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Environmental modulation of androgen levels and secondary sex characters in two populations of the peacock blenny *Salaria pavo*

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ABSTRACT

Morphology and endocrinology were studied 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, sexually dimorphic benthic fish that presents exclusive paternal care of the clutch and inhabits rocky shores of the Mediterranean and adjacent Atlantic areas. In a population from the Gulf of Trieste (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 (small, parasitic female-mimicking sneaker males that change tactic into nest holders in subsequent breeding seasons) occurs. Conversely at Ria Formosa, a coastal lagoon in Southern Portugal, where nest 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 alternative reproductive tactics is observed. Concomitantly, at Ria Formosa nest holder males are larger and present more developed secondary sex characters and higher levels of 11KT than at the Gulf of Trieste. However, the gonads of nest holders and parasitic males were larger in the Gulf of Trieste population. Competition for nests at Ria Formosa seems to promote more developed secondary sex characters in nest site scarcity conditions, while competition for females at the Gulf of Trieste seems to be spurring sperm competition among males in populations where nest sites are more abundant. 11KT was thus associated with the development and expression of secondary sex characters in contrasting environments. These results exemplify how the modulation of behavioral plasticity and secondary sex characters by the social environment can be mediated by androgens.

Introduction

Secondary sex characters (SSCs) 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 et al., 2001b). In teleost fish testosterone (T) and 11-ketotestosterone (11KT) are the major androgens that regulate the expression of SSC (Kime, 1993) and also the expression of reproductive behavior (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 those of parasitic males (*sensu* Taborsky, 1997) that exploit this investment using an alternative tactic, whereas T shows no clear pattern (Brantley et al., 1993b; Oliveira, 2006). Furthermore, the administration of 11KT to parasitic males promotes the appearance of SSCs and inhibits the expression of female-like behaviors (Oliveira et al., 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 (Hirschenhauser and Oliveira, 2006; Hirschenhauser et al., 2004; Oliveira et al., 2002). 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 SSCs (Cardwell and 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

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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 behavior.

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., unpublished data). These differences in nest site availability lead to differences in reproductive behavior between the two populations (Almada et al., 1995; Almada et al., 1994; Patzner et al., 1986; Saraiva et al., 2009; Saraiva et al., unpublished data). 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., unpublished data). Populations from coastal lagoons where males experience a high regime of male–male competition for nest acquisition and defense present sex-role reversal, with females being the leading sex in courtship, while males restrain from leaving the nest to avoid takeovers and apparently forego territories (Almada et al., 1995). The incidence of sneaker males, that lack male SSC and use female-mimicking behavior to achieve parasitic fertilizations, is also much higher in lagoon populations (Almada et al., 1995; Almada et al., 1994; Gonçalves et al., 1996; Ruchon et al., 1995; Saraiva et al., unpublished data).

The contrasting mating tactics found in these populations are a unique opportunity to study the endocrine factors underlying intraspecific sexual plasticity.

In the present paper we compare androgen levels, development of SSCs 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 intrasexual 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., unpublished data). Animals from the GT were caught with a food trap while snorkeling. A piece of food 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 SCCs as head crests and anal glands), parasitic males (smaller than nest holders, lacking or expressing only vestigial male SSCs, and releasing sperm upon a gentle abdominal pressure) or transitional males (lacking SSCs 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 anesthetized 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 gonado-somatic index (gonad weight / eviscerated weight × 100).

The morphological variables studied were: standard length (SL), crest height (inferred from the ratio head height / body height), gonado-somatic index (GSI: testes weight / eviscerated weight × 100), relative testicular gland area and relative anal gland area. Standard length and crest height were measured in the lab with a calliper to the nearest mm. The testicular gland is an accessory structure to the gonad, being the main source of androgens in this species (Oliveira et al., 2001b; Reinboth and Becker, 1986). The anal gland is a modification of the first two rays of the anal fin (Zander, 1975), that is involved in the emission of a putative sex pheromone (Barata et al., 2008). The anal gland area was determined by measuring its major and minor axis under a binocular microscope and assuming an ellipsoid shape for this structure. Relative anal gland area was obtained by dividing its area for SL. For the calculation of the relative testicular gland area we first determined each testicular gland area using the same procedure as for the anal gland area. The same was done for both testes. To finish, we divided the obtained average for the testicular gland area by the obtained average for the testes area.

