmental modulation of secondary sex characters and androgens in the peacock blenny *Salaria pavo*

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ABSTRACT

Androgens are associated with the expression of both, secondary sex characters (SSC) and behaviour. In male fish, 11-keto-testosterone (11KT) is the major functionally active androgen, promoting the development of SSC and mediating behavioural responses according to reproductive tactic. In this paper, morphological and endocrine parameters were measured in two populations of the peacock blenny *Salaria pavo* with different regimes of sexual selection imposed by differences in nest site availability. The peacock blenny is a small benthic fish with marked sexual dimorphism and exclusive paternal care of the clutch that inhabits rocky shores of the Mediterranean, as well as adjacent Atlantic areas. In a population from the Gulf of Trieste (GT) (Northern Adriatic sea) inhabiting rocky shores where nest sites are abundant, male-male competition for nests is low, males court females and a low frequency of alternative reproductive tactics (ART: small, parasitic female-mimicking sneaker males that change tactic into nest-holders in posterior breeding seasons) occurs. On the other hand, at Ria Formosa (RF), a coastal lagoon in Southern Portugal, where nests sites are scarce and highly aggregated, male-male competition for nests is very high, there is sex-role reversal with female courtship and a high frequency of ART is observed. Concomitantly, at Ria Formosa nest-holder males are larger, present more developed SSC and higher levels of 11KT than at GT. However, the gonads of nest-holders and parasitic males were larger in the GT population. Competition for nests at RF seems to promote more developed SSC in nest site scarcity conditions, while competition for females at GT seems to be spurring sperm competition among males in populations where nest sites are more abundant. Thus, 11KT was associated with the development and expression of SSC in contrasting environments. These results exemplify how the modulation of behavioural plasticity by the social environment can be regulated by androgens.

INTRODUCTION

Secondary sex characters (SSC) are generally viewed as a product of sexual selection, either associated to female preference for exaggerated traits or to male advantage in intra-sexual competition (Andersson, 1994). The expression of SSC is known to be related to the circulating levels of androgens (Borg, 1994; Liley and Stacey, 1983; Oliveira, Canario, and Grober, 2001b). In teleost fish Testosterone (T) and 11-keto-testosterone (11KT) are the major androgens that regulate the expression of SSC (Kime, 1993) and also the expression of reproductive behaviour (Oliveira and Gonçalves, 2008). In species with alternative reproductive tactics (ART), males that follow a conventional tactic, investing resources in mate acquisition and monopolization ('bourgeois' males *sensu* Taborsky, 1997), have generally higher circulating levels of 11KT than parasitic males (*sensu* Taborsky, 1997) that exploit this investment using an alternative tactic, whereas T shows no clear pattern (Brantley, Wingfield, and Bass, 1993; Oliveira, 2006). Furthermore, the administration of 11KT to parasitic males promotes the appearance of SSC and inhibits the expression of female-like behaviours (Oliveira, Carneiro, Gonçalves, Canario, and Grober, 2001c).

Androgens in general and 11KT in particular are also known to respond to the social challenges, with higher reproductive baseline levels found in polygynous species, where males are exposed to a higher rate of male-male competition, than in monogamous species (Oliveira et al., 2002; Hirschenhauser et al., 2004; Hirschenhauser & Oliveira, 2006). Acquisition of territorial status activates the hypothalamic-pituitary-gonadal axis leading to an increase in circulating androgen levels that in turn promotes the expression of status-dependent traits such as SSC (Cardwell & Liley, 1991; Oliveira et al., 1996; White et al., 2002). Hence, androgens play a key role in moderating the allocation of organism resources into reproductive effort as a function of the social environment perceived by the individual. It is therefore plausible to hypothesize that variations in the degree of male-male competition between different populations of the same species may lead to differences in androgen levels that in turn lead to differential expression of SSC and reproductive behaviour.

In this study we investigated the endocrine correlates of morphological traits in two populations of the peacock blenny (*Salaria Pavo*) that differ in male-male competition for the acquisition of nesting sites. At Gulf of Trieste *S. pavo* inhabits rocky shores where nest sites are widely available whereas at the Ria Formosa coastal lagoon, hard substrates eligible to be used as nest-sites are very scarce (Saraiva et al., in prep.1). These differences in nest site availability lead to

differences in reproductive behaviour between the two populations.(Almada, Gonçalves, Oliveira, and Santos, 1995; Almada, Gonçalves, Santos, and Baptista, 1994; Patzner, Seiwald, Adlgasser, and Kaurin, 1986; Saraiva, Barata, Canário, and Oliveira, in press; Saraiva, Pignolo, Robalo, Almada, and Oliveira, in prep). In rocky habitats, most males establish a nest and court females and defend territories around the nest, usually a hole or a crevice in the rock (Patzner et al., 1986; Saraiva et al., in prep). Populations from coastal lagoons where males experience a high regime of male-male competition for nest acquisition and defence present sex-role reversal, with females being the leading sex in courtship, while males restrain from leaving the nest to avoid take-overs and apparently forego territories (Almada et al., 1995). The incidence of sneaker males, that lack male SSC and use female-mimicking behaviour to achieve parasitic fertilizations, is also much higher in lagoon populations (Almada et al., 1995; Almada et al., 1994; Gonçalves, Almada, Oliveira, and Santos, 1996; Ruchon, Laugier, and Quignard, 1995; Saraiva et al., in press). The contrasting mating tactics found in these populations are a unique opportunity to study the endocrine factors underlying intra-specific sexual plasticity.

Here we will compare androgen levels, development of SSC and allocation of gonadal tissue in the two populations of the peacock blenny mentioned above, to test the hypothesis of the association of higher androgen levels with regimes of higher intra-sexual competition.

MATERIALS AND METHODS

Field sites and procedures

The study sites were the Ria Formosa (RF), a coastal lagoon located in Southern Portugal (36°59' N, 7°51' W) where nest sites are very scarce, and the Gulf of Trieste (GT), in the Northeastern Adriatic Sea (45°43' N, 13°45' E) with abundant nest sites (Saraiva et al., in prep). Animals from the GT were caught with a food trap while snorkelling. A piece of food (an open mussel) was put inside a transparent plastic bag, with the entrance kept open by a wire frame. The trap was placed next to nests or in areas where the abundance of *S. pavo* was known to be high. Once one or more animals entered, the trap was closed and the fish were placed in an opaque container. The trap would then be placed again successively until there were no more fish in sight. Sampling was performed in the breeding season (May to August) between 2004 and 2006.

At RF, the animals were caught during low tide, under debris scattered in the muddy intertidal plane or inside bricks delimiting clam culture fields (Almada et al.,

1994). Sampling occurred in the same breeding seasons as in the GT. Males from both populations were classified either as nest-holders (presenting well developed SCC as head crests and anal glands), parasitic males (smaller than nest-holders, lacking or expressing only vestigial male SSC, and releasing sperm upon a gentle abdominal pressure) or transitional males (lacking SSC but not releasing sperm). Laboratory dissection was used to confirm this classification: nest-holders presented a well developed testicular gland and mature testes; parasitic males also had large, mature testes but the testicular gland is vestigial or absent; transitional males had very reduced testes but a fully developed testicular gland.

Blood samples in both populations were taken in the field. Immediately after being caught, the fish were lightly anaesthetized with MS222 (Sigma-Aldrich, dilution 1:10,000). Blood was collected from the caudal vein with a heparinized needle and kept in 1.5 ml eppendorf tubes on ice until being centrifuged approximately 1 - 2 h later. Blood plasma was kept at -20 °C until further processing. After blood collection, fish were euthanized by cutting the spinal cord, near the beginning of the dorsal fin. Fish were kept in isolated plastic bags in a mixture of ice and water until dissection, which took place in a field station (RF) or the lab (GT) within 1 - 2 h. In the field station, animals were measured and weighed. The gonads were collected and gonadal weight was used to determine the gonadosomatic index ($\text{gonad weight} / \text{fresh weight} \times 100$).

Morphometric measurements were taken in the lab with a calliper to the nearest mm. The morphological variables studied were: standard length (SL), crest height (inferred from the ratio head height/body height, HH/BH), gonado-somatic index (GSI: $\text{testes weight} / \text{eviscerated weight} \times 100$), relative testicular gland (TG) area and relative anal gland (AG) area. The AG area was determined by measuring its major and minor axis under a binocular microscope and assuming an ellipsoid shape for this structure. Relative AG area was obtained by dividing AG for SL. For the calculation of the relative TG area we first determined each TG area using the same procedure as for the AG area. The same was done for both testes. To finish, we divided the obtained average for the TG area by the obtained average for the testes area.

Definition of male morphotypes

Nest-holders, bourgeois: males with developed sex characters (crest and anal gland) that acquire a nest

Transition males: males without or with vestigial secondary sex characters and very small GSI but presenting a testicular gland, assumed to be in a transitional phase between parasitic and bourgeois tactics.

