# An Experimental Test of the Immunocompetence Handicap Hypothesis in a Teleost Fish: 11-Ketotestosterone Suppresses Innate Immunity in Three-Spined Sticklebacks*

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abstract: The immunocompetence handicap hypothesis (ICHH) provides a functional explanation for how sexual ornaments can provide honest signals of male quality. A key aspect of this hypothesis is that testosterone (T) has a bimodal effect: a higher T level enhances the expression of ornaments (increasing mating success and, ultimately, fitness); however, at the same time, it suppresses immune function. Tests of the latter assumption, which have focused mainly on aspects of adaptive immunity in birds, led to equivocal results. We performed a hormone-implant experiment in male three-spined sticklebacks (*Gasterosteus aculeatus*) to test the key assumptions of the ICHH in a fish, where the dominant circulating androgen is 11-ketotestosterone (11kT) rather than T. Males were implanted with 11-ketoandrostenedione, which is a natural precursor of 11kT. Each individual's circulating 11kT level, ornamentation, and immunocompetence were measured 2 weeks later. In addition, we quantified oxidative tissue damage because the ICHH has been hypothesized to work via oxidative stress. We found that the males' 11kT levels correlated positively with ornamentation but negatively with immunocompetence, in particular, measures of innate immunity. Moreover, there was a trend for fish with high 11kT levels to suffer more from oxidative stress. Thus, our data provide support for the ICHH.

*Keywords:* immunocompetence handicap, 11-ketotestosterone, innate immunity, oxidative stress, sexual selection, teleost fish.

The evolution of elaborate traits that are involved in mate choice has been the focus of research since Darwin developed his idea of sexual selection (Darwin 1859, 1871). In many animal species, females prefer showy males as partners—for example, birds with the most beautiful feathers (Andersson 1994). In situations where females gain nothing but the males' genes, females benefit from this preference only if showy males are of superior genetic quality. Zahavi (1975) proposed that only the best males might be able to afford costly ornamental traits; Grafen's (1990) model showed that signaling high quality is more costly to the low-quality than to the high-quality male. But what, exactly, prevents inferior males from being equally showy? The immunocompetence handicap hypothesis (ICHH) proposes that the hormone that enhances ornament expression, namely, the male androgen testosterone (T), may be detrimental for immune function (Folstad and Karter 1992). As a consequence, only males with the best genes for immune defense might be able to afford the immunosuppression associated with high levels of T and still retain sufficient immunological power. This hypothesis is based primarily on observations in birds, where, as in most vertebrates, the dominant circulating androgen in males is T.

Quite a number of studies have aimed at testing the ICHH, in particular, the suppressive effect of T on immunocompetence, and several studies have experimentally manipulated circulating hormone levels, for example, using T implants. However, the empirical data obtained so far are ambiguous (Roberts et al. 2004). Recently, there have been a number of such studies in birds and reptiles

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with equivocal results. In red-winged blackbirds (*Agelaius phoeniceus*), artificially high to very high levels of T did not suppress secondary humoral immune responses (Hasselquist et al. 1999), and the same was true for primary humoral immune responses in black-headed gulls (*Larus ridibundus*; Ros et al. 1997). In contrast, T-implant experiments suppressed humoral immunity in superb fairy-wrens (*Malurus cyaneus*), dark-eyed juncos (*Junco hyemalis*), and house sparrows (*Passer domesticus*; Evans et al. 2000; Peters 2000; Casto et al. 2001); cell-mediated immunity in starlings (*Sturnus vulgaris*; Duffy et al. 2000); and both humoral and cell-mediated immunity in song sparrows (*Melospiza melodia*; Owen-Ashley et al. 2004). However, note that in all these studies, a rise in T level was accompanied by a rise in the level of corticosterone, a hormone whose long-term effect is known to be generally immunosuppressive (Besedovsky and DelRey 1996; Olsen and Kovacs 1996; Råberg et al. 1998). Hence, the action of T on immunity may be indirect (Owen-Ashley et al. 2004). In lizards, most studies have found that artificially elevated T results in suppression of immunity (Roberts et al. 2004). A recent review came to the conclusion that there is at best weak supportive evidence for T-induced immunosuppression (Roberts et al. 2004).

In teleost fish, 11-ketotestosterone (11kT), rather than T, is the dominant male androgen (Borg 1994). Fish may thus serve as particularly interesting models with which to test whether the ICHH can work with hormones other than T (Wedekind and Folstad 1994; Kurtz and Sauer 1999). However, potential effects of androgens on immunity in fish have rarely been tested. In vitro studies suggest that T binds to receptors on leukocytes from chinook salmon (*Oncorhynchus tshawytscha*) and suppresses immune functions (Slater and Schreck 1993, 1997; Slater et al. 1995). The few in vivo studies that used androgen manipulation in fish found only weak preliminary support for immunosuppression. In rainbow trout (*Oncorhynchus mykiss*), T and 11kT implants reduced mucus immunoglobulin M levels, but plasma immunoglobulin M levels were suppressed only transiently, and this effect was not dose dependent (Hou et al. 1999). In tench (*Tinca tinca*), T injection led only to a somewhat reduced spleen mass, and there was no effect on the immune functions studied (Vainikka et al. 2005). Recent studies in the Azorean rock-pool blenny (*Parablennius parvicornis*) demonstrated differences in both circulating 11kT levels and immune parameters between two male morphotypes: nest-holding males have stronger sexual characters, higher 11kT levels, and lower lymphocyte counts but not lower granulocyte counts than non-nestholders. However, there was no correlation between 11kT and immunity within each morphotype (Ros et al. 2006*a*). Implant experiments indicated that the morphological effects are mediated by 11kT, whereas immunological effects seemed mediated by T, which led the authors to suggest that these effects might be decoupled in this species (Ros et al. 2006*b*).