Hormonal assays

The hormonal parameters compared in this study were circulating plasma levels of T and 11KT. Free steroid fraction was extracted using a methodology described by Canario and Scott (1989) and Scott and Canario (1992). Steroid residues were redissolved in phosphate buffer 0.1 M, pH 7.6 containing gelatin (1 g/l), and stored again at −20 °C until assayed for 11KT and T. The antibody used for the 11KT assay was kindly donated by D. E. Kime and the cross-reactivity for T was 1.3% (Kime and Manning, 1982). The T antibody (reference: RDI-TRK2 T2) 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. The cross-reactivity with 11KT was measured in our lab with a value of 2%. For each hormone, circulating plasma levels from all animals were measured within the same assay. Intra-assay coefficients of variation for the T and 11KT 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.

Animal welfare

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

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, sneaker and transitional male). A correlation matrix of hormonal (T, 11KT) and morphometric variables (SL, relative anal gland area, GSI, relative testicular gland area) was used for the correlational analysis of all male morphs. For females, the relative anal and testicular gland 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 ± standard errors (SE) are presented throughout. Planned comparisons were used to evaluate inter-population differences among morphs.

Results

Nest holders and transition males from RF were larger than the same morphs from GT (Table 1). Sneaker males and females had similar SL in both populations (Table 1). Head crest sizes were higher in RF nest holders and females, while parasitic and transition males did not differ in head crest size between the two populations (Table 1). Nest holders from RF had also larger relative anal gland area than nest holders at GT (Table 1). GSI was higher in nest holders and parasitic males at GT, while no difference was found in transition males between the two populations (Table 1). However, the relative testicular gland area was higher in RF nest holders and transition males than in GT (Table 1).

Circulating T levels of females from GT were higher than from RF females, while no differences in T between sites were found for any of the male morphs (Table 1). Nest holder males from RF presented much higher levels of 11KT than nest holders from GT (Table 1). Neither females nor any of the other male morphs differed in 11KT 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 anal gland area in nest holders ($R=0.59$, $N=33$, $P<0.001$). In transition males SL and head crest were positively correlated ($R=0.70$. $N=10$, $P=0.024$), and the relationship between GSI and relative testicular gland 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 had larger crests, and anal gland was correlated with gonad size (GSI) (SL *Vs* crest size: $R=0.40$, $N=63$, $P<0.001$; SL *Vs* relative anal gland area: $R=0.41$, $N=46$, $P<0.01$; SL *Vs* GSI: $R=0.29$, $N=47$, $P<0.05$). Relative anal gland area also correlated 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 was also found between T and 11KT ($R=0.82$, $N=9$, $P=0.007$). In the GT parasitic males also had larger relative anal gland areas ($R=0.91$, $N=8$, $P=0.002$). In females and transition males no correlations were 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 effect of androgens in stimulating the development of SSCs has been demonstrated in teleost fish: in the midshipman *Porichthys notatus*, for example, the exogenous administration of androgens produced an increase in the sonic muscle (a structure is used to produce a sound that attracts females) in courting males (Brantley et al., 1993a).

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 et al., 2007; Oliveira et al., 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, signaling both intra- and inter-sexually. The high competition among males for access to and maintenance of nests should favor 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

Table 1
Inter-populational comparison in morphological and hormonal variables.

										Planned comparisons (RF *vs* GT)			
		Nest holders		Transition males		Parasitic males		Females		Nest holders	Transition males	Parasitic males	Females
		RF	GT	RF	GT	RF	GT	RF	GT	(*t*, *P*)	(*t*, *P*)	(*t*, *P*)	(*t*, *P*)
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	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 anal gland area	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 testicular gland 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				

GSI: gonado-somatic index; SL: standard length; RF: Ria Formosa; GT: Gulf of Trieste. Test differences at $P<0.05$ are marked with an asterisk.

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 favor males in the ecological setting of the RF population. Chemical signals released by the anal gland attract females that use this putative pheromone to locate males at a distance (Barata et al., 2008; Gonçalves et al., 2002), so there should be competition among nesting males to signal stronger and farther away from their nests, which would lead to more developed anal gland at RF.

Altogether, the available data suggest that higher male intrasexual 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 SSCs. 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 et al., 1990).

Blenny gonads exhibit accessory organs that are involved in sperm maturation and pheromone production (Oliveira et al., 2009). The testicular gland in particular is responsible for sperm storage and maturation and is the main gonadal steroidogenic site for systemic circulation (Oliveira et al., 2001a; Reinboth and Becker, 1986). The fact that nest holders and transitional males from RF have larger relative testicular gland areas than GT males is consistent with the androgen-producing role of this structure. The 11KT 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 testicular gland.