Parasitic males: small, female-like males without secondary sex characters and large GSI, assuming an alternative reproductive tactic.

Hormonal assays

The hormonal parameters compared in this study were circulating plasma levels of T and 11KT. Free steroid fraction was extracted using a previously described methodology (Canario and Scott, 1989; Scott and Canario, 1992). Steroid residues were redissolved in phosphate buffer 0.1 M, pH 7.6 containing gelatine (1 g/ L), and stored again at -20 °C until assayed for 11-KT and T. The antibody used for the 11-KT assay was kindly donated by D. E. Kime and the corresponding specificity table was published in (Kime and Manning, 1982). The T antibody (reference: RDI-TRK2T2) was purchased from Research Diagnostics Inc. (Concord, USA) and the cross-reactivity measured by the supplier varied between 16% for 5-alpha-dehydrotestosterone and 0.01% for estradiol. For each hormone, circulating plasma levels from all animals were measured within the same assay. Intra-assay coefficients of variation for the T and 11-KT assays were 2.0% and 2.3%, respectively.

The number of plasma samples from parasitic males at GT was smaller than at RF. Due to the small size of these animals and the particularly low frequency of parasitic males in the GT population, collecting blood samples with enough volume to ensure valid hormonal assays in this male morph was not always viable.

Statistical analyses

Statistical analyses were performed using Statsoft Statistica for Windows. A multi-factorial ANOVA planned comparison design was used for inter-populational analyses. Two levels were assigned to the factor 'Location' (RF and GT), and 4 levels to the factor 'sex morphotype' (Male, Female, Parasitic Male and Transitional Male). A correlation matrix of hormonal (T, 11KT) and morphometric variables (SL, relative AG area, GSI, relative TG area) was used for the correlational analysis of all male morphs. For females, the relative AG and TG areas were removed because they are not present, and GSI was not used because it varies with the female maturation state and oocyte developmental stage. Means $\pm$ standard errors (SE) are presented throughout.

RESULTS

Nest-holders and transition males from RF are larger than the same morphs from GT (Table I). Sneaker males and females have similar SL in both populations (Table I). Head-crest sizes are higher in RF nest-holders and females, while parasitic and transition males do not differ in head-crest size between the two populations (Table I). Nest-holders from RF have also larger relative AG area than nest-holders at GT (Table I). GSI is higher in nest-holders and parasitic males at GT, while no difference is found in transition males between the two populations (Table I). However, the relative TG area is higher in RF nest-holders and transition males than in GT (Table I).

Circulating T levels of females from GT are higher than from RF females, while no differences in T between sites are found for any of the male morphs (Table I). Nest-holder males from RF present much higher levels of 11KT than nest-holders from GT (Table I). Neither females nor any of the other male morphs differ in 11-KT levels.

At RF, head-crest size is positively correlated with 11KT levels (Pearson correlation $R = 0.67$, $N = 12$, $P < 0.05$), and with relative AG area in nest-holders ($R = 0.59$, $N = 33$, $P < 0.001$). In transition males SL and head crest were positively correlated ($R = 0.70$, $N = 10$, $P < 0.05$), and the relationship between GSI and relative TG area is inverse ($R = -0.77$, $N = 10$, $P < 0.01$). A strong positive correlation was also found between T and 11KT in transition males ($R = 0.92$, $N = 8$, $P < 0.001$), which was not the case for parasitic males and females.

At GT, larger males have larger crests, and anal gland is correlated with gonad size (GSI) (SL Vs crest size: $R = 0.40$, $N = 63$, $P < 0.001$; SL Vs relative AG area: $R = 0.41$, $N = 46$, $P < 0.01$; SL Vs GSI: $R = 0.29$, $N = 47$, $P < 0.05$). Relative AG area also correlates with crest size ($R = 0.38$, $N = 46$, $P < 0.01$) and GSI ($R = 0.45$, $N = 46$, $P < 0.01$). In the GT nest-holder males a correlation is also found between T and 11KT ($R = 0.82$, $N = 9$, $P = 0.007$). In the GT parasitic males also have larger relative AG areas ($R = 0.91$, $N = 8$, $P = 0.002$). In females and transition males no correlations are found between androgens and morphological traits.

DISCUSSION

The results from this study emphasize the role of androgens, and particularly 11KT, in promoting the development of SSC in males and mediating their expression according to the social environment. The most conspicuous SSC in the peacock blenny is the head crest, that increases in size in the breeding season and seems to

influence female preference (Fagundes, Goncalves, and Oliveira, 2007; Oliveira, Almada, Forsgren, and Gonçalves, 1999). The larger relative crest size in nest-holders from the RF can be explained by ecological constraint: as males cannot leave their nest due to high risk of nest takeover (Almada et al., 1995; 1994), there should be a stronger pressure for the development of visual cues, signalling both intra and inter-sexually. The high competition among males for access to and maintenance of nests should favour the appearance of visual signals advertising male competitive ability. In addition, females may also use these signals as fitness cues, especially in the RF population where little further information is available, since males hardly leave their nests to court. Another non-exclusive explanation for the difference between populations in nest-holders' body and crest size is that in RF only the larger males with the larger crests can successfully gain access to nests. This competition probably takes place in an earlier phase of the breeding season, and excludes smaller mature males from nesting.

Along with more conspicuous visual cues, more intense chemical signals should also favour males in the ecological setting of the RF population. Chemical signals released by the AG attract females that use this putative pheromone to locate males at a distance (Barata, Serrano, Miranda, Nogueira, Hubbard, and Canário, 2008; Gonçalves, Barata, Oliveira, and Canário, 2002), so there should be competition among nesting males to signal stronger and farther away from their nests, which would lead to more developed AG at RF.

Altogether, the available data suggest that higher male intra-sexual competition at RF driven by limited availability of nest-sites is associated with higher 11KT levels in nest-holder males, which in turn mediate the exaggerated expression of SSC. This is consistent with the challenge hypothesis that proposes that circulating levels of androgens respond to the social environment and that an association between the regime of male-male competition and the androgen response should be observed (Oliveira, 2004; Wingfield, Hegner, Dufty, and Ball, 1990).

Blenny gonads exhibit accessory organs that are involved in sperm maturation and pheromone production (Lahnsteiner, Nussbaumer, and Patzner, 1993; Patzner and Seiwald, 1985). The testicular gland in particular is responsible for sperm storage and maturation and is the main gonadal steroidogenic site for systemic circulation (Oliveira, Almada, Gonçalves, Forsgren, and Canario, 2001a; Reinboth and Becker, 1986). The fact that nest-holders and transitional males from RF have larger relative TG areas than GT males is consistent with the androgen-producing role of this structure. The 11-KT levels of transitional males from the RF may represent the trade-off between somatic growth and reproduction. In this particular population,

transitional males suffer a major transformation from sneaking parasitic males into nest-holders, hence the negative correlation between GSI and the development of the TG.

Interestingly, females at RF present a significant larger crest size than at GT. Since females in this population have the leading role in courtship, the higher sexual competition regime may favour the appearance of SSC (Clutton-Brock, 2007). Differences found in T levels may be related to different ovarian maturation stages, since steroid levels vary according to the development of oocytes (Berlinsky and Specker, 1991). However, since females present very different behavioural profiles in the two populations, the hypothesis of a regulatory function of T in female behaviour should not be discarded.

The sneaker-like males from GT have higher GSIs than those from RF, indicating higher sperm competition at GT (Stockley, Gage, Parker, and Moller, 1997). This may happen because females in GT do not assume an active role in courtship as in RF (Patzner et al., 1986; Saraiva et al., in prep). Since there is no female behavioural role to imitate, parasitic males from GT cannot assume an active tactic to approach nest-holders. In order to enter nests and fertilize eggs, they should either 1) rely on their female-like morphology, be courted by a nest holder and be allowed inside the nest, or 2) swiftly enter the nest while the nest-holder is away and release the maximum amount of sperm in the minimum time possible (mentioned by Gonçalves et al., (2005) as a personal observation). Either way, the predictability to be in the 'right spot at the right time' to spawn should be lower and the overall variability in reproductive success in GT should be higher than at RF. As a result, parasitic males from GT may have to invest more in spermatogenesis. The operational definition that is commonly used for female-mimicking parasitic males is 1) lack of or residual SSC, 2) small size, below the normal SL at which SSC usually appear, 3) female mimicking behaviour and colouration, 4) releasing sperm upon abdominal pressure and 5) high GSI (Gonçalves et al., 1996). However, these criteria are not always applicable in the GT population. In the Adriatic, males can develop SSC at a very small size (minimum SL of nest holder males in TS= 4.56), and so the lower male size classes overlap with those from the parasitic morph (mean SL of parasitic males in TS= 5.84) (Fig. 1A). This is not the case at RF (minimum SL of nest holder males in RF= 6.73; mean SL of parasitic males= 5.57 cm) (Fig. 1B). In addition, at GT there is no female courtship to mimic (Patzner et al., 1986; Saraiva et al., in prep), some nest-holder males also release sperm upon abdominal pressure (pers. obs.) and plotting the GSI of all male morphs does not reveal discrete groups as at RF (see Gonçalves et al., 1996), (Fig. 2). Since the GT