In general, studies of wild animals have measured effects of androgens on a limited selection of estimates of the adaptive immune system, whereas few studies to date have included properties of innate immunity. Innate immune traits, however, might be particularly rewarding for studying trade-offs with handicap expression because they are assumed to be especially costly (Råberg et al. 2002) and might give rise to oxidative stress (von Schantz et al. 1999; Kurtz et al. 2006).

We here tested the key assumptions of the ICHH in three-spined sticklebacks (*Gasterosteus aculeatus*). In this species, a number of studies have clearly demonstrated that 11kT is most potent in stimulating secondary sexual characters, including red breeding coloration and kidney hypertrophy for the production of the nest-building glue protein spiggin (Borg 1987, 1994; Borg et al. 1993; Jakobsson et al. 1999; Pall et al. 2002*a*, 2002*b*; Mayer et al. 2004; Mayer and Pall 2007). We thus here implanted males with 11-ketoandrostenedione (11kA), which is rapidly bioconverted to 11kT, in order to mimic the high natural levels of 11kT found in breeding male sticklebacks. We measured the effects of elevated circulating 11kT levels on ornamentation, nest-building behavior, innate immunity, immune system organs, and oxidative stress.

Male sticklebacks go through a number of nesting cycles during their breeding season. Each nesting cycle can be divided into an initial sexual phase (aggressive territorial behavior and nest building) and a subsequent parental phase (egg fanning is the primary behavior). As in other parental-caring fishes, and particularly pronounced in sticklebacks, plasma levels of 11kT in males are extremely high during the sexual phase (>400 ng/mL) and then decline strongly during the subsequent parental phase (~10 ng/mL; Mayer et al. 2004; Mayer and Pall 2007). This dramatic decrease might reflect both the male's decreased need for high androgen levels and protection from potential physiological damage (immunosuppression, oxidative stress) that would result if the male maintained such high 11kT levels. This pattern in 11kT levels appears to support the ICHH concept.

Three-spined sticklebacks have recently become a prime model system for studying evolutionary and ecological aspects of mate choice and parasite resistance (Milinski and Bakker 1990; Barber et al. 2001*a*; Reimchen and Nosil 2001; Reusch et al. 2001; Wegner et al. 2003; Kurtz et al. 2004; Kalbe and Kurtz 2006). In this fish species, female choice of mates is based on odor cues that reflect genetic diversity at the major histocompatibility complex (MHC), as well as the male's red throat, a carotenoid-dependent (Wedekind et al. 1998) breeding ornament that reflects

heritable parasite resistance (reviewed in Milinski 2006). Female three-spined sticklebacks distinguish between males that differ in the red breeding coloration, preferring redder males. This preference provides them with healthier and more attractive mates (Milinski and Bakker 1990). Hence, showing that 11kT has immunosuppressive properties in male three-spined sticklebacks would, together with evidence for the importance of 11kT for the expression of male secondary sexual ornaments (in particular, red coloration) and the positive effect of male redness on female mate choice, make a particularly strong case for the ICHH.

In addition, we investigated whether males with high 11kT levels also suffer from increased oxidative stress. To quantify oxidative stress, we adapted an assay for the measurement of protein-bound acrolein to stickleback tissue homogenates (Uchida et al. 1998), which relates to biologically relevant, accumulated long-term tissue damage, rather than the short-term oxidative stress that is measured with other methods. We were interested in oxidative stress because it has been suggested that carotenoids or other compounds that protect against oxidative stress could represent a direct link between carotenoid-based ornaments and immunity (von Schantz et al. 1999). This idea is based on the fact that certain immune reactions, such as the respiratory burst, give rise to reactive oxygen species (ROS) that can lead to oxidative stress. In three-spined sticklebacks, we recently found that immune activation was associated with oxidative stress (Kurtz et al. 2006). If 11kT causes males to transfer their carotenoids into ornaments, thereby reducing carotenoid availability for oxidative-stress protection, we might expect that males with high levels of 11kT would show increased levels of oxidative stress. Based on such a scenario, oxidative stress might be the missing link in explaining the honesty of sexual signals (von Schantz et al. 1999; Kurtz et al. 2006; Alonso-Alvarez et al. 2007). Therefore, we studied simultaneously the effects of 11kT on ornamentation, immunocompetence, and oxidative stress.

