

Alternative male reproductive tactics and the immunocompetence handicap in the Azorean rock-pool blenny, *Parablennius parvicornis*

Albert F. H. Ros^{1,*}, Niels Bouton², Ricardo S. Santos² and Rui F. Oliveira¹

¹Unidade de Investigação em Eco-Etologia, Instituto Superior de Psicologia Aplicada, Rua Jardim do Tabaco 34, 1149-041 Lisboa, Portugal

²Departamento de Oceanografia e Pescas, Universidade dos Açores, 9901 862 Horta, Portugal

In the Azorean rock-pool blenny (*Parablennius parvicornis*) reproductively active males display alternative morphotypes, which differ in the expression of secondary sexual characters (SSC). Males expressing SSC, the M+ morphotype, have high androgen levels and compete for crevices that will be visited by females to spawn. M+ males holding nests court females and care for the eggs. Males with low expression of SSC, the M- morphotype, have low levels of androgens and reproduce by stealing fertilizations from the M+ males. Based on the hypothesis that androgens are immunosuppressive, we expected these morphotypes to differ in immunocompetence. To test this hypothesis, we conducted a field study in which we collected repeated blood samples to monitor leukocyte populations (blood smears), and to measure the primary antibody response of males that were experimentally challenged with a foreign non-pathogenic antigen (sheep red blood cells). Circulating levels of 11-ketotestosterone and testosterone were higher in M+ males than in M- males. Neither granulocyte nor thrombocyte counts did covariate with androgens or male tactic. In contrast, lymphocyte counts and humoral antibody response were negatively correlated with body size, and as expected, both were lower in M+ than in M- males. Interestingly, in M+ males androgen levels decreased after immunization, and this was less in nest-holder males than in M+ males that were floating around in the pools. Within each morphotype we found no relationship between androgens and immunocompetence. The latter result is not supportive for androgen regulated immunosuppression in M+ males. A possible alternative is enhancement of immunity in M- males. These males had relatively high levels of injuries in comparison with M+ males. High immunity might be a consequence of high infection rate because of such injuries.

Keywords: alternative reproductive tactics; leukocytes; 11-ketotestosterone; testosterone; sheep red blood cells; haemagglutination

1. INTRODUCTION

In many teleost fishes, it is essential for males to attain relative big size and fully developed secondary sexual characters (SSC) in order to compete for primary access to reproduction (e.g. Côté & Hunte 1989; Oliveira *et al.* 2000; Taborsky 2001). In some species, less competitive males successfully reproduce by adopting alternative tactics in which they steal fertilizations from resource holding males (parasitic versus bourgeois males *sensu* Taborsky 1997). For example, small parasitic males may move between nests of the bourgeois males and sneak into these nests to release their sperm ('sneaker' males), or they may associate with a particular nest and wait for an opportunity to enter this nest and release sperm ('satellite' males; Santos & Almada 1988; Taborsky 1994; Oliveira *et al.* 2002a). Teleost fishes are particularly rich in such alternative reproductive tactics (Taborsky 1994, 1998).

In species with sequential reproductive tactics, the transition between tactics can be mediated by social factors through a cascade of internal processes (e.g. Semsar & Godwin 2003; Scaggiante *et al.* 2004). One possible process is a change in circulating androgen levels,

which are known to be regulated by social stimulation (Wingfield *et al.* 1990; Oliveira *et al.* 2002b). In fact, 11-ketotestosterone (KT), which is the most potent androgen among teleosts (Borg 1994), shows higher levels in bourgeois than in parasitic males, and experimental manipulations of KT levels in parasitic males induce the development of SSC typical for the bourgeois phenotype (reviewed in Brantley *et al.* 1993; Oliveira 2005). Less clear is the effect of androgens on the behavioural changes associated with tactic switch (Semsar & Godwin 2003; Oliveira 2005). However, in bourgeois males androgens have been shown to facilitate the expression of courtship and aggressive behaviours (e.g. Kindler *et al.* 1991; Salek *et al.* 2001; Páll *et al.* 2002; Ros *et al.* 2004; Oliveira *et al.* 2005).

Different levels of androgens in alternative male types might indicate the occurrence of differential costs associated with the alternative tactics, since androgens have been proposed to be immunosuppressive (Folstad & Karter 1992; but see Roberts *et al.* 2004 for a critical review in non-teleost species). Androgen regulated immunocompetence has been shown most convincingly in salmonids in which male-male competition, androgen levels and mortality are elevated during the reproductive

* Author for correspondence (aros@ispa.pt).

period (Gross 1984; Slater & Schreck 1998). In these species, testosterone (T) treatment has been shown to suppress immunocompetence (Slater *et al.* 1995a), and a specific androgen receptor has been identified on leukocytes (rainbow trout, *Oncorhynchus mykiss* and Chinook salmon, *Oncorhynchus tshawytscha*: Slater *et al.* 1995b; Slater & Schreck 1998). *In vitro* treatment of leukocytes with T either killed lymphocytes or decreased their antibody production (Slater *et al.* 1995a; Slater & Schreck 1997). Such androgen-regulated immunosuppression might be a functional mechanism to free resources required for reproduction (Wedekind & Folstad 1994; Kortet *et al.* 2003).