population is not limited by nest-site availability as in RF (Almada et al., 1995; Almada et al., 1994; Saraiva et al., in prep), the presence of condition-dependent parasitic tactic in smaller males is somehow surprising, suggesting that the pay-off matrix for smaller males is favourable to the sneaker tactic in comparison to nest-defence. A possible explanation may be that these males mature too late in the season to grow SSC and directly compete for females. Unlike sneakers from RF, where size is an essential factor in determining the reproductive tactic (only the largest males occupy nests and exclude a wide array of size classes, see results above and also Gonçalves et al., (1996), at GT even very small males can acquire a nest and develop SSC. The only apparent reason for a male to assume a parasitic tactic thus seems to be an incomplete maturation state at the onset of the breeding season. At this point, only a very reduced percentage of males find themselves caught in an inappropriate time window to fully develop the bourgeois SCC.

It is interesting to note that GSIs from both nest-holders and parasitic males from GT are higher than at RF (Table I; see also Fig. 2). This can again be explained by the differences in mating system between the two populations. At RF males fiercely compete for nest sites, but once they acquire a nest spawning becomes facilitated due to the female-biased OSR. Nest-holder males from RF thus have a high resource holding potential but apparently there is no pressure for sperm competition. Although there is a high prevalence of sneakers, nest-holders do not easily discriminate them from females (Gonçalves et al., 2005) and so their perception of reproductive competitors is reduced. This set of data suggests that there is a threshold size for males to breed as nest-holders in RF, and once achieved the reproductive success is high and apparently guaranteed. In contrast at GT, three factors may contribute to a higher sperm competition regime:

- 1) The availability of nests sites enables virtually every male to have a nest, balancing the OSR with a consequent rise in male inter-sexual competition for spawning;
- 2) Males spend a lot of time outside the nest, increasing the opportunity for nest intrusions (be it for egg predation or stealing fertilizations) either by the nest holder or by its neighbours;
- 3) Parasitic males do not have an active female courtship tactic to mimic, resulting in a much higher reproductive unpredictability. For these animals, producing and releasing more sperm increases the probability of a successful spawning.

In conclusion, our data indicates that differences in mating behaviour between two populations of the peacock blenny under different regimes of sexual selection are accompanied by differences in the expression of SSC and allocation of gonadal

tissue, and that androgens mediate the effects of the ecological conditions on morphological traits.

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FIGURE & TABLE LEGENDS

Table I – Inter-population comparison in morphological and hormonal variables.

NH: nest-holders; TM: transition males; PM: parasitic males; F: females; RF: Ria Formosa; GT: Gulf of Trieste. Test differences at $P < 0.05$ are marked with *.

Figure 1A- Histogram of size class frequencies of nest-holders (M) and parasitic males ('sneakers': Sn) at Ria Formosa (RF). Lines represent models of normality.

Figure 1B- Histogram of size class frequencies of nest-holders (M) and parasitic males ('sneakers': Sn) at Gulf of Trieste (GT). Lines represent models of normality.

Figure 2 – Scatterplot of standard length (SL) Vs gonado-somatic index (GSI) of all male morphs in both populations. RF: Ria Formosa; GT: Gulf of Trieste.

TABLE I

		NHRF	NHGT	TMRF	TMGT	PMRF	PMGT	FRF	FGT	Planned comparison NHRF vs NHGT (<i>t</i> , <i>P</i>)	Planned comparison TMRF vs TMGT (<i>t</i> , <i>P</i>)	Planned comparison PMRF vs PMGT (<i>t</i> , <i>P</i>)	Planned comparison FRF vs FGT (<i>t</i> , <i>P</i>)
SL (cm)	Mean	9.44	8.21	6.67	5.47	5.53	5.78	6.54	6.70	5.37, 0.000*	2.80, 0.005*	-0.77, 0.442	-0.71, 0.478
	SE	0.21	0.13	0.26	0.13	0.13	0.24	0.17	0.09				
	N	33	111	10	27	30	23	31	158				
Crest size (HH:BH)	Mean	1.29	1.12	1.00	0.93	0.99	0.96	1.00	0.89	5.91, 0.000*	-1.40, 0.161	0.899, 0.369	4.24, 0.000*
	SE	0.02	0.02	0.02	0.05	0.01	0.01	0.04	0.01				
	N	33	63	10	27	30	23	31	111				
Relative AG area (AG area:SL)	Mean	1.94	1.27							5.69, 0.000*			
	SE	0.10	0.08										
	N	33	46										
GSI	Mean	1.19	1.64	0.40	0.80	3.57	5.48			2.08, 0.039*	1.14, 0.254	6.26, 0.000*	
	SE	0.06	0.11	0.07	0.10	0.24	0.49						
	N	24	47	10	15	21	13						
Relative TG area (TG area: testis area)	Mean	0.27	0.16	0.37	0.15	0.04	0.05			-5.38, 0.000*	-6.38, 0.000*	0.14, 0.885	
	SE	0.01	0.01	0.04	0.02	0.01	0.00						
	N	32	46	10	14	27	12						
T (ng/ml)	Mean	1.96	2.08	0.78	0.98	0.41	0.70	0.60	1.74	0.266, 0.791	-1.00, 0.319	-0.41, 0.686	-2.32, 0.024*
	SE	0.46	0.45	0.40	0.21	0.20		0.13	0.42				
	N	12	9	8	6	9	1	10	9				
11KT (ng/ml)	Mean	4.09	1.46	1.20	0.68	0.75	0.04	0.52	0.60	-2.90, 0.005*	0.14, 0.890	0.22, 0.825	0.26, 0.798
	SE	1.26	0.37	0.70	0.30	0.20		0.06	0.26				
	N	12	9	8	6	9	1	10	9				