## Material and Methods

### *Fish and Experimental Setup*

The fishes used in this experiment were seine-netted in Lake Vierer See, Schleswig-Holstein, Germany, in autumn 2001, and to our knowledge they were unrelated individuals. Fish were kept in groups in 190-L tanks with a continuous supply of aerated tap water and fed three to five times per week with frozen mosquito larvae. They experienced a temperature of 18°C on a 16L : 8D cycle until late November/early December, when they were moved to "autumn conditions": 12°C, 12L : 12D. In late January 2002, after approximately 10 weeks in autumn conditions, they were moved to 16-L tanks with a continuous supply of aerated tap water and held together in mixed-sex groups of three to eight fish experiencing "winter conditions" (6°C, 8L : 16D) for 6 weeks. In early to mid-March, they were moved to "spring conditions" (12°C, 12L : 12D), which they experienced for approximately 2 weeks. In mid- and late March, fish were then transferred to "summer conditions" (18°C, 16L : 8D) in two batches. In one batch (which spent 5 weeks in summer conditions; $n = 33$), fish were first kept for approximately 2 weeks in small mixed-sex groups (without nesting substrate) before males where housed individually, and in the other batch (which spent 3 weeks in summer conditions; $n = 27$), males were immediately housed singly. We chose to include males from these two batches in order to get a larger sample size and hence increase the power of our tests. We had no reason to believe that these two batches of males would differ much because the only difference between the groups was that the first batch had spent 2 weeks longer in a mixed-sex group under summer conditions. However, males from both batches did not have access to nesting material and did not progress with nest building. Still, when setting up the androgen-implant experiment, we balanced the design with respect to weeks in summer conditions so that the two batches were equally represented in the control and the two experimental groups.

The transfer to individual tanks took place on March 25–27 for all 60 males included in the experiment. Opaque partitions between tanks prevented fish from seeing each other. After spending one week singly, all males were implanted with a Silastic capsule (see "Hormone Implantation"). After yet another week, the males were provided with a Petri dish containing sand suitable for building a nest on and a bundle of nesting material, 7–10-cm-long green cotton threads, which sticklebacks readily accept as material for nest building. We then allowed the implanted males to commence nest building for 1 week before they were killed, dissected, and sampled for further analyses.

### *Hormone Implantation*

We implanted stickleback males with 11-ketoandrostenedione (11kA; Sigma), which is readily bioconverted to 11kT, in order to mimic the high natural levels of 11kT measured in the plasma of breeding male sticklebacks. All males were implanted with Silastic tube capsules of similar sizes (7.5 mm long, 1.1 mm in outer diameter; Degania Silicone). The implant study consisted of three experimental groups ($n = 20$/group). A sham-operated control group was implanted with empty Silastic capsules, a high-dose group was implanted with capsules filled with crystalline 11kA, and a low-dose group was implanted with

capsules filled with cocoa butter containing approximately half the amount of 11kA as the high-dose capsules. To make the low-dose capsules, crystalline 11kA was first dissolved in melted cocoa butter (as a neutral vehicle), and while still molten, was then injected into a length of Silastic tubing. Once the cocoa butter had solidified (at room temperature), the length of tubing was cut into 7.5-mm lengths. All Silastic capsules were sealed at both ends with silicone glue. The controls were implanted with empty capsules rather than with cocoa butter–filled capsules because no differences had been observed between these two capsules in previous studies (I. Mayer, personal observation). The high-dose capsules were each pierced three times with a fine needle to facilitate 11kA release. Particular attention was made to matching the three groups so that they contained fish of the same size and the same degree of body redness. This matching was successful; there were no significant differences in body coloration, body length, or mass between the three groups (table 1).

The hormone 11kA has been used in hormone implant experiments in three-spined sticklebacks to modulate androgen levels that had effects on the redness of body coloration in males (a secondary sexual trait; Borg 1987, 1994) and reproductive behaviors such as nest building and courtship (Borg et al. 1993; Jakobsson et al. 1999; Pall et al. 2002*a*, 2002*b*). Based on previous experience (I. Mayer, personal observations), the low dose of 11kA dissolved in cocoa butter should result in physiological levels of 11kT, whereas the high dose of crystalline 11kA should result in supraphysiological levels of 11kT in sticklebacks.

Fish were implanted using the following procedure. In lightly anesthetized fish (MS 222), a small (1-mm) incision was made into the abdominal cavity on the lower part of the side of the body, close to the cloaca. We first gripped and lifted a small piece of the skin with fine forceps and then made the small incision using fine surgical scissors. A capsule was then inserted into the abdominal cavity and pushed to the side to prevent it from protruding out of the incision. Because of the small size of the incisions, no sutures were required, and all fish retained their implants to the end of the experiment.

### *Blood Sampling and Hormone Assay*

Fish were killed by a blow on the head, the tail was cut off, and a blood sample was immediately collected from the caudal vein using a heparinized microcapillary that was then stored on ice. Within a few hours, blood samples were centrifuged at 3,000 rpm for 7 min and the plasma extracted and frozen at −70°C for later analyses. Plasma samples were shipped on dry ice to Stockholm in May 2002 and analyzed for levels of 11kT by I. Mayer in June 2002, using a radioimmunoassay as described by Pall et al. (2002*a*). This method is highly repeatable, with intra- and interassay coefficients of variance of a pooled plasma sample containing 200 ng/mL of 5.4% and 7.0%, respectively. The effective lower limit of detection for 11kT in individual samples is approximately 2 ng/mL (Pall et al. 2002*a*).

### *Measuring Male Color Ornaments and Nesting Behavior*

Just before and 14 days after the end of the implant treatment period, all males were photographed on the left lateral side with a digital camera (Olympus E-20P, 5.0 megapixels, using identical, manually chosen settings for all exposures). The male was put inside a small quartz glass cuvette, with a moist sponge kept gently to the side, and water was added to a level of ~3 cm. The cuvette was then put in a dark box and illuminated by a two-arm cold-light source (Schott KL 1500 LCD, 150 W) for digital photography. The digital images were analyzed with the software Adobe Photoshop 7.0. Using the polygonal tool, we selected an area (identical in all fish) covering the majority of the red parts of the lateral side of the fish. Intensity of the red coloration of the defined area was measured using the RGB mode (8-bit; i.e., values 0–255 for each of the color channels red, green, and blue) as the part of the total color intensity that came from the red color (R/RGB; Frischknecht 1993).