The Azorean rock pool blenny, *Parablennius parvicornis* (Vallenciennes 1836) is a relatively long-living species, reaching ages of more than 6 years and showing two distinct male morphotypes expressing alternative reproductive tactics (Santos 1985; Santos & Almada 1988; Santos *et al.* 1995; Oliveira *et al.* 2001a). Males of the M- morphotype have a small genital papilla and undeveloped anal glands while these are large and developed in males of the M+ morphotype (Santos 1995). Generally, males first express the M- morphotype and switch to the M+ morphotype during the second or third year and this is associated with an increase in KT levels (Oliveira *et al.* 2000, 2001b). Males of the M- morphotype may use two tactics to reproduce. Either they act as satellites attached to the territories of nest-holder males and helping to defend these territories against conspecifics while occasionally entering the nest to release sperm, or alternatively they act as sneakers, visiting various nests and trying to steal fertilizations without acting as satellites to any of the nests (Santos & Barreiros 1993; Oliveira *et al.* 2002a). However, some doubt has been raised about whether these alternatives might be considered different tactics (Oliveira *et al.* 2001a). Within the M+ morphotype a distinction is made in two ethotypes: nest-holder males which defend a territory with a cavity for brooding; and floaters which do not care for eggs and roam around in the pools (Santos 1985; Santos & Barreiros 1993). In comparison to nest-holder males, the floater males and M- males are not submitted to the high energetic stress of simultaneously showing parental care and territorial behaviour and M- males suffer less loss in body condition during the reproductive season than M+ males (Santos & Barreiros 1993; Santos 1995). However, M- males risk injuries as a result of aggressive interactions with other parasitic males and with the nest-holders (Santos & Nash 1996).

Our aim was to study how alternative reproductive tactics interact with immunocompetence and whether such interaction is correlated with the behavioural and hormonal differences between alternative male morphotypes. This article addresses the questions whether males of different tactic differ in immunosuppression, and whether these differences are correlated with differences in levels of androgens. To address these questions we have conducted a field study in an Azorean population of *P. parvicornis*. We used two techniques to measure immunocompetence. Firstly, blood smears were collected to monitor different cell populations of leukocytes in the blood. Since high counts of leukocytes could reflect the status of infections, we also counted the number of injuries of each subject. Secondly, males were immunized with

sheep red blood cells (SRBC) to measure their primary antibody response to this foreign non-pathogenic antigen.

2. MATERIAL AND METHODS

(a) *Study area and subjects*

This study was conducted on *P. parvicornis* males in rock intertidal pools (maximum depth at low tide 0.75 m) on a flat basaltic intertidal platform at Feteira on the south coast of Faial Island, Azores (38°31' N; 28°27' W). Blood collection and immunization were carried out in the field during the breeding seasons (June–July) of 2002 and 2003. To characterize males, the following measurements were taken: body mass (to the nearest 0.1 g), standard length, the length and width of the genital papilla and the diameter of the first anal gland (to the nearest 0.1 mm).

All subjects (2002: $N=51$, 2003: $N=80$) were individually tagged with plastic beads inserted at the base of the dorsal fin (as in Oliveira *et al.* 2001b) and were returned to the place of capture. Six males died due to experimental procedures. All other males resumed to normal activities (foraging, nest-defence, fanning) within minutes after release. During low tide the pools were scanned for marked subjects and 82% of the tagged subjects were spotted more than once in the pool area. This is likely to be an underestimate of the percentage of subjects that roamed around in the area since four subjects were spotted more than 10 m away from the pool of capture and some were spotted in the sub-tidal zone, where it was difficult to observe and catch them. Eighty-four per cent of the subjects stayed in the pool of capture until the end of the experiment. Re-capture rate over 12 days (± 1 day) was 64%.

(b) *Sampling procedures*

Fish were captured with a hand net during low tide. Immediately after catching fish were anaesthetized with MS222 (tricaine methanesulfonate, Sigma-Aldrich; dilution 1 : 10 000, 1 min) and a blood sample was drawn from the caudal vein with a 1 ml heparinized syringe fitted with a 25 g needle. Subjects were then examined for the presence of fungi and injuries on the skin. The number of cuts on the body was recorded according to Santos & Nash (1996), which considered such cuts a consequence of the aggressive encounters of M- males while they are helping defending a territory of a nest-holder. Additionally, wounds may be inflicted by nest-holder males when M- males try to establish themselves as satellite males on nest-holder territories or when they attempt to sneak into the nest of a nest-holder to release sperm (e.g. Santos & Barreiros 1993). In addition, we recorded all scratches on the body that were likely to have been inflicted during such agonistic interactions. Scratches were characterized by a series of parallel superficial cuts. These scratches were likely the result of intra-specific fighting, since the width and length matched the size and dental pattern of the mouth of the species. Predator species like moray eels were observed to inflict deep cuts rather than scratches.

