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# Fish & Shellfish Immunology

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## Short communication

# Immune activation is inversely related to, but does not cause variation in androgen levels in a cichlid fish species

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## ARTICLE INFO

### Article history:

Received 20 December 2011

Accepted 5 March 2012

Available online xxx

### Keywords:

Testosterone

11-Ketotestosterone

Immunocompetence

Sexual selection

SRBC challenge

## ABSTRACT

Studies on birds and mammals indicate that sexual traits may signal superior health because active immunity, like inflammatory responses to infections, is suppressive to the production of androgens that facilitate the expression of these traits. Here we test this possible pathway for honest signaling in a teleost species, *Sarotherodon galilaeus*, by activating the immune system with sheep red blood cells (SRBC), which is a non-pathogenic T- and B-cell stimulating antigen. Two weeks after the start of treatment adult males injected with SRBC showed a significant increase in antibody production in comparison with control males. The variation in specific antibody production was negatively related with variation in both testosterone and 11-ketotestosterone levels. This suggests that investment in immune protection is incompatible with increased activity of the hypothalamic-pituitary-gonadal axis. However, opposite to our expectation no difference in androgen levels was found between placebo and SRBC treatment suggesting that immune activation did not cause androgen suppression in our studied species.

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## 1. Introduction

Elevated levels of androgens play an important role in reproduction as they are involved in spermatogenesis and facilitate many sexual displays [1]. In order to propose a mechanism for parasite mediated sexual selection, Folstad and Karter postulated the Immunocompetence Handicap Hypothesis [2,3]. The major premise of this hypothesis is that androgens suppress immunity so that only those individuals with superior immune systems would be able to express the exaggerated sexual traits that play a role in sexual competition. However, there is only moderate support for an effect of androgens on immunosuppression [4–7]. Reversing the chain of causation others have suggested that immune activation suppresses androgen production, which might similarly result in parasite mediated sexual selection of androgen dependent sexual traits as only animals in good health will fully express these sexual traits and sexually compete [8–10]. Suppression of androgen dependent reproductive investments might assist in recovery by

releasing metabolic resources that can be allocated to the healing process [11].

A recent meta-analysis of the effect of immune activation on testosterone levels showed a strong negative effect, but the range of species tested so far is limited to few bird and mammalian species [8]. The aim of the present study was to test this androgen-suppression variant of the immunocompetence handicap hypothesis in a teleost species, the St. Petersfish (*Sarotherodon galilaeus*). We chose this species because it is easy to keep in captivity and because it has been shown that the androgens testosterone and 11-ketotestosterone play an important role during sexual competition [18]. Fish were injected with different doses of sheep red blood cells, which result in a T- and B-cell dependent antibody response but without being pathogenic [25]. We measured changes in androgen levels to test the hypothesis that manipulation of the adaptive immune response in fish would reduce/suppress testosterone levels.

## 2. Methods

Animals at the aquarium facilities of the institute of TG, Haren, the Netherlands were second and third generation offspring of a stock that originated from fish bred at the Experimental Station

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for Aquaculture (Min. of Agriculture, Dept. of Fisheries, Israel) at Lake Kinnereth, Israel. Fish were fed every day with Tetra Pond® Sticks (Tetra GmbH, Melle, Germany), supplemented once per week with a mixture of ground peas and shrimps. Aeration was provided and temperature kept at  $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ . The photoperiod regime was 12L:12D.

Two experiments were carried out with a similar basic design: Two weeks before the start of the experiment, experimental fish (experiment 1,  $n = 20$ , bodyweight =  $69.3 \pm 2.7$ ; experiment 2,  $n = 28$ , bodyweight =  $53.9 \pm 2.6$ , mean  $\pm$  s.e.) were transferred to 750–800 l aquaria with transparent dividers so all fish could interact with conspecifics. At the start of the experiment a pre-immunization blood sample (see 2.2) was drawn of all animals for detecting initial titers of antibodies against SRBC antigens and androgens. A recovery period of 1 week was allowed before the subjects were either immunized with SRBC in saline (the SRBC group) or received the same volume of placebo (the saline group). A post-immunization sample was drawn two weeks after immunization at around the time of the peak of the primary antibody response [19,20]. The effect of immunization was tested by comparing androgen levels of post and pre-immunization plasma samples.

For immunization we used freshly purchased SRBC as antigen (in alsever's solution, Harlan Laboratories B.V., Venray, the Netherlands). These cells were washed three times in sterile saline and suspended with saline to the desired concentration. For intraperitoneal injection, the concentration of the SRBC suspension was adjusted to the body mass of the fish. We based our immunization regime on different concentrations of SRBC used in the literature [17,21]: in the first experiment we used low doses, and in the second experiment we used higher doses. Only at the highest dose fish showed visual signs of a "sickness" syndrome, being relatively immobile with increased respiration rates. This lasted for about one day after immunization. In experiment 1 the dosage was 0.1 ml of a 2% SRBC suspension (about  $0.8 \times 10^8$  cells) per 50 g of body mass [17]. In experiment 2 a high dosage of SRBC was used: 0.3 ml of a 25% SRBC suspension (about  $30 \times 10^8$  cells) per 50 g body mass [21].