We scored male nest building every day for the first week of the experiment using a scale from 0 to 3 (0 = no signs of nest building, 1 = nest building just started, 2 = nest under construction but tunnel not complete, 3 = nest complete with full tunnel). In the analyses, we

**Table 1:** Comparison of preexperimental breeding coloration, length, and mass of fish designated for the different experimental treatment groups (sham, low, and high dose of 11-ketoandrostenedione implant)

| | 3 weeks in breeding conditions | | | | | 5 weeks in breeding conditions | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sham | Low | High | *F* | *P* | Sham | Low | High | *F* | *P* |
| Breeding coloration (R/RGB) | .367 | .366 | .371 | .404 | .672 | .373 | .370 | .378 | .253 | .778 |
| Body length (mm) | 52.1 | 51.8 | 51.8 | .077 | .926 | 49.9 | 49.5 | 49.5 | .065 | .937 |
| Body mass (g) | 1.35 | 1.30 | 1.29 | .432 | .654 | 1.16 | 1.16 | 1.21 | .128 | .880 |

Note: R/RGB indicates the part of the total color intensity that came from the red color.

used a measure of the males' nest-building speed that was based on the number of days it took for a male to complete his nest from the start of the experiment.

### *Body Size and Reproductive Status*

We measured body length (from snout to tip of tail, to the nearest millimeter) and body mass of fish (to 0.1 mg) before and after the experiment. After the experiment, fish were dissected, and we then measured the mass of the testes (to 0.1 mg) for calculation of testes mass according to body mass (testes index $I_T$ = testes mass/fish mass).

### *Immune Defense: Estimates of Immunity of Sticklebacks*

We analyzed the activity of the immune system of the sticklebacks. As an estimate of overall immune activity, including aspects of both innate and adaptive immunity, we calculated a head kidney index, that is, the number of leucocytes obtained from the head kidney according to individual body weight. The head kidney is the major immune organ of bony fish (Zapata et al. 1996). We further determined a spleen index, that is, the mass of the spleen according to body mass ($I_S$ = 100 × spleen mass/fish mass). In bony fish, the spleen is a major lymphoid organ; that is, it is involved mainly in adaptive immunity. There are so far no techniques that allow us to directly measure the responsiveness of the adaptive (humoral) immune defense in sticklebacks.

As functional estimates of the level of activity of the innate immune system, we quantified the respiratory burst reaction of head kidney cells in two independent in vitro assays. During the respiratory burst, reactive oxygen species (ROS) are generated to kill pathogens. We isolated leucocytes from the head kidneys of fresh dissected fish as described previously (Kurtz et al. 2004, 2006). We first analyzed the respiratory burst associated with phagocytosis of zymosan particles in vitro in a lucigenin-enhanced chemiluminescence (CL) assay (Kurtz et al. 2004, 2006). For this assay, the cell density was adjusted to $1.25 \times 10^6$ live cells/mL, corresponding to $2 \times 10^5$ cells per well of a 96-well plate. We further analyzed the respiratory burst in a nonphagocytic context, using a photometric assay with nitroblue tetrazolium salt (NBT; Sigma), as described in detail by Scharsack et al. (2004). Head kidney cells ($4 \times 10^5$ live cells per well of a 96-well plate) either were stimulated with phorbol myristate acetate (PMA), an unspecific inducer of ROS production, or did not receive this stimulator and thus give an estimate of the baseline ROS production of the cells without any additional stimulus.

### *Oxidative Stress: Measuring Protein-Bound Acrolein in Spleen and Liver Tissue*

To measure oxidative stress in stickleback tissue, we adapted a technique that is based on the detection of protein-bound acrolein, a marker of oxidative protein damage (Uchida et al. 1998; Uchida 1999). Acrolein ($CH_2{=}CH{-}CHO$) is ubiquitously generated in biological systems through lipid peroxidation, which proceeds by a free-radical chain reaction. It is accumulated as a stable product, covalently bound to protein, mainly as an acrolein-lysine adduct (FDP-lysine). We quantified acrolein in liver and spleen tissue with an enzyme-linked immunosorbent assay using the monoclonal antibody mAb5F6, which was raised against acrolein-treated keyhole limpet hemocyanin (KLH; Uchida et al. 1998).

To produce tissue homogenates, previously weighed stickleback livers and spleens were placed in 0.01 M phosphate-buffered saline, pH 7.4 (Sigma, P-3813). The buffer volume was adjusted to 10 mg fresh liver and 1 mg fresh spleen per milliliter. We processed these samples as described by Kurtz et al. (2006). For analysis, we used means of the measurements obtained from spleen and liver as a combined estimate of oxidative stress of each fish.

### *Statistical Analyses*

To achieve normality for statistical analyses, spleen index, head kidney index, testes index, and the measures of phagocytosis (CL) and respiratory burst (NBT with and without PMA) were Box-Cox transformed. However, we show untransformed values in the figures. All other variables were normally distributed without transformation (Shapiro-Wilk $W$ test, $P > .2$), except for nesting behavior, which was analyzed with nonparametric statistics. We calculated general linear models with interaction terms. The original sample size of 60 fish was slightly reduced in cases where the corresponding measurements could not be obtained (e.g., three fish in which T levels could not be measured), as can be seen from the degrees of freedom of the analyses. Test statistics refer to two-tailed tests, and we considered effects significant at a level of $P < .05$. Analyses were performed with SPSS 11.04 and JMP 6.0 for Mac OS X.