Blood was placed in 1 ml Eppendorf tubes and kept on ice before it was taken to the laboratory for further processing. This was done because occasional rainy and stormy weather prevented us from processing blood samples in the field.

(c) *Leukocyte counts*

In 2003 blood smears were made of the first blood sample of each male that was caught ($n=80$). Directly after returning to

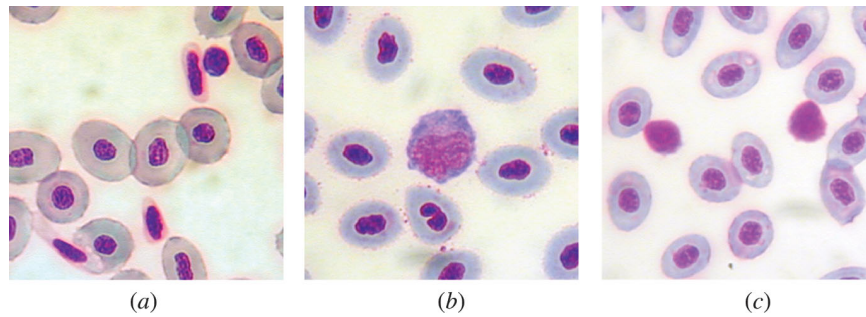


Figure 1. Examples of the different cell types in blood smears of *P. parvicornis*. (a) Erythrocytes, a lymphocyte and several thrombocytes; (b) erythrocytes with a granulocyte; (c) erythrocytes with two lymphocytes.

the laboratory (within 1–3 h after taking the blood samples) a drop of blood was smeared over a slide and dried on air. Blood smears were stained for 20 min with Giemsa's azur eosin methylene blue solution (Merck KGaA, Germany) at 1 : 10 dilution in distilled water. Blood smears were observed under a microscope (BX-50, Olympus, Japan) at 100 \times magnification. For each subject 25 fields were counted from randomly chosen places on the smear (these contained about 150 cells per field). The following cells were counted (figure 1): (i) erythrocytes: elongated, elliptical cells with an oval, centrally located nucleus. These cells were counted to calculate relative numbers of leucocytes; (ii) thrombocytes: oval or spindle-shaped cells. The primary function of these cells is haemostasis, while some evidence exist that thrombocytes play a role in phagocytosis and inflammation (Meseguer *et al.* 2002); (iii) granulocytes: large spherical cells of which the cytoplasm contains numerous fine granules. The primary function of these cells is phagocytosis; (iv) lymphocytes: small spherical cells with little cytoplasm. These comprise a variety of cells involved in the production of specific antibodies, like T and B cells. The mean within subject coefficient of variation for the relative counts of leukocytes was 4.1%.

(d) Measurement of antibody titres

Sheep red blood cells (SRBC) from one donor sheep were collected and used as antigens. The cells were washed three times in phosphate-buffered saline (PBS) and re-suspended in PBS at 5×10^8 cells ml⁻¹ (2% SRBC). In 2002 and 2003, each subject ($n=131$) was immunized with an intraperitoneal injection of a life antigen suspension. A two-way analyses of covariance with year as independent factor did not indicate significant differences between years ($F_{1,75}=2.17$, $p=0.15$) and thus we pooled the data of both years for further analyses. The amount of SRBC suspension was adjusted to the body mass of the fish (0.1 ml SRBC suspension per 50 g body mass, Aitken & Parry 1974). Fish were recaptured 12 days (± 1 day) later and a second blood sample was drawn for measuring the specific antibody response to SRBC ($n=80$). For the estimation of antibody levels, the following procedure was used. To prevent lyses of sheep red blood cells by complement, the plasma was heated to 56 °C for 30 min (Collazos *et al.* 1994). Thereafter, plasma was diluted 1 : 1 in PBS and then serially diluted in PBS in U-shaped microtitre plates. An equal volume of 0.2% SRBC was added to these dilutions, and the plates were incubated at 37 °C for 60 min. Antibody titres were scored visually as the highest twofold dilution of plasma showing haemagglutination.

To save plasma for hormonal analyses, the plasma of 15 subjects, which we could not recapture, was tested for naturally occurring antibodies. None of these subjects

showed measurable haemagglutination titres to SRBC. In order to check whether blood sampling influenced haematocrit values in subsequent blood sampling we used the following method: Eppendorf vials were marked and weighed at the nearest 0.01 g. After blood collection, Eppendorf vials were measured again, first with whole blood and a second time after centrifuging and taking of the plasma fraction with only the red blood cell fraction. This allowed us to calculate the weight percentage of red blood cells in the blood. Using this method we collected data of 67 initial blood samples and of 27 matched initial and final blood samples. Haematocrit values showed a non-significant decrease from 40 to 37% of red blood cells (matched pairs t -test, $t_{26}=1.78$, $p=0.087$). Haematocrit values did not differ between males of different morphotype (t -test, M+ versus M-, $t_{66}=1.29$, $p=0.20$).