FIGURES

Figure 1A

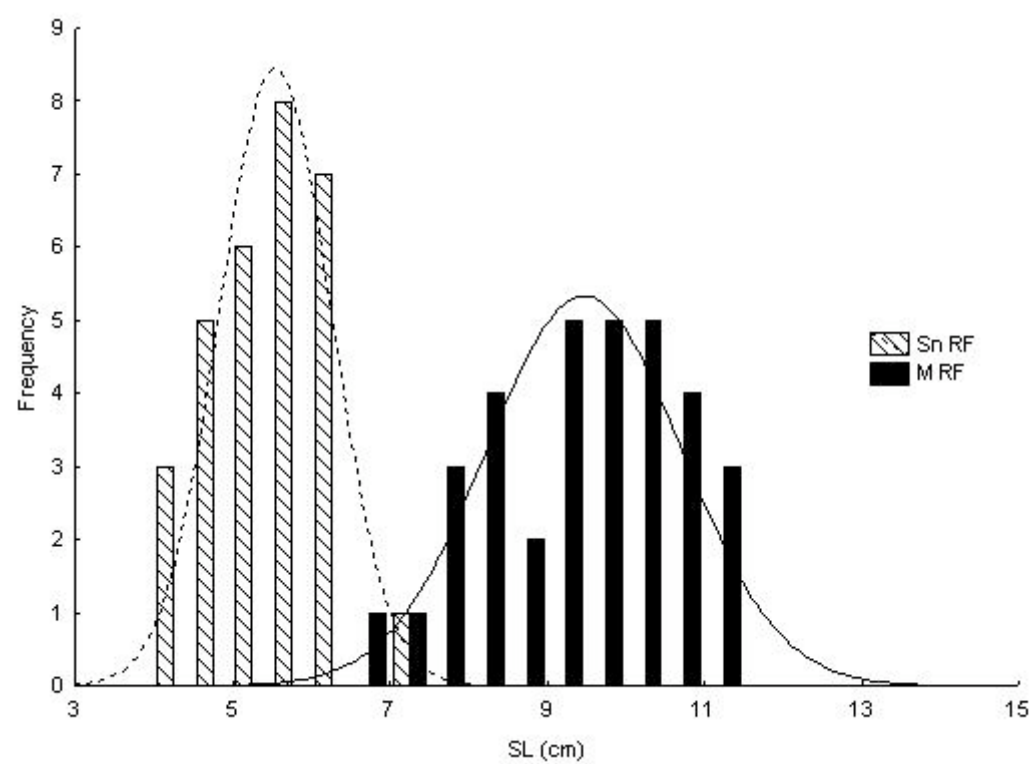


Figure 1B

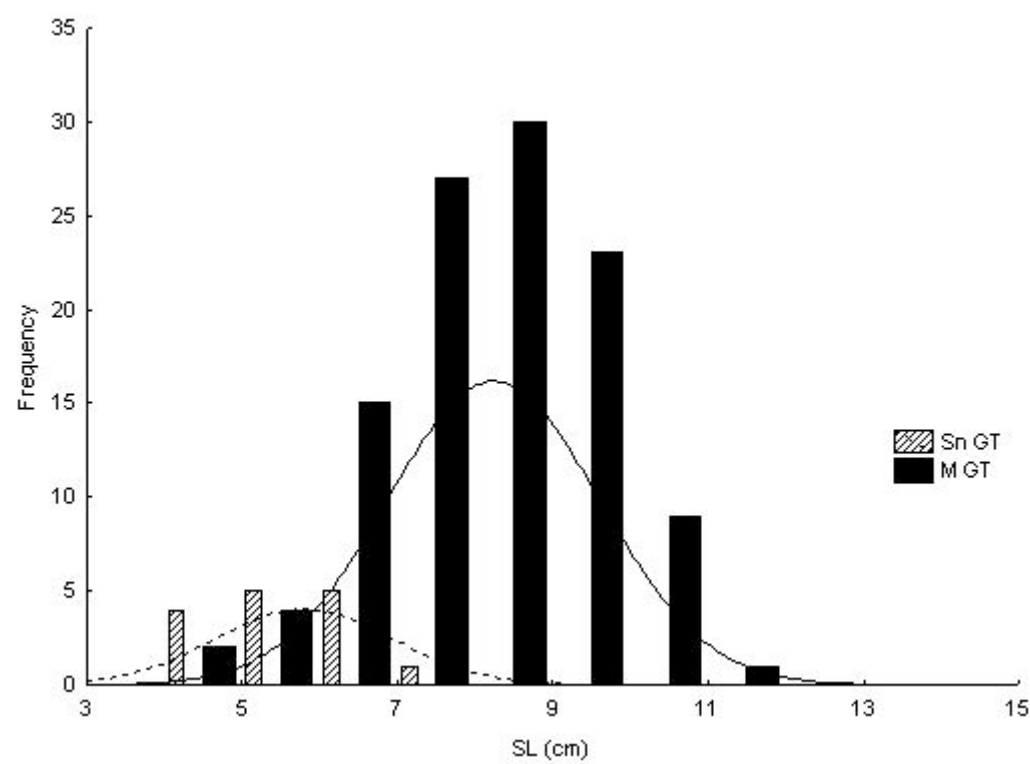
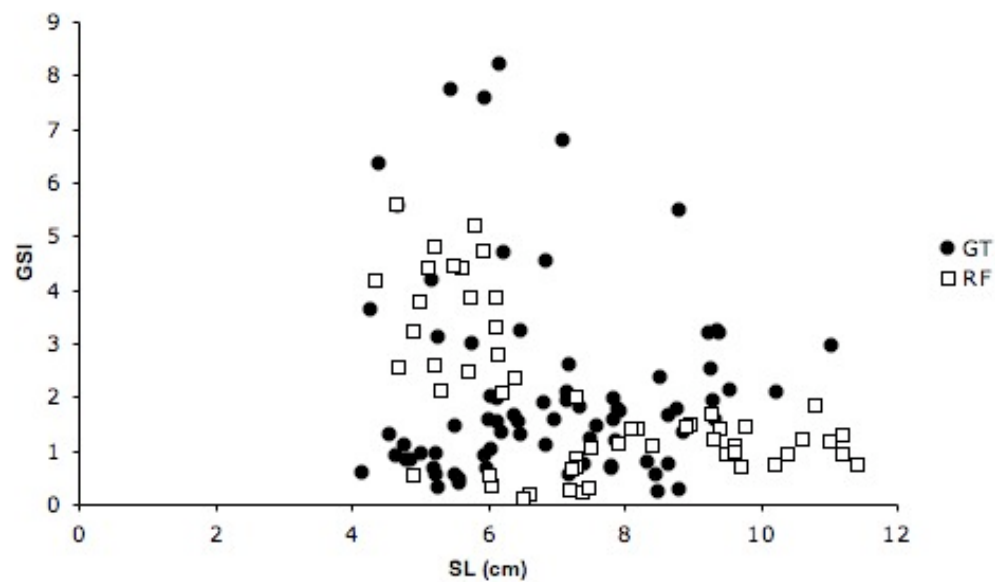


Figure 2



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CHAPTER V

Brain aromatase mRNA expression in two populations of the peacock blenny *Salaria pavo* with divergent mating systems

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ABSTRACT

By catalyzing the conversion of androgens into estrogens, aromatase regulates the availability of these hormones in tissues and controls many physiological and behavioral processes. In fish and other vertebrates, the regulation of aromatase expression in the brain has been suggested to be implicated in the modulation of male sexual and aggressive behaviors. Here, the pattern of mRNA expression of the brain aromatase isoform (codified by the *cyp19b* gene) was quantified in brain macroareas of males and females of the blenny *Salaria pavo* from two populations that differ in their mating systems, and for which male alternative reproductive tactics have been described. In Trieste (Adriatic) males take the initiative in courtship while at Ria Formosa (Southern Portugal) the sex-roles are reversed due to a shortage of appropriate nesting sites. Males from Ria Formosa had overall higher levels of brain aromatase mRNA expression. Because testosterone levels do not differ between males of the two populations, this suggests a higher brain estrogen synthesis in the Ria Formosa males. At the peak of the breeding season these males have a reduced expression of sexual displays, due to a female-biased operational sex ratio, and also of aggressive behaviors, due to nest shortage and aggregation. In fish, exogenous estradiol administration has been shown to decrease sexual and agonistic behaviors in males, and thus the higher brain aromatase levels in Ria Formosa males in comparison with males from Trieste may relate with an estrogen inhibition of these behaviors. In alternative, the higher brain aromatase levels in males from Ria Formosa could be a mechanism to decrease the putative androgen-induced activation of aggressive and sexual displays by reducing the local availability of androgens through their metabolism into estrogens. Although females and parasitic female-like males also differ in their displays between populations, the interpopulational pattern of brain aromatase mRNA expression was similar, suggesting that other neuroendocrine agents mediate the expression of female and female-like behaviors. The neuropeptide arginine vasotocin (AVT) was shown in previous studies to activate female and female-like displays in females and parasitic males. In conclusion, the regulation of brain aromatase availability seems like a probable mechanism to regulate the effects of steroids in the brain circuits underlying the expression of sexual and agonistic displays in *S. pavo*.

Keywords: aromatase; reproductive behavior; *Salaria pavo*; peacock blenny; alternative reproductive tactics; sex-role reversal

INTRODUCTION

In mammals, the activation of sexual behavior in adults has long been known to be facilitated by gonadal estrogens in females and by androgens in males (see (Oliveira, 2004) for an historical overview). However, the paradoxical observation that estrogens could also promote male sexual displays (e.g. (Beach, 1942)) suggested that the effects of androgens could be at least partially dependent on their local conversion into estrogens in the brain. This was solved with the discovery that local conversion of androgens into estrogens, already well established for peripheral tissues such as the placenta (e.g. (Ryan, 1959)), also occurred in the brain ((Naftolin, Ryan, and Petro, 1971; Naftolin, Ryan, and Petro, 1972)). Aromatase was identified as the enzyme responsible for this conversion and thus the bioavailability of androgens and estrogens in target tissues is partially dependent on aromatase levels. It is now well established that the balance between androgens and estrogens determines many physiological and behavioral processes, including brain and gonadal sexual differentiation and the activation of sexual behavior in adults, and that aromatase plays a key role in the regulation of this balance.