## Results

### *Effects of 11kA Implants on 11kT Levels and Nesting Behavior*

The 11-ketoandrostenedione (11kA)-implant experiment resulted in clear effects on the measured levels of circulating 11-ketotestosterone (11kT) in male three-spined sticklebacks. Males given empty implants had the lowest 11kT levels, males with medium-sized implants had intermediate 11kT levels, and males with large implants had

the highest 11kT levels. Moreover, the two groups of fish that differed in the time they had spent in summer conditions (i.e., suitable temperature conditions for breeding when exposed to high 11kT levels produced endogenously or administered from the 11kA implants) differed significantly in the mean levels of circulating 11kT. Males that spent 3 weeks in summer conditions had higher 11kT levels than males that spent 5 weeks in summer conditions (general linear model, 11kA-implant size: $F = 11.92$, $df = 2, 51$, $P < .001$; weeks in summer conditions: $F = 53.94$, $df = 1, 51$, $P < .001$; 11kA-implant size × weeks in summer conditions: $F = 0.07$, $df = 2, 51$, $P = .933$; fig. 1). Thus, we have included the time spent in summer conditions (i.e., 3 or 5 weeks) as a factor in all further analyses, and we also present data separately for these two groups in all figures. We also calculated separate analyses for these two groups of fish when the interaction term was significant.

Because we did not castrate the fish before 11kA implantation, circulating 11kT levels in all fish were dependent on both exogenously administered and endogenously produced androgens. We found a tendency for reduction of the testes in fish with high-dose 11kA implants compared to controls, which was significant in fish that had spent 5 weeks in summer conditions (least significant difference [LSD] post hoc test, $t = -2.40$, $df = 30$, $P = .023$). This could indicate that 11kA-implanted fish downregulate their endogenous 11kT production. Thus, in all further analyses of the effects of 11kT, we use the measured levels of circulating 11kT, rather than implant group, as the independent factor. This should be the most relevant estimate of the circulating 11kT levels with direct functional effects on the dependent factors, rather than implant size per se.

Nest-building activity was higher in males that had spent only 3 weeks in summer conditions; these males took less time to construct a complete nest (Wilcoxon test, $Z = -3.83$, $P < .001$). In males that had spent 5 weeks in summer conditions, nest building was faster for males with higher 11kT levels (Spearman rank correlation, $r_S = -0.47$, $P = .008$), while this correlation was not significant in males that had spent only 3 weeks in summer conditions (Spearman rank correlation, $r_S = -0.15$, $P = .467$).

### *11kT Levels and Male Red Body Coloration*

The change in redness of the body coloration over the implant experiment period was predicted by weeks spent in summer conditions but only weakly by 11kA-implant size (general linear model, 11kA-implant size: $F = 1.42$, $df = 2, 54$, $P = .252$; weeks in summer conditions: $F = 6.81$, $df = 1, 54$, $P = .012$; 11kA-implant size × weeks in summer conditions: $F = 0.51$, $df = 2, 54$, $P = .604$). Males that had spent 3 weeks in summer conditions showed more intense reddening over the course of the experiment than those that had spent 5 weeks. In the latter group, low-dose 11kA implants significantly intensified reddening compared to results in control fish (LSD post hoc test, $t = 2.83$, $df = 30$, $P = .008$), while this tendency was not significant for males with high-dose implants (LSD post hoc test, $t = 1.20$, $df = 30$, $P = .241$).

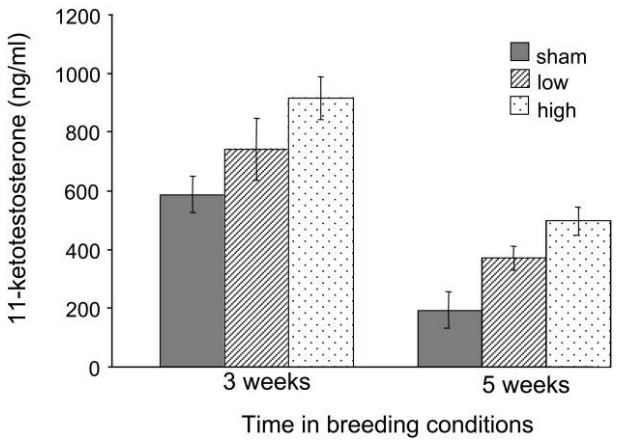


**Figure 1:** Effects of androgen implants (11-ketoandrostenedione) on the level of 11-ketotestosterone in circulating blood of male three-spined sticklebacks. Data are shown separately for the two groups of fish that differed in time spent in summer conditions (3 weeks vs. 5 weeks).

There was a positive relationship between circulating 11kT levels and redness of male body coloration at the end of the 11kA-implant experiment (general linear model, 11kT level: $F = 7.67$, $df = 1, 53$, $P = .008$; weeks in summer conditions: $F = 0.73$, $df = 1, 53$, $P = .396$; 11kT level × weeks in summer conditions: $F = 6.53$, $df = 1, 53$, $P = .013$; fig. 2). This relationship appeared to be strong in fish that had spent 5 weeks in summer conditions (11kT level: $F = 14.60$, $df = 1, 29$, $P = .001$), whereas it was not detectable in fish that had spent only 3 weeks in summer conditions (11kT level: $F = 0.022$, $df = 1, 24$, $P = .882$).