(e) Radioimmunoassays

Plasma was stored at -20 °C. To assess circulating levels of androgens, the free and not conjugated steroid fraction was extracted with diethyl ether from plasma using the methodology described in Scott & Vermeirssen (1994). Steroid residues were re-suspended in 1 ml assay buffer and stored again at -20 °C until assayed for T and KT. The radioimmunoassays and cross reactions for T and for KT were described, respectively, in Scott *et al.* (1984) and Kime & Manning (1982). Intra- and inter-assay coefficients of variation were 7.5 and 12.4% for T, and 8.2 and 11.6% for KT.

(f) Data analyses and statistics

The following morphometric indexes were calculated: condition index (K) = body mass \times total length⁻³; the genital papilla area was calculated assuming a rectangular shape (for comparison with Oliveira *et al.* 2001b). After transformation, distributions of the data for body measurements (using log transformation) and blood cell percentages of the different leukocytes (using arcsin transformation) did not significantly deviate from normality (Kolgomorov–Smirnov, $p>0.10$) and parametric statistics were used. Measurements of SRBC antibody titres, anal gland length and androgen levels showed skewed distributions that were significantly deviating from normality (Kolgomorov–Smirnov, $p<0.01$). For statistical testing of these parameters we used non-parametric statistics (e.g. table 1). Where possible Spearman was used for testing simple correlations. ANCOVA was used to test for differences between tactics while correcting for allometric relationships. In all presented ANCOVA analyses homogeneity tests did not reject equality of error variances across groups (Levine's tests: $p>0.12$). All analyses were carried out with the SPSS 11.0 package (SPSS Inc., Chicago, USA) and p -values represent two-tailed probabilities.

Table 1. Comparison of morphological and immune parameters between *P. parvicornis* males of different tactics. (*F* statistics apply to ANOVA analyses (Levine's tests: $p > 0.08$), χ^2 statistics apply to the Kruskal–Wallis test. Small letters refer to *post hoc* Newman–Keuls tests with different letters notifying significant differences between groups ($p < 0.05$).

morphotype		M–			M+						one-way ANOVA	
		satellite			nest-holder			floater			statistics	<i>p</i>
ethotype		mean	s.e.	<i>n</i>	mean	s.e.	<i>n</i>	mean	s.e.	<i>n</i>		
total length	TL (cm)	11.1 ^a	0.2	51	13.9 ^b	0.2	34	13.6 ^b	0.4	21	<i>F</i> =44.04	<0.001
body mass	<i>W</i> (g)	14.8 ^a	0.7	51	26.0 ^b	1.3	34	24.9 ^b	2.2	21	<i>F</i> =33.32	<0.001
condition index	$W \times TL^{-3} \times 100$	1.06 ^a	0.02	51	0.95 ^b	0.02	34	0.95 ^b	0.02	21	<i>F</i> =12.90	<0.001
anal gland length	mm	1.57 ^a	0.05	51	5.21 ^b	0.17	34	4.05 ^c	0.19	21	$\chi^2=82.53$	<0.001
papilla area	mm ²	5.3 ^a	0.3	51	14.0 ^b	0.9	33	10.0 ^c	0.6	21	$\chi^2=60.27$	<0.001
KT 1st sample	ng ml ⁻¹	0.05 ^a	0.01	43	1.87 ^b	0.32	26	2.04 ^b	0.67	10	$\chi^2=53.87$	<0.001
KT 2nd sample	ng ml ⁻¹	0.06 ^a	0.02	26	0.68 ^b	0.20	21	0.14 ^b	0.07	7	$\chi^2=19.60$	<0.001
T 1st sample	ng ml ⁻¹	0.30 ^a	0.04	43	0.92 ^b	0.15	26	0.63 ^{ab}	0.23	10	$\chi^2=20.36$	<0.001
T 2nd sample	ng ml ⁻¹	0.06 ^a	0.02	26	0.54 ^a	0.27	21	0.04 ^a	0.00	7	$\chi^2=4.20$	0.11
thrombocytes	%	0.14 ^a	0.02	44	0.14 ^a	0.04	26	0.13 ^a	0.04	10	<i>F</i> =0.57	0.57
granulocytes	%	0.20 ^a	0.02	44	0.21 ^a	0.04	26	0.17 ^a	0.03	10	<i>F</i> =0.20	0.82
lymphocytes	%	2.64 ^a	0.23	44	1.05 ^b	0.12	26	1.39 ^b	0.27	10	<i>F</i> =21.04	<0.001
antibody response	log ₂	1.76 ^a	0.29	33	0.55 ^b	1.06	29	1.44 ^a	1.65	18	$\chi^2=11.79$	0.002
cuts	count	0.67 ^a	0.12	51	0.42 ^b	0.12	33	0.48 ^a	0.15	21	<i>F</i> =0.94	0.39
scratches	count	5.49 ^a	0.43	51	1.42 ^b	0.41	33	2.62 ^b	0.50	21	<i>F</i> =27.89	<0.001
total injuries	count	6.16 ^a	0.50	51	1.85 ^b	0.46	33	3.10 ^b	0.52	21	<i>F</i> =24.21	<0.001