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 favor the appearance of SSCs (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 behavioral profiles in the two populations, the hypothesis of a regulatory function of T in female behavior should not be discarded.

The sneaker-like males from GT have higher GSIs than those from RF, indicating higher sperm competition at GT (Stockley et al., 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., unpublished data). Since there is no female behavioral 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 SSCs usually appear, 3) female-mimicking behavior and coloration, 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., unpublished data), 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., unpublished data), the presence of condition-dependent parasitic tactic in smaller males is somehow surprising, suggesting that the pay-off matrix for

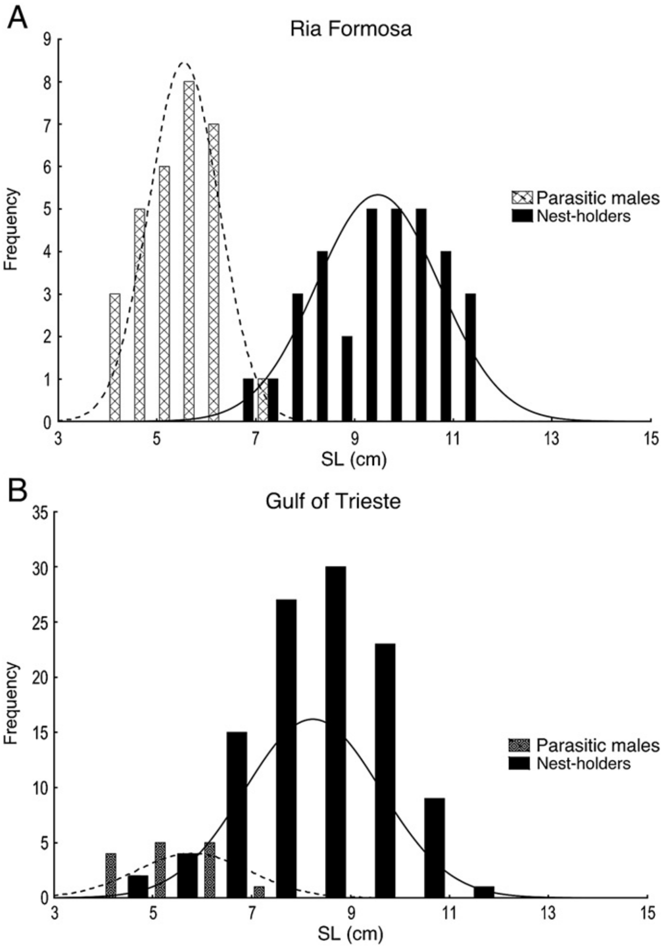

Fig. 1. (A) Histogram of size class frequencies of nest holders and parasitic males at Ria Formosa. Lines represent models of normality. (B) Histogram of size class frequencies of nest holders and parasitic males at Gulf of Trieste. Lines represent models of normality.

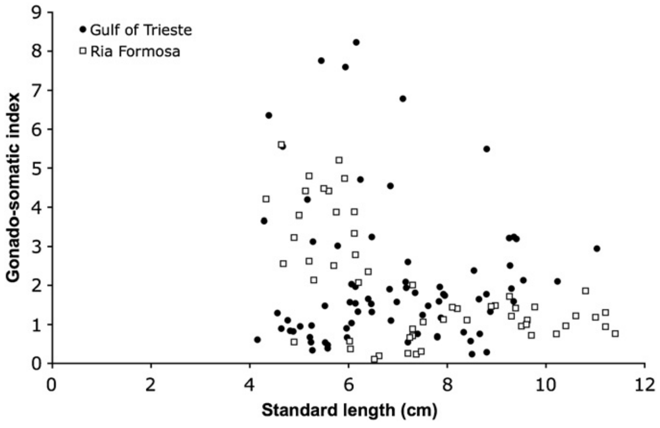

Fig. 2. Scatterplot of standard length vs gonado-somatic index (testes weight / eviscerated weight × 100) of all male morphs in both populations.

smaller males is favorable to the sneaker tactic in comparison to nest defense. A possible explanation may be that these males mature too late in the season to grow SSCs 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 SCCs.

It is interesting to note that GSIs from both nest holders and parasitic males from GT are higher than at RF (Table 1; 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 nest 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 neighbors;
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 increase the probability of a successful spawning.

In conclusion, our data indicate that differences in mating behavior 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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