More recent experimental studies in mammals in birds have confirmed that the activation of at least some aspects of male sexual displays in adults depends on brain aromatization of androgens into estrogens (reviewed in Ball and Balthazart, 2004; Baum, 2003). In fishes, the role of brain aromatization in the regulation of sexual displays has been poorly investigated. This is surprising as fishes have the highest levels of brain aromatase across all vertebrate classes ((Callard, Schlinger, and Pasmanik, 1990)) and aromatase has been shown to occur in brain areas related to the control of reproduction (e.g. Forlano et al., 2001; Menuet et al., 2003; Menuet et al., 2005) and to peak during the reproductive period in seasonal breeders (Forlano and Bass, 2005; Gelinas et al., 1998; Gonzalez and Piferrer, 2003). In a study on guppies, aromatase activity was pharmacologically inhibited with the drug fadrozole, administered in the water, and the frequency of two of three male sexual displays decreased ((Hallgren, Linderöth, and Olsen, 2006)). This suggests that, similarly to what has been found for birds and mammals, estradiol (E2) locally synthesized in the brain from testosterone (T) also facilitates some aspects of male sexual behavior in fishes. In contrast, in five fish species for which exogenous E2 was administered to males, sexual displays were reduced in 4 cases and remained unchanged in one case (see Table 3.1 in (Oliveira and Gonçalves, 2008)). This contradictory findings show that more data is necessary in order to understand the role played by androgens and estrogens in the regulation of male sexual displays in fishes. A study in the plainfin midshipman suggests that brain aromatase may also be implicated in both intra and

intersexual behavioral differences. In this species, male alternative reproductive tactics (ART) occur, with type I males reproducing by courting females into their nests and type II males reproducing by trying to approach the nests of type I males inconspicuously and achieve parasitic fertilizations of eggs ((Brantley and Bass, 1994)). Type I males emit “hum” vocalizations in order to attract females, while both females and type II males do not emit these vocalizations ((Bass and McKibben, 2003; Brantley and Bass, 1994)). When compared with type I males, both females and type II males have high levels of aromatase activity in a hindbrain region where a motor nucleus controlling vocalizations occurs (Schlinger et al., 1999). The high levels of aromatase activity in females and type II males was interpreted as a possible mechanism to avoid the testosterone-induced masculinization of the vocal motor nucleus through an increase in androgen to estrogen conversion (Schlinger et al., 1999). Taken together the available data suggests that brain aromatase may play a significant role in the regulation of sexual displays in fish.

Here, the brain levels of aromatase mRNA expression were characterized in two populations of the peacock blenny *Salaria pavo* differing in their mating systems. A population at the Adriatic, in Trieste, occurs in an area with a rocky bottom where nests are available in abundance. Males establish nests in rock crevices or holes and defend a territory around the nest. Males take the initiative in courtship and the frequency of male courtship displays is higher than the frequency of female courtship displays (Saraiva, Pignolo, Robalo, Almada, and Oliveira, in prep-b). In contrast, a population at the Ria Formosa (southern Portugal) occurs in a mudflat area where the only substrate available for nesting are artificial materials such as bricks and tiles used by clam culturists to delimit concessions. The scarcity of nest sites promotes a strong male–male competition for nests and only large competitive males are able to acquire a nest (Almada, Gonçalves, Santos, and Baptista, 1994). At the peak of the breeding season most nests are filled with eggs and nest space seems to become a limiting factor for female reproduction (Almada et al., 1994). Females compete for the access to nests and the sex-roles are reversed with females taking the initiative in courtship and displaying courtship displays more often than males (Almada, Gonçalves, Oliveira, and Santos, 1995a; Saraiva et al., in prep-b). Small males are unable to acquire nests and reproduce by mimicking the females’ appearance and courtship displays in order to approach nesting males and parasitically fertilize eggs (Gonçalves et al., 2005; Gonçalves et al., 1996). Parasitic males switch into nesting males from their second breeding season onwards (T. Fagundes, D. Gonçalves, J. Saraiva and R.F. Oliveira, unpublished data), thus suffering major morphologic and behavioral modifications.

Parasitic males have also been described for the Trieste population but in a much lower frequency (Saraiva et al., in prep-b), suggesting that nest availability mediates the frequency of male ART. Interestingly, parasitic males seem to adjust the frequency of female-like displays to the frequency of female courtship behavior in the population. In the Ria Formosa, where females are most actively engaged in courting, female-like displays towards nesting males by parasitic males are very common. In Trieste, where males take the initiative in courtship and females assume a more passive role, parasitic males do not court males and for now it is unclear how they reproduce (Saraiva, Gonçalves, Simões, and Oliveira, in prep-a).

Sex steroids have been shown to influence sexual displays in *S. pavo*. Administration of both T and 11-ketotestosterone (11KT) to parasitic males decreases the frequency of the female-like sexual displays and promotes the development of male secondary sexual characters (Gonçalves et al., 2007; Oliveira et al., 2001). This is consistent with the fact that circulating levels of both T and 11KT are higher in nesting males than in parasitic males (Gonçalves et al., 2008), and in fish species with male ART higher levels of 11KT (but not T) have consistently been found in nesting males (Brantley et al., 1993; Oliveira, 2006). Brain aromatase activity is lower in parasitic than in nesting males (Gonçalves et al., 2008), and from the above it seems possible that this difference is related to the divergent sexual displays exhibited by the two male morphs.

Here, it was hypothesized that the above described differences in the reproductive behavior of females, nesting males and parasitic males across the Ria Formosa and the Trieste population could correlate with brain aromatase mRNA expression levels. Unlike most mammals, for which only one aromatase coding gene has been described ((Conley and Hinshelwood, 2001)), two aromatase isoforms have been found in fish, one preferentially expressed in the ovary and encoded by the *cyp19a* gene and another preferentially expressed in the brain and encoded by the *cyp19b* gene (e.g. Chang et al., 1997; Tchoudakova and Callard, 1998). In this study the *cyp19b* mRNA levels were measured in brain macroareas of females and parasitic, transitional and nesting males of *S. pavo* captured at Trieste and in the Ria Formosa.

METHODS

Fish collection

Females (N = 11), nesting males (N = 12), parasitic males (N = 12) and transitional males (N = 10) were collected at Culatra island (southern Portugal, 36°59'N;7°51'W) during the peak of the breeding season. The criteria to discriminate the various morphotypes were as follows. Nesting males had fully developed male secondary

sexual characters and were defending eggs. Females had swollen abdomens, an indicator of ripeness, and after dissection their ovaries were confirmed to have fully developed oocytes. Parasitic males lacked male secondary sexual characters, sperm could be easily extruded from their vas deferens by gently pressing the abdomen and had enlarged testes (confirmed during dissection). Parasitic males have testes proportionally more developed than nesting males (Gonçalves, Almada, Oliveira, and Santos, 1996). Transitional males were beginning to develop secondary sexual characters. These males have never been observed guarding eggs or courting nesting males with female-like displays and most likely they do not reproduce. Their testes are usually very small when compared with both parasitic and nesting males and this was confirmed during dissection (see Gonçalves et al., 2008). Fish were collected during low-tide with a hand-net from bricks used as nests. Animals were immediately lightly anaesthetized with MS222 (Sigma-Aldrich, dilution 1:10,000) and euthanized by sectioning the spinal cord. Fish were kept in isolated plastic bags in a mixture of ice and water (approx. 0°C) until dissection, which took place in a field station within 1–2 h. In the field station, animals were measured, weighed and dissected. Brains were divided into five macroareas during dissection: telencephalon, optic tectum, cerebellum, diencephalon (excluding the pituitary) and brainstem (Fig. 1).

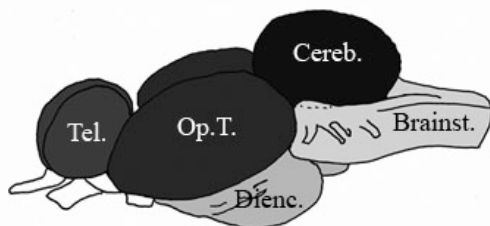


Figure 1. Division of the brain into five macroareas for the quantification of *cyp19b* mRNA expression. Tel.: telencephalon; Op.T.: optic tectum; Dienc.: diencephalon; Cereb.: cerebellum; Brainst.: brainstem.

The brain macroareas were first briefly homogenized in a fixed volume (20–50 µl, depending on the macroarea) of 0.1M RNase free chilled phosphate buffer (pH 7.5) with a Teflon homogenizer by applying two 15 s pulses separated by 30 s intervals. Half of this volume was transferred to a tube with 300 µl of Tri reagent (Sigma, Spain) for RNA extraction and the other half to another tube for an enzymatic aromatase activity assay. Aromatase activity data has been published elsewhere (Gonçalves et al., 2008). Because aromatase activity and *cyp19b* mRNA expression were measured in the same samples, it is possible to correlate these different measures. Briefly,

aromatase mRNA expression values measured in this study were positively and significantly correlated with aromatase activity measured by Gonçalves et al. (2008) (Pearson correlation coefficient: telencephalon, $r = 0.71$, $N = 32$, $P < 0.001$; optic tectum, $r = 0.54$, $N = 32$, $P = 0.001$; diencephalon, $r = 0.54$, $N = 36$, $P = 0.001$; cerebellum, $r = 0.57$, $N = 35$, $P < 0.001$; brainstem, $r = 0.72$, $N = 38$, $P < 0.001$).

The same general procedure was applied for fish collected at the Trieste (northern Adriatic sea, $45^{\circ}40' \text{ N}$, $13^{\circ}35' \text{ E}$) with the difference that males were caught in food traps or fish nets while snorkelling. Females ($N = 9$), nesting males ($N = 10$), parasitic males ($N = 5$) and transitional males ($N = 5$) were processed exactly as in Ria Formosa.