3. RESULTS

(a) *Within morphotype correlations*

About 6% of the leukocytes had typical variable spindle-shaped cell structures and were categorized as thrombocytes (figure 1*a*). Within morphotype no significant correlations were found between thrombocyte count and body size condition index, androgen levels or number of injuries (figure 2; all comparisons $|r_s| < 0.27$, $p > 0.10$). Nine per cent of the relative leukocyte population in the blood were classified as granulocytes (figure 1*b*). Less than 0.1% of the leukocytes had monocyte-like appearance, but since classification of these cells was difficult we pooled them with the granulocytes. Within each morphotype no significant correlations were found among blood cell percentages of these cells and body size, condition index, or number of injuries (figure 2; all comparisons: $|r_s| < 0.30$, $p > 0.07$). In M– males granulocyte count showed a positive correlation with T ($r_s = 0.33$, $n = 43$, $p = 0.032$) but not with KT ($r_s = 0.068$, $n = 43$, $p = 0.67$). No such significant correlation was found in M+ males (all comparisons: $r_s < 0.086$, $n = 36$, $p > 0.6$). Lymphocytes constituted the largest population of leukocytes in the blood (85%). In both morphotypes a negative correlation was found with body size (figure 2; linear regression over transformed data; M–: $r = -0.42$, $n = 44$, $p = 0.0045$; M+: $r = -0.38$, $n = 36$, $p = 0.022$). Within each morphotype, neither condition index nor the number of injuries was significantly correlated with lymphocyte counts (all comparisons: $|r_s| < 0.15$, $p > 0.35$), or androgen levels (figure 2; all comparisons: $|r_s| < 0.086$, $p > 0.62$).

Antibody titres, quantified by means of the haemagglutination assay, were not significantly correlated with body size, condition index, androgen levels or number of injuries (all comparisons $|r_s| < 0.30$, $p > 0.12$).

(b) *Comparisons between alternative male tactics*

Blood plasma levels of androgens were significantly higher in M+ males than in M– males (Kruskal–Wallis test: KT: $\chi^2 = 53.87$, $p < 0.001$; T: tactic, $\chi^2 = 20.36$, $p < 0.001$; see figure 2). Posthoc Bonferroni tests indicated that M– males had lower androgen levels than M+ males ($p < 0.05$), whereas within M+ males no difference was found between floater and nest-holder males ($p > 0.2$).

Total length ranges showed considerable overlap between males of the different morphotypes (M–: 8.1–13.8 cm; M+: 10.6–17.4 cm), and M+ males (nest-holder and floater) were significant larger than M– males (*t*-test, $t_{77} = 4.55$, $p < 0.001$). Therefore, to compare different male types while statistically correcting for possible allometric relationships, we carried out analyses of covariance with total length as cofactor and tactic as the independent variable (three levels: M–, floater, nest-holder). This analysis showed a significant effect of male tactic on the number of injuries (figure 2; ANCOVA: tactic, $F_{2,101} = 20.76$, $p < 0.001$; total length, $F_{1,101} = 3.35$, $p = 0.070$). Posthoc Bonferroni tests indicated that M– males had more injuries than floater or nest-holder males ($p < 0.001$), whereas the latter two male types did not differ significantly ($p > 0.08$).

Lymphocyte count differed significantly among alternative tactics (table 1; ANCOVA: tactic, $F_{2,76} = 3.39$, $p = 0.039$; total length, $F_{1,76} = 13.89$, $p < 0.001$). *Post hoc* Bonferroni tests indicated a difference between nest-holder and M– males ($p = 0.04$), while floater males did not differ significantly from the other two groups ($p > 0.29$). Neither thrombocyte nor granulocyte counts showed significant differences among alternative tactics (table 1; ANCOVA: tactic, $F_{2,76} < 0.52$, $p > 0.6$; total length, $F_{1,76} < 0.28$, $p > 0.6$).