### *11kT Levels and Immunocompetence*

We found negative relationships between circulating 11kT levels and three out of four measures of immunity. There was a significant negative relationship between 11kT levels and respiratory burst upon phagocytosis (CL), a functional activity measure of innate immunity (general linear model, 11kT-level: $F = 5.51$, $df = 1, 53$, $P = .023$; weeks in summer conditions: $F = 3.15$, $df = 1, 53$, $P = .082$; 11kT level × weeks in summer conditions: $F = 0.35$, $df =$

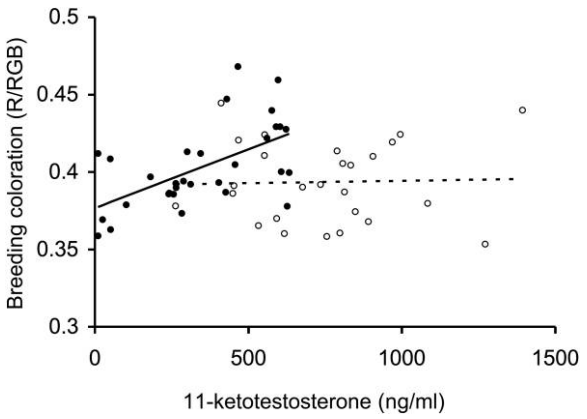


**Figure 2:** Red breeding coloration in relation to circulating 11-ketotestosterone levels of three-spined sticklebacks (*open circles, dashed line* = males that spent 3 weeks in summer conditions; *filled circles, solid line* = males that spent 5 weeks in summer conditions). R/RGB indicates the part of the total color intensity that came from the red color.

1, 53, $P = .558$; fig. 3*A*). However, there was no relationship between circulating 11kT levels and respiratory burst in the absence of phagocytosis (general linear model, NBT assay with PMA, 11kT level: $F = 0.17$, df = 1, 52, $P = .686$; weeks in summer conditions: $F = 0.29$, df = 1, 52, $P = .590$; 11kT level × weeks in summer conditions: $F = 2.27$, df = 1, 52, $P = .138$; fig. 3*B*; NBT assay without stimulation, 11kT level: $F = 0.82$, df = 1, 52, $P = .371$; weeks in summer conditions: $F = 0.23$, df = 1, 52, $P = .630$; 11kT level × weeks in summer conditions: $F = 1.26$, df = 1, 52, $P = .266$).

We then investigated whether circulating 11kT levels had an effect on the size of immune system organs important for innate and acquired immunity. Circulating 11kT levels showed a significant negative relationship with head kidney index (general linear model, 11kT level: $F = 7.67$, df = 1, 53, $P = .008$; weeks in summer conditions: $F = 0.63$, df = 1, 53, $P = .433$; 11kT level × weeks in summer conditions: $F = 0.03$, df = 1, 53, $P = .867$; fig. 3*C*). The negative relationship between circulating 11kT level and spleen mass (adjusted for body mass) showed a tendency to be significant (general linear model, 11kT level: $F = 3.18$, df = 1, 53, $P = .080$; weeks in summer conditions: $F = 0.06$, df = 1, 53, $P = .805$; 11kT level × weeks in summer conditions: $F = 0.78$, df = 1, 53, $P = .382$; fig. 3*D*).

### *11kT Levels and Oxidative Stress*

There was a tendency for fish with high circulating 11kT levels to experience more oxidative stress. The two groups of fish that differed in the time they had spent in summer conditions differed significantly in oxidative tissue damage (general linear model, 11kT level: $F = 3.27$, df = 1, 53, $P = .076$; weeks in summer conditions: $F = 9.85$, df = 1, 53, $P = .003$; 11kT level × weeks in summer conditions: $F = 2.50$, df = 1, 53; $P = .120$; fig. 4). Fish that had spent 3 weeks in summer conditions showed more oxidative damage (which coincides with higher 11kT levels in these fish) than fish that had spent 5 weeks. When the two groups of fish were analyzed separately, there was a significant increase in oxidative damage with 11kT level ($F = 4.61$, df = 1, 29, $P = .040$) in fish that had spent more time in summer conditions (and thus had been exposed longer to breeding-condition temperatures).

## Discussion

We found support for an immunosuppressive action of 11-ketotestosterone (11kT; the dominant male androgen in teleost fishes) for three out of four of our immune function measures in male three-spined sticklebacks. This confirms a key assumption of the immunocompetence handicap hypothesis (ICHH; Folstad and Karter 1992). Moreover, we can here corroborate previous studies of 11kT in three-spined sticklebacks that have found positive effects on breeding performance, nest building, and the red breeding coloration of males (Borg 1987; Borg et al. 1993; Jakobsson et al. 1999; Pall et al. 2002*a*, 2002*b*), which is an important sexually selected ornament of sticklebacks (Milinski and Bakker 1990; Bakker 1993). Thus, the available data from these latter studies, together with our own findings of 11kT-induced immunosuppression in male three-spined sticklebacks, clearly support the ICHH as a functional mechanism for honest secondary sexual signals in this system.

While the ICHH was originally supported by observations in birds, based on effects of testosterone (T), this study for the first time comprehensively demonstrates that the ICHH can also be applied to teleost fishes, where rather than T it is 11kT that has a bimodal effect, enhancing the expression of secondary sexual ornaments while at the same time suppressing immune function. This would explain why plasma levels of 11kT decline after the sexual phase in all fishes that display parental care (Mayer et al. 2004).

