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Homeostasis of Circulating Androgen Levels in the Breeding Male Three-spined Stickleback *Gasterosteus aculeatus*

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Yi-Ta Shao, Rüdiger W. Schulz, and Bertil Borg (2012) Homeostasis of circulating androgen levels in the breeding male three-spined stickleback *Gasterosteus aculeatus*. *Zoological Studies* **51**(8): 1282-1289. Androgens are important for stimulating male characters, but excessively high levels may suppress the immune system, and in many animals, circulating levels are homeostatically controlled by feedback mechanisms. However, it was shown that there is no compensation of plasma androgen levels in hemi-castrated three-spined stickleback *Gasterosteus aculeatus*. In this study, we investigated to what extent androgens levels are homeostatically regulated in the breeding male three-spined stickleback, and whether aromatization of androgens plays a role in this. To that end, breeding male sticklebacks were either completely castrated, hemi-castrated, or sham-operated, and then implanted with different doses of 11-ketoandrostenedione (11KA) and testosterone (T) or with the aromatase inhibitor (AI), fadrozole. Hemi-castration alone diminished androgen levels, while complete castration almost completely removed them. Low doses of 11KA and T increased plasma androgen levels in castrated but not in sham-operated fish. Both low and high doses of 11KA increased plasma 11-ketotestosterone (11KT) in hemi-castrated fish, whereas only the high dose of 11KA did so in sham-operated fish. If aromatization plays a role in homeostatic mechanisms, androgen levels would be expected to rise in sham-operated fish treated with the AI. However, this was not the case. The reduction in plasma androgen levels in fully mature hemi-castrated fish suggests that the remaining testis was unable to further increase its steroidogenesis. However, both 11KA and T treatments increased plasma levels much less in sham-operated fish than in castrated ones, indicating that homeostatic mechanisms are present and act to prevent excessively high plasma androgen levels. http://zoolstud.sinica.edu.tw/Journals/51.8/1282.pdf

Key words: Steroid, RIA, Homeostasis, Feedback, Stickleback.

Androgens are key hormones in male reproduction, which stimulate male sex characters and gonad development. However, high plasma androgens levels may have severe consequences for other physiological processes, e.g., immune suppressive effects, as was also observed in the three-spined stickleback *Gasterosteus aculeatus* (Kurtz et al. 2007) and in a cichlid species, *Pundamilia nyererei* (Dijkstra et al. 2007). Thus, it is critical for males to keep circulating androgens at suitable levels. In teleosts, 11-ketotestosterone (11KT; 4-androstene-17βol-3,11-dione) is found at higher levels in plasma of males than in females, whereas this is usually not the case for testosterone (T). 11KT is generally more effective than T in stimulating secondary sexual characters and reproductive behavior (see review in Borg 1994). T can be converted to estrogen via aromatase. Aromatization was found in the brain and/or pituitary of many vertebrates (see review in Balthazart and Foidart 1993), including the stickleback (Borg et al. 1987). Although 11KT shows stronger stimulatory effects on many male characters than T in fishes, T was often found

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to be more effective than 11KT, or other non-aromatizable androgens, in feedback mechanisms on the brain-pituitary-gonad (BPG) axis (see review in Borg 1994). A number of studies indicated that aromatization can be important in feedback mechanisms on the BPG axis and in controlling maturation in different fishes, including the three-spined stickleback (Bornestaf et al. 1997).