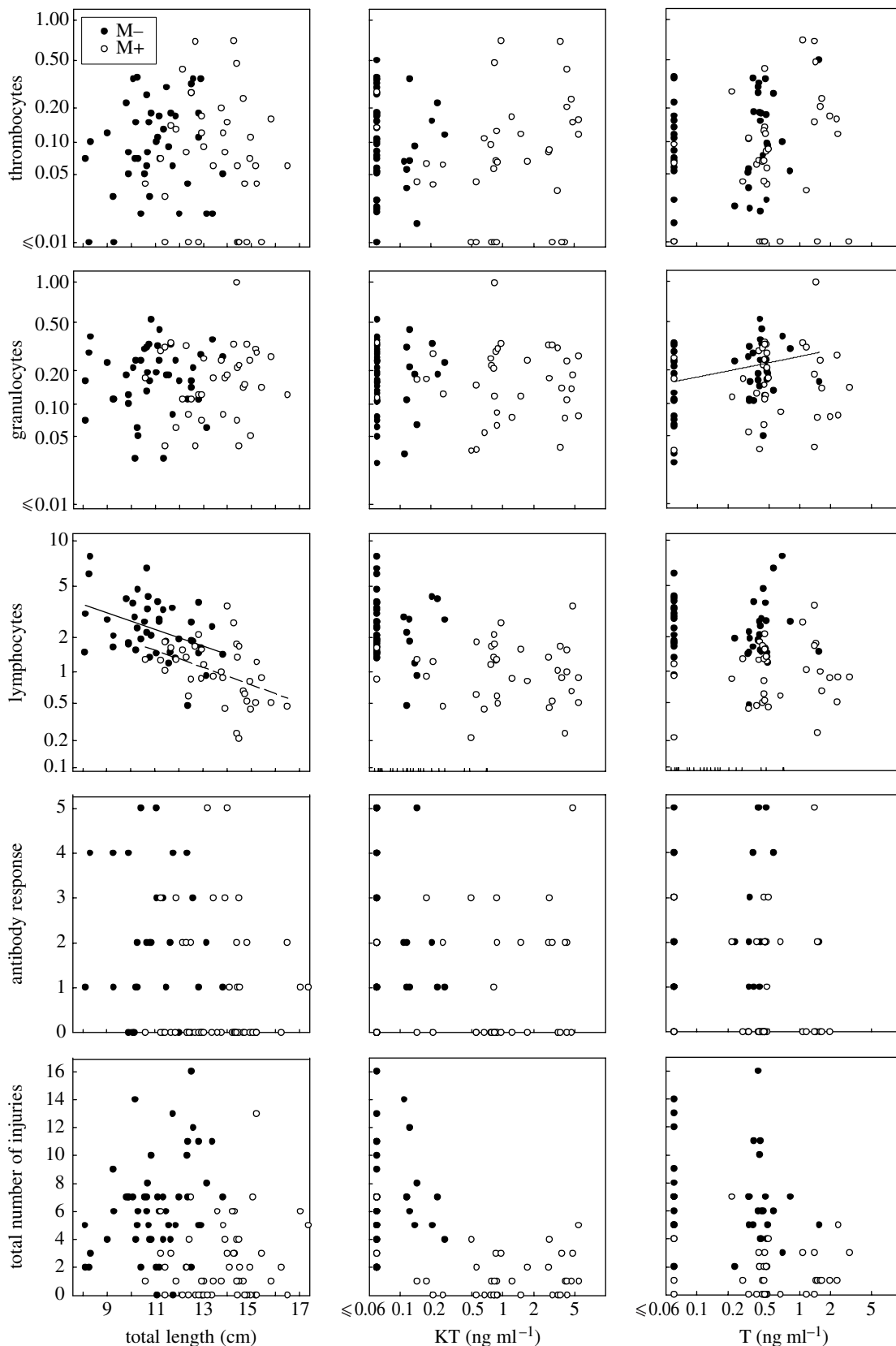


Figure 2. Correlations between individual scores of leukocyte counts (percentage of total blood cell count), the antibody response to SRBC immunization, or incidence of injuries (cuts and scratches), and total length or the level of androgens (KT and T) in the two morphotypes of *P. parvicornis*. M-: reproductively active males that did not express developed secondary sexually characters; M+: reproductively active males are males that fully expressed secondary sexual characters. Regression lines were drawn in cases where the correlation was tested significant.

A two-way repeated ANOVA (factors tactic (three levels: M-, floater, and nest-holder); sampling time (two repeated levels: initial and final samples)) was carried out to test changes in androgen levels between the samples

drawn before and after SRBC immunization (table 1). Plasma levels of KT and T were significantly lower after immunization in both floater and nest-holder males (factor sampling time: $F_{1,51} > 68.3$, $p < 0.001$). After

immunization, androgen levels decreased in all tactics, but decreased significantly more in floater than in nest-holder males. This difference was only significant for the levels of KT (KT: interaction tactic and time, $F_{2,51}=52.8$, $p<0.001$; T: interaction tactic and time, $F_{2,51}=1.9$, $p=0.16$), and *post hoc* Newman–Keuls tests indicated that after immunization KT levels were still increased in nest-holder males, while no significant difference existed between M– and floater males.