During the experimental procedure the “ASAB guidelines for the use of animals in research” were followed.

Cyp19b and 18S sequences

For partial cloning of the *cyp19b* cDNAs, one male and two females were deeply anaesthetized, euthanized by sectioning of the spinal cord and their brains pooled and homogenized in TRI reagent (Sigma, Spain) for total RNA extraction according to the manufacturer's instructions. RNA was treated with DNase I (Ambion, Portugal) and for cDNA synthesis 5 μg of total RNA (quantified spectrophotometrically) was reverse transcribed using oligo-dT primers and M-MLV reverse transcriptase (Promega, Spain) in a 60 μl reaction. For the *cyp19b* sequence, a predicted 450bp fragment was amplified in a 50 μl PCR reaction, using primers designed from the tilapia *Oreochromis mossambicus* *cyp19b* sequence (forward: 5'-TCTTAGCAGGACTCGGTCCAAT - 3'; reverse: 5'- AGATGTCCAACACGATGGCTCT - 3'). The PCR mix contained 1X reaction buffer, 1.5 mM MgCl_2 , 200 μM of each dNTP, 20pmol of each primer, 1U of *Taq* DNA polymerase (Promega) and 2 μl of first-strand cDNA. Cycling conditions were 2min at 95 $^{\circ}\text{C}$, 35 cycles of 1min at 95 $^{\circ}\text{C}$, 1min at 59 $^{\circ}\text{C}$ and 1min at 72 $^{\circ}\text{C}$, followed by 5min at 72 $^{\circ}\text{C}$. PCR products were run on a 2% agarose gel to confirm predicted fragment size and the fragment corresponding to the putative aromatase cDNA was cut from the gel, eluted (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences, Piscataway, USA) and cloned into pGem-T-easy vector (Promega). Two clones with the predicted insert were sequenced by automatic sequence analysis (Macrogen Inc., South Korea) and confirmed to contain a match to other teleosts' *cyp19b* sequences. For the 18S sequence, total RNA was extracted from the brain of a male and a female and reversed transcribed as above. Primers were designed for conserved regions of other teleosts in order to amplify a 407bp fragment (forward: 5'-

GTTCCGACCATAAACGATGC - 3'; reverse: 5'- CTCAATCTCGTGTGGCTGAA -3'). The PCR conditions were as above. The PCR fragment size was confirmed by gel electrophoresis, purified and directly sent for automatic sequencing (Macrogen Inc., South Korea). The sequence highly matched the 18s sequences of other teleosts.

Cyp19b relative mRNA expression

Sequencing information was used to design specific primers for relative quantitative real-time PCR (QRT-PCR) (cyp19b, forward: 5' – TATGGCAGCATTACCAGGGT – 3', reverse: 5' - GCCGAATCTTGACGTGTACTG – 3', fragment size: 111bp; 18S, forward: 5' – GCATGGCCGTTCTTAGTTGGT – 3', reverse: 5' – TTAGCAAGCCGGAGTCTCGTT – 3', fragment size: 73bp). The identity of the resulting PCR products was confirmed by DNA sequencing of PCR products.

The expression of aromatase measured during QRT-PCR was normalized to the expression of 18S to account for variation in total RNA levels between samples. Total RNA from each brain macroareas was extracted, DNase treated and reversed transcribed (1 µl) into cDNA as described above except that random hexamers (Promega, Spain) were used during reverse transcription. QRT-PCR reactions (20 µl) were run in a Stratagene MX3000p thermocycler with Stratagene's Brilliant SYBR green QRT-PCR Master Mix (Stratagene, Spain) and primers at 0.5 µM. Thermocycling conditions were equal for both reactions and were as follows: 10min at 95°C, 40 cycles of 95°C for 30s, 59°C for 30s and 72°C for 30s. After PCR, a melting curve program from 55°C to 95°C with 0.5°C change in 10s intervals was applied and the presence of a single reaction product in each tube confirmed. For the same animal, reactions for each macroarea and for both 18S and cyp19b were run in duplicate in a single PCR. Controls without template were included for both primer sets and a set of samples were included in all reactions to determine interassay variation. Raw fluorescence data was submitted to PCR Miner (<http://miner.ewindup.info/miner>, (Zhao and Fernald, 2005)) to calculate reaction efficiencies and cycle thresholds from individual wells during the reaction. The average reaction efficiencies (E) were 1.8 for 18S and 1.9 for aromatase and the average intrassay and interassay coefficient of variation in cycle threshold (CT) were 1.7% and 5.3%, respectively. For each sample, the mean CT of 18S and aromatase was calculated and the relative initial template concentration (R0) of both genes determined from $1/(1+E)^{CT}$ ((Zhao and Fernald, 2005)). The relative aromatase mRNA expression was thus given by the ratio between the aromatase and 18S R0s. The 18S average R0s did not differ between macroareas, morphotypes or populations (data not presented), suggesting this is an appropriate reference gene.

Statistical analyses

A three-way ANOVA with factors morphotype (four levels), population (two levels) and brain macroarea (five levels) was applied to test differences in *cyp19b* relative mRNA expression. Data was squared-root transformed to comply with normality and homocedasticity assumptions. When the ANOVA results were significant, relevant differences were tested with contrast analysis.

RESULTS

Overall, aromatase mRNA expression differed between the morphotypes ($F_{3, 255} = 21.6$, $P < 0.001$). Females and nesting males had higher values than both transitional and parasitic males ($P < 0.001$). The remaining comparisons were non-significant ($P > 0.14$). The overall pattern of aromatase mRNA expression was similar between populations ($F_{1, 255} = 0.00$, $P = 0.984$). However, there was a significant location * morphotype interaction ($F_{3, 255} = 4.5$; $P = 0.004$, Fig. 2). In Ria Formosa, males had overall higher aromatase mRNA expression values than in Trieste ($P=0.001$). Females, transitional males and parasitic males did not differ between locations ($P>0.17$).

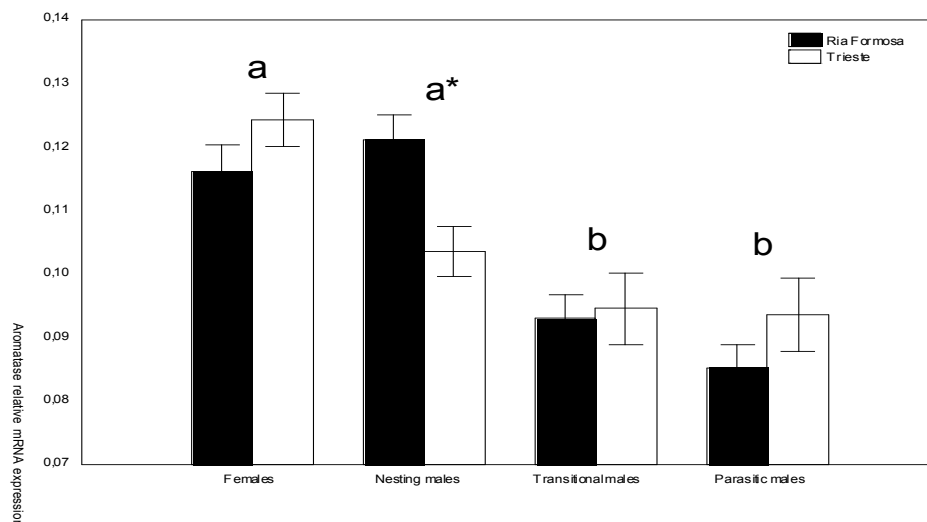


Figure 2. Average $\pm$ S.E. aromatase (cyp19b isoform) relative mRNA expression in the brain of females and nesting, transitional and parasitic males of *S. pavo* collected in the Ria Formosa and in Trieste. Different letters indicate significant differences ($P < 0.05$) between morphotypes (average of both populations). Significant differences between populations for the same morphotype are marked with *.

Aromatase mRNA expression differed between macroareas ($F_{4, 255} = 21.6$, $P < 0.001$, Fig. 3). The cerebellum had the lowest expression ($P < 0.001$ in the comparison with

the other macroareas) followed by the brainstem (marginally different from the diencephalon, $P = 0.078$, and significantly different from the other macroareas, $P < 0.03$). The optic tectum had significantly higher levels than the diencephalon ($P = 0.025$) and marginally than the telencephalon ($P = 0.066$). The diencephalon and telencephalon did not differ ($P=0.99$). Differences between macroareas were similar between locations (location * macroarea interaction: $F_{4, 255} = 1.6$, $P = 0.117$, Fig. 3).