It is interesting that we found aspects of innate immunity to be suppressed by high circulating 11kT levels. Previous studies in vertebrates have concentrated on the effect of T on measures of acquired immune function (Ros et al. 1997; Hasselquist et al. 1999; Duffy et al. 2000; Evans et al. 2000; Peters 2000; Casto et al. 2001). In this study, however, we also found androgen-induced immunosuppression of innate immunity. This deserves further study in the future.

To our surprise, we found a striking difference between

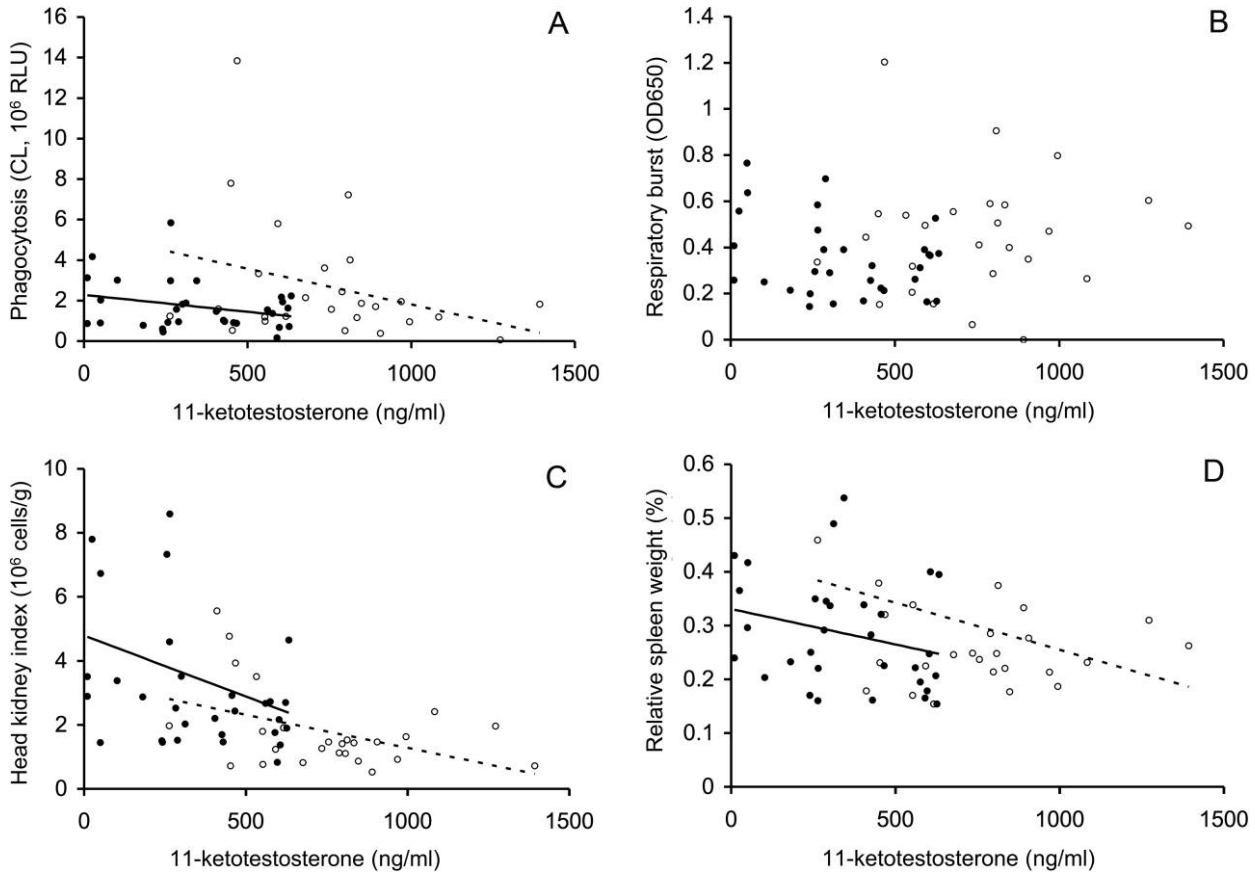


**Figure 3:** Measures of immune function in relation to circulating 11-ketotestosterone levels of three-spined sticklebacks (*open circles*, *dashed line* = males that spent 3 weeks in summer conditions; *filled circles*, *solid line* = males that spent 5 weeks in summer conditions). Functional activity measures of innate immunity: *A*, respiratory burst upon phagocytosis (quantified as relative luminscence units [RLU] in a chemiluminescence assay [CL]); *B*, respiratory burst index (quantified as change in optical density at 650 nm [OD650], in a nitroblue tetrazolium salt reduction assay, phorbol myristate acetate–stimulated). Size of immune system organs important for innate and acquired immunity: *C*, head kidney index; *D*, spleen mass (adjusted for body mass).

males that had spent 3 and 5 weeks in summer conditions. Interestingly, males that had spent more time in summer conditions had lower circulating 11kT levels, built nests slower, and showed less intense reddening over the course of the experiment. These data suggest that these males, which did not get positive feedback by courting receptive females, might have already started to shut down their breeding activity. Effects of 11kT on ornamentation and oxidative stress tended to be more pronounced in these fish. It seems that experimental effects of 11kA implants were obscured by endogenously high 11kT levels in fish that spent less time in summer conditions and therefore were still in the peak reproductive state. With the 11kA implants, these fish might have reached unnaturally high 11kT levels, preventing us from detecting any strong effects of hormone levels on immunity and oxidative stress in this group. Moreover, this may suggest that male three-spined sticklebacks incur substantial costs by being in the reproductive state, that is, when keeping up high levels of circulating 11kT.