Reduced negative feedback on the BPG axis in hemi-castrated mammals leads to steroidogenic compensation, e.g., more androgens are secreted from the remaining testis to maintain levels in the plasma (Lindgren et al. 1976, Berger et al. 1978, Walton et al. 1978, Barnes et al. 1983). In fishes, unlike in mammals, a number of previous studies suggested that androgen level compensation may be limited or absent. In goldfish, *Carassius auratus*, spawning tubercles (an androgen-dependent secondary sexual character) were found in only 1/2 of partly castrated males, whereas they were observed in all intact males (Tozawa 1923). Weaker breeding colors were observed in hemi-castrated male sticklebacks than in intact fish (Bock 1928). Furthermore, plasma levels of 11KT and T in hemi-castrated sticklebacks were only about 1/2 those found in sham-operated individuals (Hellqvist et al. 2002). A lack of complete compensation of androgen levels in hemi-castrated animals was also reported in Atlantic salmon, *Salmo salar*, where respective plasma T and 11KT levels in hemi-castrated males were only 75% and 63% of those in intact fish (Hellqvist et al. 2002). In African catfish, *Clarius gariepinus*, on the other hand, sex steroid plasma levels did not differ between sham-operated and hemi-castrated males (Schulz et al. 2012), and a similar observation was made in female European sea bass, *Dicentrarchus labrax* subjected to a unilateral ovariectomy (García-López et al. 2011). Thus, hemi-castration studies on different fishes have given conflicting results on whether there is homeostatic control of androgen levels.

In this study, we wanted to test the hypothesis that circulating levels of androgens are homeostatically regulated in male sticklebacks, and determine whether aromatization possibly plays a role in this homeostatic system. Androgen treatments were found to increase plasma androgen levels to a lesser extent in controls than in castrated males, indicating the presence of homeostasis. A lack of an effect of the aromatase inhibitor (AI), fadrozole, suggested that aromatization is not involved.

MATERIALS AND METHODS

Experimental animals

Adult, non-breeding male three-spined sticklebacks were collected from the Öresund during the winters of 2008 and 2010. Fish were kept in 1200- or 700-L aquaria with filtered and aerated artificial seawater (at 0.5% salinity), under winter conditions (4-8°C and a light: dark (L: D) photoperiod of 8: 16 h) before the experiments. The aquaria contained sand and ceramic hiding places. The fish were fed daily with frozen blood-worms, Artemia, or mysids.

Fish were moved from the winter condition, and kept separately in 50-L aquaria under summer conditions (20°C and an L: D photoperiod of 16: 8 h) after 1 d of accommodation to the increased temperature. The bottoms of the aquaria were covered with sand, and algae were present as nest-building material. The water was filtered with a small underwater pump. When a nest was found in an aquarium, the fish was considered to be in full breeding condition; only such males were used. Androgen levels in the male stickleback are at their highest in breeding males with no eggs in the nest (Mayer et al. 1990, Páll et al. 2002); in other conditions, levels are too low for this type of study.

Implants

Capsules were made from 5-mm-long medical-grade Silastic tubes (with an inner diameter of 0.64 mm and an outer diameter of 1.19 mm), which were filled with crystalline 11-ketoandrostenedione (11KA; 4-androstene-3,11,17-trione; Sigma, St. Louis, MO, USA), the main androgen produced by the testes in breeding stickleback males (Borg et al. 1989), which is converted to 11KT extratesticularly (Mayer et al. 1990) or an aromatase inhibitor (AI, fadrozole: 4-(5,6,7,8-tetrahydroimidazo[1,5-*a*]pyridin-5-yl) benzonitrile; a generous gift from Novartis, Basel, Switzerland), and sealed with silicon glue. In a previous study (Páll et al. 2002), the type of 11KA implant used in the present study increased plasma 11KT levels in castrated male sticklebacks to around 300 ng/mL, similar to levels found in breeding males. For the high dosage of 11KA (11KA(H)), ordinary (low dose) 11KA implants (11KA(L)) were perforated with a 31G syringe needle to make 6 holes. For T treatments, however, dosages of the crystalline T implant were far higher than normal circulating levels in

breeding males. In the present study, another type of implant was used. It was made of Silastic tubes filled with 1% or 2% T dissolved in cacao butter and cut at a 5-mm length, but not sealed. The cacao-butter implants were stored at 4°C. Empty implants (5 mm) were used as controls in the 11KT experiment, and Silastic tubes (5 mm) filled with only cacao butter were used as controls in the T experiment.