According to standard laboratory procedures at the end of both experiments the animals were sacrificed with a lethal dose of anesthesia (1 g/l MS-222) and sex was confirmed by inspection of the gonads.

Blood samples were drawn in the morning hours between 10:00 h and 12:00 h. Fish were gently netted and transferred to a bucket with a general anesthetic (100 mg/l MS-222 buffered with  $\text{CaCO}_3$ ). When fish reached anesthesia (1–2 min), we collected 0.3 ml of blood (about 0.5% of fish weight) from the caudal vasculature, and fish were allowed to recover (5–10 min) before being returned to their original aquarium. Blood was centrifuged at 800 g and plasma stored at  $-70\text{ }^{\circ}\text{C}$  until further processing.

For the estimation of antibody titers in the blood plasma samples, the following procedure was used [22]: To prevent lyses of SRBC by complement, the plasma was heated to  $56\text{ }^{\circ}\text{C}$  for 30 min [23]. Thereafter, 40  $\mu\text{l}$  plasma was diluted 1:1 in sterilized PBS and then serially diluted in PBS in U-shaped micro-titer plates. An equal volume (40  $\mu\text{l}$ ) of 0.2% SRBC was added to these dilutions, and the plates were incubated at  $37\text{ }^{\circ}\text{C}$  for 60 min. Antibody titers were scored visually as the highest twofold dilution of plasma showing hemagglutination. The scores were carried out on plates that allowed for direct comparison between individuals without knowing which dilution series belonged to which group. This was done so to prevent possible observer bias.

The radioimmunoassay for testosterone for experiment 1 and 2 was carried out in the laboratory of TG. In short, 40  $\mu\text{l}$  of plasma was extracted twice with 70% diethyl ether/30% petroleum benzine followed by overnight incubation with 70% methanol. The extract was

dried and dissolved in assay buffer (PBS-G). Testosterone levels were quantitated by means of a commercial competitive binding assay using  $^{125}\text{I}$ -radioactive labeled testosterone as antigen (DSL-4000 kit, Diagnostic Systems Laboratories, Webster, Texas). In our assays, the lower detection level was 0.05 ng/ml. The assay has 100% cross-reactivity with testosterone and low cross-reactivity with other steroids (<6%). Intra- and inter-assay coefficients of variation were 4.9% and 6.1%, respectively.

The radioimmunoassay for 11-ketotestosterone for experiment 1 was carried out at the Endocrinology & Metabolism unit of the Biology department of the University of Utrecht [24]. The effective lower limit of detection for 11-ketotestosterone in individual samples was 0.48 ng/ml plasma. The intra-assay coefficient of variation of a pooled plasma sample containing 10 ng/ml was 13%. All plasma samples were analyzed in a single assay. The individual samples (40  $\mu\text{l}$  of plasma) were diluted with a twofold volume of an aqueous sodium azide solution (0.05%, w/v), incubated at  $80\text{ }^{\circ}\text{C}$  for 1 h to liberate protein-bound steroid hormones and then centrifuged at 14,000 rpm for 30 min at room temperature. The supernatant was harvested and stored at  $4\text{ }^{\circ}\text{C}$ . The same day, 50  $\mu\text{l}$  of the supernatant was analyzed for its content in 11-ketotestosterone using a RIA. The details this analysis and of the antibody specificity and cross-reactivities are given in Schulz [25].

Except for the SRBC antibody response, in which individuals treated with Saline had the expected zero antibody response scores, variables did not significantly deviate from normality (Kolmogorov–Smirnov test, NS) and we used ANOVA to include both experiments in a single model. For testing the SRBC antibody response we used non parametric tests. All statistical analyses were carried out with the SPSS 15.0 package (SPSS Inc., Chicago, USA) and  $p$ -values represent two-tailed probabilities.

In experiment 1, one animal of the SRBC group showed no detectable antibody response (hemagglutination) two weeks after the injection of the antigen. Apparently this animal did not respond to our treatment. Because our aim was to study the effects of activation of the immune system by SRBC on androgen levels, this individual was left out of the analysis.

We followed the CCAC guidelines (<http://www.ccac.ca>). All experiments were carried out with consent of the institutional animal care and use committee of the University of Groningen (DEC license D4335).