We included the quantification of tissue damage through oxidative stress in a test of the ICHH. As expected, there was a trend for males with high circulating 11kT levels to suffer more from oxidative stress, which was significant in the group of fish that had spent more time in summer conditions and thus had been longer exposed to endogenously differing 11kT levels. Potential effects of 11kT appeared to be rather weak, although one should keep in mind that the method we used to measure oxidative stress detects protein damage, which may take some time to build up from the exposure to the agent generating the oxidative stress.

Why should there be a relationship between androgens, such as 11kT, and oxidative stress? At this point, a closer

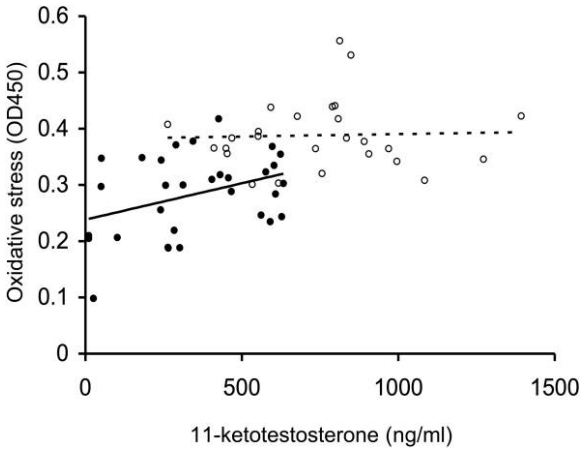


**Figure 4:** Oxidative stress (quantified by measuring the amount of acrolein in the spleen and liver using an enzyme-linked immunosorbent assay; OD450 = optical density at 450 nm) in relation to circulating 11-ketotestosterone levels of three-spined sticklebacks (*open circles*, *dashed line* = males that spent 3 weeks in summer conditions; *filled circles*, *solid line* = males that spent 5 weeks in summer conditions).

look at the assumptions and potential mechanisms behind the ICHH may be fruitful. Ultimately, the core assumption of the ICHH is an androgen-mediated trade-off between the expression of an ornament trait and immune defense (Wedekind and Folstad 1994; Sheldon and Verhulst 1996) such that there is a limiting common resource for these conflicting demands. This resource could simply be the energy needed for both the expression of a costly ornament and immune defense (Sheldon and Verhulst 1996). Alternatively, carotenoids or other compounds that protect against oxidative stress could be a more specific limited resource (Lozano 1994; von Schantz et al. 1999).

Oxidative stress, which is a major contributor to aging and degenerative diseases, occurs as a consequence of reactive oxygen species (ROS; free radicals) that are produced as by-products of mitochondrial respiration and also during immune reactions such as the respiratory burst, where ROS are used to kill pathogens and parasites (Chanock et al. 1994; Bogdan et al. 2000; Finkel and Holbrook 2000; Fang 2004). Antioxidants, such as carotenoids, that alleviate oxidative stress are also used for sexual signaling (Lozano 1994; Wedekind et al. 1998; Grether et al. 1999; Blount et al. 2003; Faivre et al. 2003). Individuals can thus be expected to adjust their investment of carotenoids to the conflicting demands of sexual signaling and oxidative stress protection, for example, during immune defense. Such adjustment might be through T, which has recently been shown to increase the bioavailability of carotenoids in birds (Blas et al. 2006). Experimentally (through androgen implants) shifting this balance toward sexual signaling could then lead to depressed immunity (with unaltered oxidative stress), increased oxidative stress (with unaltered immunity), or a combination of both. This might explain why T suppressed immunity in some studies but not in others (Roberts et al. 2004).

Our data indicate that elevated circulating 11kT levels lead to both immunosuppression and (slightly) increased oxidative stress in sticklebacks. The only other test of the ICHH that included a measure of oxidative stress supports this view but did not find simultaneous effects of T on ornamentation of male zebra finches (*Taeniopygia guttata*; Alonso-Alvarez et al. 2007). Clearly, more work on the relevance of oxidative stress for the ICHH is needed. Ideally, these studies should engage more than just one technique for measuring oxidative stress.

In conclusion, our 11kA-implant experiment clearly shows that male three-spined sticklebacks with high levels of circulating 11kT experience suppressed phagocytosis activity (an important part of innate immunity) and somewhat regressed immune organs (spleen and head kidneys, which are important for both innate and adaptive immunity) and potentially experience high oxidative stress. On the other hand, males with high 11kT levels showed redder body coloration and faster nest building than controls. Together with data from earlier studies showing the importance of these traits for female mate choice and sexual selection (Milinski and Bakker 1990; Bakker 1993; Frischknecht 1993; Barber et al. 2001*a*, 2001*b*), our data provide strong support for the ICHH. This study, together with prior work (Kurtz et al. 2006), thus completes our understanding of mate choice in sticklebacks, which use multiple signals to assess their partners' immune system, including odor cues, which directly signal MHC genetics (Milinski 2006), and breeding coloration, an indicator of immunocompetence and oxidative stress resistance.

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