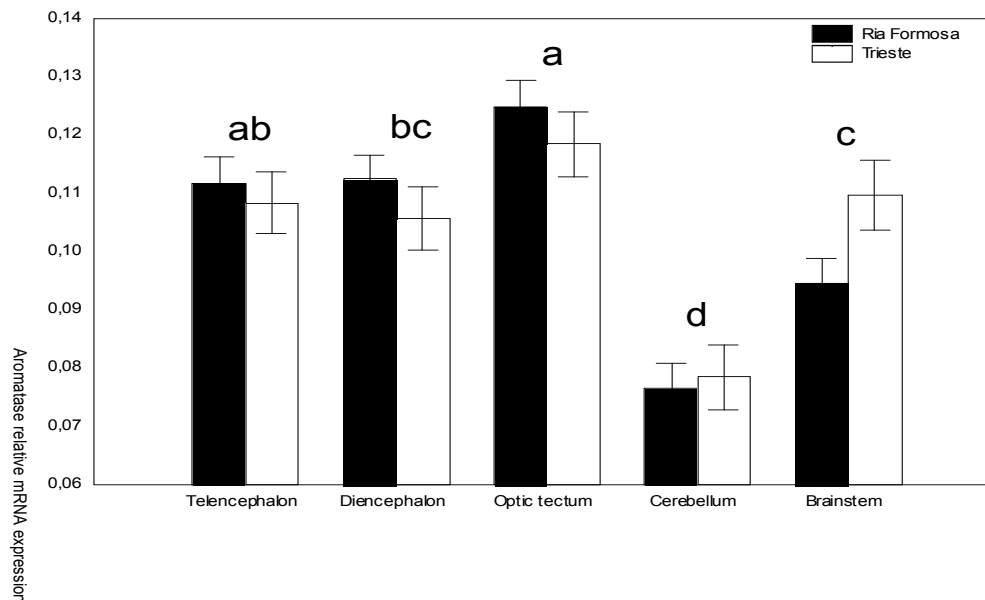


Figure 3. Average $\pm$ S.E. aromatase (cyp19b isoform) relative mRNA expression measured in the five brain macroareas in Ria Formosa and in Trieste. Different letters indicate significant differences ($P < 0.05$) between macroareas (average of all morphotypes).

The morphotype * macroarea interaction ($F_{12, 255} = 1.6$, $P = 0.076$) and the morphotype * macroarea * location interaction ($F_{12, 255} = 1.1$, $P = 0.372$) were not significant.

DISCUSSION

Differences between populations

The pattern of aromatase (cyp19b isoform) mRNA expression in the brain was similar between the two populations, with the exception of nesting males which presented higher levels in Ria Formosa. Aromatase has been implicated in the regulation of sexual behaviors in vertebrates (Ball and Balthazart, 2004; Baum, 2003) and thus it seems possible that the measured interpopulational difference in brain aromatase expression relates to the divergence in the nesting males' behaviour. Previous studies have shown that the reproductive behaviour of nesting males, but also of females and parasitic males, differs between populations. In a lab experiment, run during the peak

of the breeding season, nesting males from the two populations were sequentially presented with a female and a parasitic male. As expected, females from the Ria Formosa displayed more often courtship behavior towards the nesting male than females from the Adriatic (Saraiva et al., in prep-a). Males, however, did not differ in their frequency of courtship displays. This was interpreted as a consequence of males not having any eggs in the nest during the experiment. In fish, male courtship behavior has been shown to decrease when the percentage of nest egg coverage increases (Shibata and Kohda, 2006). Thus, when females were introduced in the aquaria they assumed the typical population courtship pattern based on the nest egg coverage expectation: females from Ria Formosa actively courted males in order to be accepted into their nests while females from Trieste waited to be courted. Males were given a nest without eggs, and this is particularly atypical for nesting males of Ria Formosa at the peak of the breeding season. An increase in courtship behavior from these males may thus have accounted for the absence of differences in the frequency of courtship behavior between populations (Saraiva et al., in prep-a). This hypothesis is reinforced by the fact that field observations have shown the expected significant differences in both male and female courtship displays between populations, i.e., a higher frequency of male courtship displays in Trieste and a higher frequency of female courtship displays in the Ria Formosa (Saraiva et al., in prep-b). Although the only experimental study testing the effects of aromatization in fish male sexual displays found a significant decrease of these behaviors with aromatase blockage (Hallgren et al., 2006), in 5 studies where E2 was administered to males sexual displays were reduced in four cases and remained unchanged in one (Oliveira and Gonçalves, 2008). Also, in the plainfin midshipman, courting males presented lower levels of aromatase in brain regions implicated in the control of courtship displays when compared with non-courting males (Schlinger et al., 1999). These data suggests that in most cases estradiol will reduce male sexual displays in fish. The higher aromatase mRNA expression levels measured in the brain of nesting males from Ria Formosa could thus relate to the lower expression of sexual displays by these males in that population. Testosterone levels did not differ between males of both populations and thus the higher brain aromatase levels will induce a higher local T to E2 synthesis in males from Ria Formosa. This could be a possible mechanism mediating the reduction in sexual displays by these males. Experiments testing the effects of T and E2 in male sexual displays are ongoing. A second not exclusive hypothesis, relates to the expression of aggressive behavior. In Trieste males aggressively defend an area around the nest from other competitors (Saraiva et al., in prep-b), while in the Ria Formosa males do not defend territories and very often have one or more males nesting in adjacent brick

holes (Almada et al., 1994). Estrogens have been shown to reduce aggression in fish (Bell, 2001; Munro and Pitcher, 1983) and increases in aggression were negatively correlated with brain aromatase activity in a sex-changing goby ((Black, Balthazart, Baillien, and Grober, 2005)). Importantly, E2 administration to parasitic males of *S. pavo* from the Ria Formosa population was also shown to inhibit aggression (Gonçalves et al., 2007). Thus, the higher brain aromatase levels in males from Ria Formosa increase local E2 synthesis and this may induce the necessary decrease in aggression due to the scarceness and aggregated nature of the available nests.

Although females and parasitic males exhibit notorious quantitative differences in sexual displays between these populations, the pattern of brain aromatase mRNA expression was similar. Females from Ria Formosa court more often nesting males than females from Trieste (Saraiva et al., in prep-b) and laboratory observations show that parasitic males follow the same pattern, courting very often nesting males in the Ria Formosa and not courting at all in Trieste (Saraiva et al., in prep-a). For now it is unclear how parasitic males reproduce in Trieste as field observations in this area focusing on the parasitic males' behavior are lacking. The absence of interpopulational differences in brain aromatase levels is more unexpected for females than for parasitic males. Estradiol administration to parasitic males from Ria Formosa had no effect in the female-like displays (Gonçalves et al., 2007) and thus interpopulational differences are likely to be regulated by other neuroendocrine agents. For females however, preliminary data of a lab experiment performed with animals from Ria Formosa suggest that E2 may promote female sexual displays. In that experiment, ovariectomy reduced to 50% female sexual displays while E2 administration to ovariectomized females partly recovered sexual behaviours (M. Teles, S. Costa, D. Gonçalves and R.F. Oliveira, unpublished data). Thus, we would expect females from Ria Formosa to present higher brain aromatase levels if E2 promotes sexual displays. However, in the Ria Formosa female-female aggression is apparently elevated as a consequence of female competition for nests (Almada, Gonçalves, Oliveira, and Santos, 1995b). Brain aromatase levels have been shown to correlate negatively with aggression in males (Black et al., 2005) and, if the same applies to females, the higher aggression levels in females from Ria Formosa could be modulated by lower brain aromatase production. This might explain the similar pattern in brain aromatase levels between females from the two populations. Also, both female and parasitic female-like courtship behaviors in *S. pavo* have been shown to depend on the action of the brain neuropeptide arginine vasotocin (AVT), the fish homologous of the mammalian arginine vasopressin (AVP). Females and parasitic males have higher preoptic levels of AVT mRNA than nesting males (Grober, George, Watkins, Carneiro, and Oliveira, 2002) and exogenous AVT

administration to females and parasitic males promoted female courtship displays (Carneiro, Oliveira, Canário, and Grober, 2003). This suggests that AVT generally promotes female and female-like displays, and interpopulational differences in female and parasitic male sexual behaviour may be more related to variations in central AVT levels than to the direct action of steroids in the CNS.