### 3. Results

Immunization using SRBC resulted in a dose-dependent activation of specific antibody production two weeks after immunization (Fig. 1; Mann–Whitney  $U$ -test control vs. SRBC group

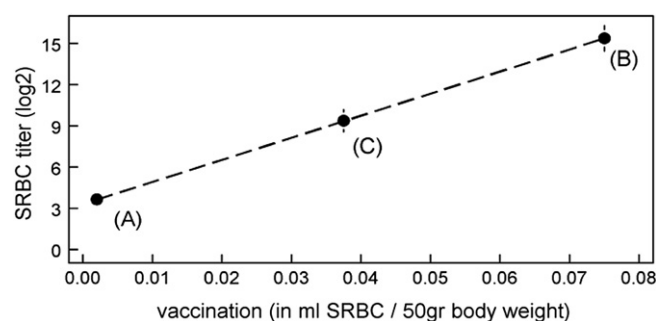


Fig. 1. Relationship between injected dosage of SRBC and the SRBC antibody titer (hemagglutination test: post-minus pre-immunization). Immunization: A = SRBC treatment low dosis in experiment 1, B=SRBC treatment high dosis in experiment 2, C = intermediate dosis that was given to the control group of experiment 2.

immunization experiment 1:  $n_c = 9$ ,  $n_t = 11$ ,  $U = 4.5$ ,  $p < 0.001$ ; experiment 2:  $n_c = n_t = 14$ ,  $U = 0$ ,  $p < 0.001$ ). This activation was long-lasting as repeated sampling in experiment 2 showed that SRBC antibody titers were still detectable 56 days after immunization (day 35:  $13.4 \pm 0.6$  (mean  $\pm$  sem), Mann–Whitney  $n_t = n_c = 14$ ,  $U = 0$ ,  $p < 0.001$ ; day 56:  $8.8 \pm 0.6$  (mean  $\pm$  sem), no control group).

Contrary to expectation, immune activation with SRBC antigen did not have a significant effect on plasma levels of testosterone (Fig. 2; repeated measures ANOVA: SRBC immunization [factors: sample{before vs after immunization}, treatment{control vs SRBC}, nested in experiment], treatment:  $F_{1,45} = 0.003$ ,  $p = 0.96$ , treatment  $\times$  sample  $F_{1,40} = 0.46$ ,  $p = 0.50$ ) or 11-ketotestosterone (only measured in experiment 1; repeated measures ANOVA: SRBC immunization [factors: sample{before vs after immunization}, treatment{control vs SRBC}], treatment:  $F_{1,16} = 0.36$ ,  $p = 0.56$ , treatment  $\times$  sample  $F_{1,16} < 0.001$ ,  $p = 0.99$ ).

Although the SRBC immunization treatment did not affect androgen levels relative to the control animals, within the SRBC group there was a negative relationship between antibody titers and the change in testosterone levels from pre- to post-immunization samples (ANCOVA [factor: experiment, covariate: antibody titer],  $p < 0.001$ ). The slope of this effect was significantly different for the two experiments (ANCOVA interaction:  $p = 0.008$ ) and was not significant in experiment 2 (Fig. 3; Spearman rho, experiment 1:  $r_{10} = -0.86$ ,  $p = 0.0027$ ; experiment 2:  $r_{14} = -0.35$ ,  $p = 0.23$ ). In order to collect more data on this relationship, at the end of experiment 2 the control animals were treated with an intermediate dose of SRBC (see Fig. 1). The results of these extra measurements were in line with the trend shown by experiment 1 and 2: a significant negative correlation between SRBC titers and changes in T-levels with an intermediate slope (Fig. 3; Spearman rho,  $r_{13} = -0.57$ ,  $p = 0.045$ ). Changes in levels of 11-ketotestosterone showed a highly significant positive relationship with levels of testosterone (Experiment 1: Spearman rank correlation:  $r_{19} = 0.70$ ,  $p = 0.0010$ ). As found for levels of testosterone, the relationship between antibody responses and changes in 11-ketotestosterone levels was negative (Experiment 1: Spearman rank correlation:  $r_{10} = -0.62$ ,  $p = 0.054$ ).

#### 4. Discussion

Here we present the first study in a teleost species in which we test the effects of immune system activation on androgen levels via

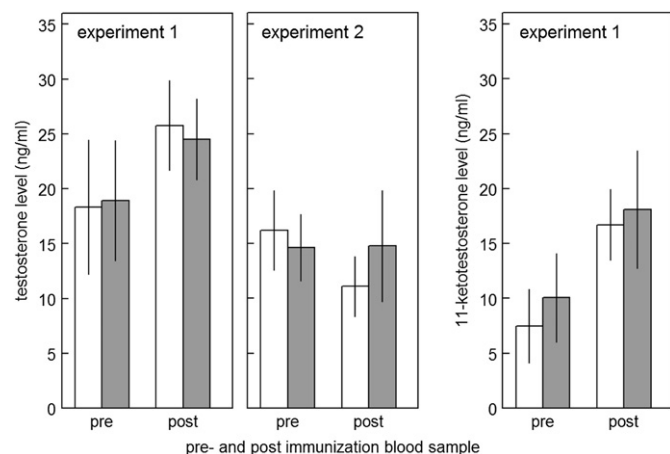


Fig. 2. Effect of the SRBC treatment on blood plasma levels of testosterone and 11-ketotestosterone. White bars: control treated fish; Gray bars: SRBC immunized fish.