The haemagglutinin response to SRBC immunization was shown by 24.1% ($n=29$) of the males showing the nest-holder tactic, significantly less than in the non-parental floater males and M– males in which about two thirds of the animals responded to immunization (respectively, 61.1%, $n=18$; 69.7%, $n=33$; $\chi^2_2=13.7$, $p=0.0010$, for absolute values see table 1). Absolute antibody titres within responding animals did not differ between tactics (mean \pm s.e. = 2.44 ± 0.20 ; Kruskal–Wallis ANOVA: $\chi^2_2=0.18$, $p=0.9$), nor did responding males differ in body size compared to males not showing this response (*t*-test; M–: $t_{31}=1.41$, $p=0.16$; M+: $t_{46}=0.77$, $p=0.44$).

4. DISCUSSION

(a) *Alternative male tactics and immunocompetence*

In an increasing number of species it has been demonstrated that sex steroid levels and behavioural traits do not only show inter-sexual but also distinct intra-sexual differences (for recent reviews see Rhen & Crews 2002; Knapp 2004). The aim of this paper was to determine whether intra-sexual differences occur in immunocompetence in a species with alternative reproductive tactics, the Azorean rock pool blenny. Such a difference was expected based on two facts: (i) androgen levels differ between alternative male morphs, being higher in the bourgeois tactic (Oliveira *et al.* 2001b); (ii) androgens have been found to be immunosuppressive (Folstad & Karter 1992; Slater *et al.* 1995a). In accordance with this expectation lymphocyte count and antibody responsiveness were found to be elevated in males expressing the parasitic tactic (M– morphotype) in comparison with males expressing the bourgeois tactic (nest-holder males of the M+ morphotype). No such difference was found for thrombocytes and granulocytes. This suggests that males of different tactics also differ in their capacity to mount ‘specific’ immune responses (i.e. acquired immunity) but are comparable in their ability to show the ‘non-specific’ type of defence to pathogens (i.e. innate immunity).

P. parvicornis males typically showed many injuries on their body and fins, which were likely to be a consequence of aggressive encounters either with conspecifics during territorial interactions, or with nest-holder males during sneaking attempts (see also Santos & Nash 1996). Since males in *P. parvicornis* have small home ranges and population density is high (Santos 1985; Santos *et al.* 1994), it is conceivable that such injured males are repeatedly exposed to the same types of pathogens. Acquired immunity, although usually acting together with innate immunity, is an efficient system in quickly responding and neutralizing such repeated exposures to pathogens. Thus, elevated levels of lymphocytes and

humoral immune responsiveness may importantly contribute to survival of injured males.

M+ males are exposed to injuries during a relatively short period at the start of their reproductive period when they fight over crevices in the rocky substrate (Santos & Nash 1996). Once most territories of M+ males (i.e. nest-holders) are established, M+ males are less exposed to injuries than the territorial M– (satellite) males which have exposed territories in front of the crevices of nest-holder males and which engage in sneaking activities. During our sampling period, most nest-holder males and many floaters did not show any injury. It may come to mind that under the condition in which such males are not parasitized at the start of the reproductive season, low immunocompetence does not necessarily compromise their survival. Our data show that M+ (in particular nest-holder) males had low specific immunity but comparable levels of thrombocytes and granulocytes to those of M– males. Moreover, lymphocyte numbers negatively correlated with body size and, therefore, with competitive ability of the males (Oliveira *et al.* 2000), while neither thrombocytes nor granulocytes showed such a correlation. Because thrombocytes mediate wound healing by blood clotting, and granulocytes detect and neutralize a wide range of antigens, these cells are important in the first line of protection of the organism to invasion by pathogens. Furthermore, some evidence exists in birds that individuals compromised with one of the two components of immunity (i.e. specific versus non-specific) may try to compensate by switching their immune response to the other component (reviewed by Norris & Evans 2000).

Thus, M– males might have used both cellular non-specific and specific mechanisms to cope with invasions with pathogens, while M+ males might rely more on avoiding infections either by being less exposed to injuries (as a side effect of staying inside crevices brooding eggs) or by cellular non-specific mechanisms. Nest-holder males carry out both parental and territorial activities, which do not allow them to forage far from their nest. In contrast, M– males can often be observed foraging. Both elevated behavioural activity and limited forage opportunity make it functionally important to downregulate specific immunity in order to shunt energetic resources to reproductive activities. Studies mainly on birds have shown that humoral and cellular immunocompetence are costly (Martin *et al.* 2003) and trade-off with reproduction (Sheldon & Verhulst 1996; Deerenberg *et al.* 1997; Norris & Evans 2000; Cichoń *et al.* 2001). Despite the assumed implications for reproduction, surprisingly little is known about the costs associated with the maintenance of immunocompetence in fish. R. F. Oliveira and co-workers found that in *P. parvicornis* at the end of the reproductive season M+ males did show more parasites than M– males (66.7 versus 43.8%, respectively, R. F. Oliveira 1999, unpublished data). The latter data suggest that long lasting suppression of specific immunocompetence might compromise survival.