Differences between morphotypes

Females and nesting males had higher brain aromatase mRNA levels than parasitic and transitional males. Transitional males had intermediate values between parasitic and nesting males, although the difference was only significant for nesting males. This is in accordance with what has been described in a previous study measuring brain aromatase activity in similar samples (Gonçalves et al., 2008). In that study, the increase in brain aromatase activity during the transition suggested a role for estrogen activation of male sexual displays, as demonstrated for other vertebrates. However, the data in this study argues against that hypothesis as males from Trieste, expressing more courtship behavior, had lower brain aromatase mRNA expression. One hypothesis is that male-sexual displays are modulated by the direct action of androgens and not by estrogens. Androgens have been shown to promote male-sexual displays in fish (reviewed in Borg, 1994; Liley and Stacey, 1983). In *S. pavo*, levels of both 11KT and T are higher in nesting males (Gonçalves et al., 2008), and, in general, in species with male ART nesting males have higher levels of 11KT, a non-aromatizable androgen, but not of T when compared with parasitic males (Brantley et al., 1993; Oliveira, 2006). The administration of both T and 11KT to parasitic males inhibited the expression of female-like displays, although it did not induce male-like sexual behaviors (Gonçalves et al., 2007; Oliveira et al., 2001). Those experiments only lasted for 8 days and it is possible that a longer time frame would be necessary for the putative masculinizing behavioral effects of androgens to be observed. Under that scenario, the higher aromatase levels in males of the Ria Formosa population, where sex-roles are reversed, could be a mechanism to reduce the androgen-induced activation of male sexual displays. A similar mechanism was proposed for the plainfin midshipman where the higher levels of aromatase in the brains of females and parasitic males in comparison with nesting males was suggested to prevent the masculinization by androgens of brain regions implicated in the production of mating calls (Schlinger et al., 1999). Experiments are ongoing to test the effects of androgens in *S. pavo* male sexual behavior.

Differences between brain macroareas

Aromatase levels were highest in telencephalon, diencephalon and optic tectum and lower in the cerebellum and brain. This is in agreement with the results reported for fish where aromatase levels have been reported to be higher in forebrain regions known to regulate reproductive behaviors, particularly the preoptic and hypothalamic periventricular nuclei, central telencephalon and optic tectum (e.g. Forlano et al., 2001; Gelinas and Callard, 1993, 1997; Melo and Ramsdell, 2001). In the Gonçalves et al. (2008) study, the pattern of aromatase activity across brain macroareas differed between morphotypes, with females presenting higher levels in posterior brain regions when compared to males, a pattern also described for the medaka *Oryzias latipes* (Melo and Ramsdell, 2001). In this study the interaction between the macroarea and morphotype factor was marginally non-significant ($P = 0.07$), but females also presented the highest values of aromatase mRNA expression in posterior brain regions (i.e. cerebellum and brainstem, data not shown).

In conclusion, the correlational data presented in this study suggests a major role for brain aromatase in the regulation of sexual displays in *S. pavo*. High aromatase mRNA expression levels were recorded in brain macroareas containing nuclei associated with the control of sexual displays. Regulation of aromatase levels may be used as a mechanism to control the availability of both estrogens and androgens in local brain regions and, consequently, the effects of these steroids in the brain circuits underlying the expression of sexual and also aggressive behaviors. The described differences in the pattern of aromatase mRNA expression across the two populations and across morphotypes raises a number of hypotheses on the effects of estrogens and androgens in the regulation of behaviors in *S. pavo* that can be experimentally tested.

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GENERAL DISCUSSION

GENERAL DISCUSSION

In this research, a transversal approach using different levels of analysis was used to assess the causes and consequences of the inter-population variation in reproductive behaviour in the peacock blenny *Salaria pavo*. With the present data, a model for ecological modulation of behaviour can now be proposed.

In the first chapter, we asked a fundamental question: if the mating system reported for Ria Formosa (namely sex-role reversal) could be experimentally modified by manipulating the spatial distribution of nest sites. The data collected showed that aggregation of nests may bias the OSR towards females and decreases the number of males qualified to mate, thereby creating a competitive mating situation for females. This apparently leads to predominant female courtship and intra-sexual competition. The results found in this work suggested that nest aggregation has a clear impact in the dynamic of sex-roles. In other terms, the data show that a) the reproductive behaviour is plastic and b) it may be modulated by ecological conditions.

In the following chapters, we studied the mating system in populations where the ecological conditions were suspected to differ: Ria Formosa, with scarcity and aggregation of nest sites, and Gulf of Trieste (Adriatic sea, Italy and Slovenia) with excess of nest sites. Differences were found not only in behaviour, occurrence of ART and occupation of nests, but also in morphology and hormonal regulation.

The results found in Chapters 2 and 3 suggest that the mating system differs according to the ecological conditions. In the RF population (nest scarcity scenario) only the largest and more competitive males get to occupy a nest (Almada et al. 1994). Those who acquire a nest face competition from floater males in search for nests, and nest-holders apparently restrain from leaving the nest to court females or attack intruder males. Staying inside the nest in high-density situations may work as a mechanism to prevent nest take-overs (Kotrschal 1988). In fact, our data show that at RF, the denser are distributed the nests, the less active are the nest-holders. They receive more visits from possible intruders than nest-holders from GT, but there are no differences in the expression of agonistic behaviours; this differential inter-male aggression is evidence pointing to behavioural plasticity in contrasting ecological situations, as suggested by Almada et al. (1994). The control of agonistic behaviour may be regulated in the brain by an increase in aromatase expression in nest-holders from RF (Chapter 5). Aromatase is the enzyme responsible for the conversion of testosterone into estradiol, and estradiol has been reported to be linked to inhibition of aggressive and courtship behaviours (Gonçalves et al. 2007). Thus, higher brain levels of aromatase allow RF

nest-holders to tolerate the presence of neighbours, as well as keeping courtship behaviours reduced.

As the number of males qualified to mate (Kvarnemo et al. 2001) is reduced at RF, the OSR shifts towards females who take on the leading role in courtship. In this population, females visit nests and court nest-holders more frequently than at GT (Chapter 2), which suggests high competition among females for mating opportunities. Due to the high aggregation of nest sites, many females can be found simultaneously visiting nests in the same patch. In such high densities females increase their courtship rate as a competitive mechanism (Chapter 1).

The available data suggests that at RF the mating system probably has two distinct phases:

- 1-At the onset of the breeding season, there is high male intra-sexual competition for access to nest sites.
- 2-Once the larger males establish nests and the smaller males are excluded, the intra-sexual competition decreases in males and increases in females, leading to sex role reversal.

Previous research demonstrates that sneakers (small males without SSC that follow a parasitic tactic) not only mimic female behaviour and morphology, but also copy females when it comes to choose which nest-holders to court (Gonçalves et al. 2003). The sneaking tactic probably appears in high frequencies at RF because only the largest males can acquire and secure nests. While floaters (i.e. males slightly smaller than nest-holders with fully developed SSC, but that could not secure a nest) swim around ready to take over vacant nests (Almada et al. 1995; Almada et al. 1994), the only option for sexually mature males in lower size classes is to compromise the growth of SSC and invest in growing large testis that allow them to spawn parasitically (Taborsky 1998).

At GT, there is virtually an excess of nesting sites for every mature male during the whole breeding season (Chapter 2). This reduces male intra-sexual competition for nests and allows even small mature males to acquire a nest. Apparently only a very reduced number of males 'choose' to follow an ART at GT (Chapter 2), but this tactic is not likely to be based on female mimicking (Chapter 3). Given the availability of potential nests, it is surprising that these parasitic males even exist at all. One hypothesis is that these males mature very late in the breeding season, too late to grow SSC and adopt a bourgeois tactic. At this point, they invest in large testis that still allow them to spawn parasitically. Developing a parasitic tactic in this case should not be an effect of size, since even very small males are capable of finding a nest. Instead, this may represent an extreme example of a birthdate effect in a condition-dependent ART

(Taborsky 1998), with only a very small proportion of males being caught in this ontogenic trap.

This model is supported by the analysis of morphology and androgens in Chapter 4. Bourgeois males from RF are larger, have more developed SSC and higher circulating levels of 11KT than at GT, suggesting:

- i) a highly competitive social environment, and
- ii) a mediation from the endocrine system responding to (and feeding back into) this competitive environment.

However, the difference in GSI between populations both in bourgeois and parasitic males indicates that this competition should be focused more on resources than on reproduction. In fact, both these male morphs from GT have less developed SSC but higher GSIs, suggesting higher sperm competition (Chapter 4). At GT, three factors may contribute to this situation:

- 1) The availability of nests sites enables virtually every male to have a nest, balancing the OSR with a consequent rise in male intra-sexual competition for spawning;
- 2) Males spend a lot of time outside the nest, increasing the opportunity for nest intrusions (e.g. for egg predation or stealing fertilizations) either by the nest holder or by its neighbours;
- 3) Parasitic males do not have an active female courtship tactic to mimic, resulting in a much higher reproductive unpredictability. For these animals, producing and releasing more sperm increases the probability of a successful spawning.

In the future long-term common garden experiments (i.e. allowing individuals from the two populations to grow in the same environment) or translocation experiments (i.e. growing RF juveniles in GT conditions and vice-versa) should help to clarify these issues. Nevertheless, these populations represent a rare case where the assumptions for ecological modulation of behaviour can be tested at a large scale.

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