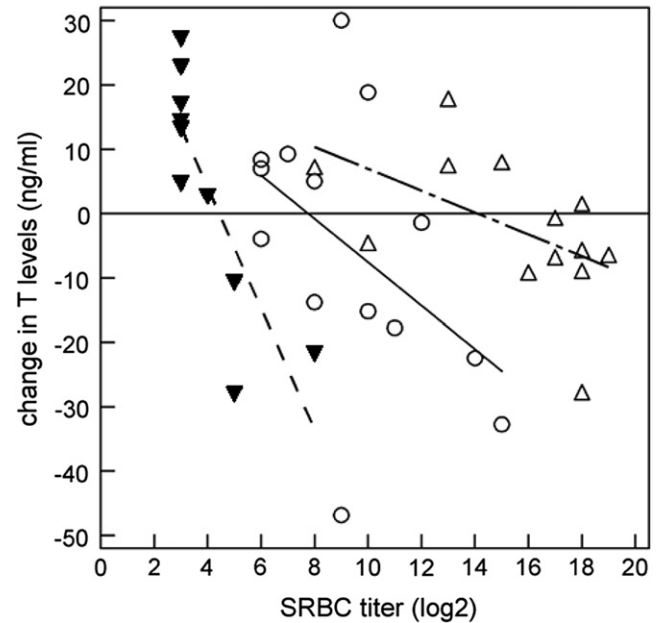


Fig. 3. SRBC immunized fish: Relationship between changes from pre-immunization to post-immunization samples in SRBC antibody titers and blood plasma levels of testosterone. The dark triangles and dashed line gives the relationship for the low dosage in experiment 1; The open triangles and dash-dotted line for the high dosage in experiment 2; And the open circles and the solid line for the intermediate dosage in experiment 2.

experimental manipulation. Contrary to expectation, there was no effect of our immune activation treatment on androgen levels. Vainikka and co-workers [26] tested the effect of injecting the natural polysaccharide  $\beta$ -glucan tench (*Tinca tinca*), which is a molecule that is known to enhance the response of phagocytic leukocytes to antigens. As in our experiment the immune system enhancement did not change androgen levels. Although  $\beta$ -glucan does not necessarily lead to immune activation, it likely increases immune responses to naturally occurring antigens. Therefore, their result is consistent with ours, that immune activation does not affect androgen production in fish. Thus until now the reverse immunocompetence handicap pathway has only been found in mammals and birds [8,27], and it might be interesting to test whether mechanisms playing a role in endothermic immune traits like fever play a causal role in the decrease in androgen levels [e.g. [28,29]].

Although there was no experimental decrease in androgen levels, the results did show a negative relationship between variation in antibody responses and changes in androgen levels: animals that showed a larger immune activation (SRBC antibody titers) decreased in plasma levels of testosterone and 11-ketotestosterone and vice versa. The variation in testosterone levels did not differ between experiments in which animals were treated with different SRBC dosages. Similar negative relationships have been reported in literature (e.g. peacock [30]; Azorean rock pool blenny [17]). It might be that the SRBC challenge revealed some pre-existing relationship between the hypothalamic-pituitary-gonadal axis (testosterone production) and immune activation, the nature of which we can only speculate.

#### 5. Conclusion

The experiment did not provide positive evidence for an effect of immune activation on androgen levels in a fish species.

## Acknowledgments

We thank Prof. Dr. Rüdiger Schulz of the University of Utrecht for analyzing levels of 11-ketotestosterone; Bonnie de Vries, Maarten Lasthuizen and Jelle Boonekamp for their discussions and assistance in the laboratory; Roelie Veenstra-Wiegman, Sjoerd Veenstra and Monique Huizinga for their assistance with fish care. Maarten Voordouw gave valuable comments on the manuscript. A.F.H.R. was being supported by a postdoctoral fellowship from the Portuguese Foundation for Science and Technology (FCT) (SFRH/BPD/7143/2001) and is currently receiving a grant of the Swiss National Science Foundation. The research of A.F.H.R. and R.F.O. is additionally supported by FCT's Plurianual Program to R&D Unit 331/94 and R&D Unit 531/96. The research of P.D.D. was financed by a NWO (SLW) grant 810.64.013. The research was carried out with an animal experiment license (DEC D4335) from the University of Groningen and complied with current laws in The Netherlands and Portugal.

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