(b) *The immunocompetence handicap*

Testosterone has been proposed to be a causal link between changes in immunocompetence and the reproductive status of an animal (‘immunocompetence

handicap hypothesis': Folstad & Karter 1992; see also Slater *et al.* 1995b; Skarstein & Folstad 1996). In *P. parvicornis*, the switch from the M- to the M+ morphotype involves the development of SSC like the head-hump and pheromone producing anal glands, which have been shown to be mediated by an increase in KT (Oliveira *et al.* 2001b,c) but not T (A. F. H. Ros & R. F. Oliveira 2004, unpublished data). At the time of first sampling plasma circulating levels of T and KT were elevated in males of the M+ morphotype. The measurements of specific immunity (lymphocyte counts and antibody responsiveness) follow the expectation based on the immunocompetence handicap hypothesis if compared at the level of male alternative tactics. However, neither of our measures of immunocompetence showed the expected negative correlation with actual circulating levels of androgens within each of the tactics. This might be explained in one of the following ways. Androgens modulate immunocompetence, but changes in circulating levels of androgen are more rapid than changes in immunocompetence; or alternatively, androgens do not modulate immunocompetence, but other mechanisms associated with the different life styles of males expressing different reproductive tactics downregulate immunocompetence. One of these mechanisms might be a direct regulation of lymphocyte activity by the hypothalamic-pituitary-interrenal axis, in particular by cortisol that has been shown to be immunosuppressive (reviewed by Weyts *et al.* 1999).

Within M+ males, nest-holder males were found to be suppressed in their humoral immunity (antibody responsiveness to immunization with SRBC), whereas floater males were not. After immunization, plasma levels of androgens decreased in both types of males but stayed elevated in nest-holder males (especially KT). Floater males are likely more flexible in androgen production than nest-holder males. Nest-holding *P. parvicornis* males vigorously defend a parental territory against intrusions by other males using behaviours that are mediated by KT (Ros *et al.* 2004). Furthermore, exposure to agonistic challenges likely feedbacks to KT production (Hirschenhauser *et al.* 2004). Floater males showed SSC and may challenge nest-holder males but are also often found in parts of the pools where they are not engaged in territorial interactions.

The relationship between androgen hormones and immunocompetence has been most extensively studied in Salmonidae. In particular semelparous salmon of the genus *Oncorhynchus* have dramatic life histories in which reproductive animals seem to allocate all resources to competitive behaviour and spawning, and in which the immune system degenerates (Robertson & Wexler 1960; Robertson *et al.* 1961). T has been shown to suppress the immune response in Chinook salmon, *O. tshawytscha* (Slater & Schreck 1993), and receptors with high affinity to T have been found in the cytosol of leukocytes of this species (Slater *et al.* 1995b; Slater & Schreck 1998). Furthermore, T treatment of *in vitro* cultures of leukocytes reduced both the antibody production of lymphocytes (Slater *et al.* 1995a) and the number of leukocytes (Slater & Schreck 1997). This indicates a direct pathway for T suppressing immunocompetence. Functionally, changes in T levels during the reproductive season have been shown to correlate with changes in energy allocation

(Leonard *et al.* 2002). Less is known about the relationship between levels of KT and immunocompetence in Salmonidae. Binding affinity of KT to leukocytes of rainbow trout (*O. mykiss*) has been shown to be similar to that of T (Slater *et al.* 1995a), although the affinity of an androgen receptor that was isolated from these leukocytes was found to be much lower for KT than for T (Slater *et al.* 1995b).

To our knowledge, the only other teleost in which the relationship between alternative reproductive tactics and immunocompetence has been investigated is the corkwing wrasse, *Symphodus melops* (Uglem *et al.* 2001). Contrary to our results, between tactics in the corkwing wrasse no differences were found in blood lymphocytes. In this species two male morphotypes are present: large territorial males that exhibit male SSC and smaller female mimics that try to sneak fertilizations from the territorial males. These male morphotypes are thought to be fixed strategies in contrast to the sequential morphotypes in *P. parvicornis* in which M- males have been observed to switch to M+ males later in life (Santos *et al.* 1995; A. F. H. Ros & R. F. Oliveira 2003, unpublished observations of individually tagged subjects). In *S. melops*, female mimics in comparison to territorial males were found to have higher levels of T and 17 β -oestradiol while having lower levels KT (Uglem *et al.* 2002). In *P. parvicornis*, M- males in comparison to M+ males have similar to lower T levels and lower KT levels (Oliveira *et al.* 2001a; this study). Interestingly, we also found similar tactic associated differences in the percentage of lymphocytes in males of another blenny, *Salaria pavo* (A. F. H. Ros & R. F. Oliveira 2004, unpublished data), a species that also has flexible alternative reproductive tactics associated with differences both in KT and in T such as those found in *P. parvicornis* (Oliveira *et al.* 2001a). Comparative studies of teleost species living in different social environments with different life history traits might help in understanding how intra-sexual differences in immunocompetence have evolved and what their fitness consequences are.

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