Operations

Breeding fish were either completely castrated, hemi-castrated, or sham-operated, and then different types of implants were put in place. Before the operation, fish were anesthetized with around 0.1% 2-phenoxyethanol (in 2008 and 2009) or 0.025% buffered MS-222 (ethyl 3-aminobenzoate, methanesulfonic acid salt) solution (in 2011) (Molinero and Gonzalez 1995). About a 1.5-mm-long incision was made on each side of the abdominal cavity, and 1 or both testes were excised with fine forceps. Sham-operated fish were treated similarly, but their testes were not removed. The incisions were closed with BV-2 (0.4 Ph. Eur) sutures after capsule implantation. After the operation, the fish was released back into its aquarium.

Several sets of experiments, each using 10-25 individuals were carried out in 2008 and 2009 (with 11KA and AI treatments) and in 2011 (with T and AI). In total, 196 fish were used. Numbers of fish in the different treatments are shown in table 1.

Dissection and sample treatment

Twelve days after the operation, each fish was netted and immediately put into an anesthetic solution. The caudal peduncle was severed, and blood was collected from the caudal artery in micro hematocrit tubes (Na-hep. cat no. 7493 11; BRAND, Wertheim, Germany). After centrifugation for 2 min at 13,000 rpm (hematocrit rotor, 185 mm in diameter), plasma samples were transferred to another set of Eppendorf tubes, which had been weighted and labeled. The volume of a plasma sample was calculated from the tube weights (± 0.01 mg) before and after the sample was added, with the density of plasma assumed to be 1. Plasma samples were stored at -70°C before the steroid measurement. Castrated fish were inspected with a stereomicroscope for remnants of the testes at dissection; if any were found, then the sample was discarded. The kidneys and

Table 1. Sample number and the kidney-somatic index (KSI) of each treatment group

Operation	Implant	Sample *n*	Body weight (g)
2008-2009			
Sham	Empty	29	1.91 ± 0.15
	11KA(L)	12	1.92 ± 0.14
	11KA(H)	12	2.07 ± 0.14
	AI	12	1.66 ± 0.51
Hemi	Empty	16	2.04 ± 0.13
	11KA(L)	4	2.18 ± 0.12
	11KA(H)	13	2.06 ± 0.14
Castr	Empty	15	1.49 ± 0.08
	11KA(L)	6	1.46 ± 0.06
	11KA(H)	5	1.46 ± 0.16
2011			
Sham	Empty	13	1.49 ± 0.04
	1% T	10	1.49 ± 0.02
	2% T	11	1.59 ± 0.05
	AI	10	1.47 ± 0.04
Castr	Empty	10	1.57 ± 0.07
	1% T	10	1.47 ± 0.04
	2% T	8	1.60 ± 0.11

Sham, sham-operated; Hemi, hemi-castrated; Castr, completely castrated. Empty implants or implants with 1% or 2% of testosterone (T) in cacao butter; 11KA(H)/(L), high/low dosages of 11-ketoandrostenedione; AI, aromatase inhibitor (fadrozole). Values are shown as the mean ± SEM. *$p < 0.05$.

body of the fish were weighed (± 0.01 g), and the kidney-somatic index (KSI) ((kidney weight / body weight) × 100) was calculated. Since the kidneys of male sticklebacks hypertrophy under androgen stimulation at breeding and begin to produce spiggin (Jakobsson et al. 1999), a protein used in nest-building, the KSI was used to indicate male maturation.

Steroid measurement

Each plasma sample was diluted with 100 µl radioimmunoassay (RIA) buffer (Schulz 1985) and vortexed for 20 s. Following heat treatment at 80°C for 60 min, tubes were centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were pipetted into a new set of tubes, which were stored in -70°C until being measured.

Both T and 11KT levels of samples were measured via the RIA (Schulz 1985) to quantify levels from heat-treated samples following the protocols in Schulz et al. (1994).

Statistical analysis

Data were analyzed using SPSS vers. 14 (SPSS, Chicago, IL, USA) with a two-tailed Student's *t*-test with Tukey's post-hoc test for independent samples after a normality test ($p < 0.05$) for comparisons between 2 groups after a one-way ANOVA for comparisons between multiple groups.

RESULTS

Body weights of completely castrated fishes were lower than those of sham-operated fish in 2008 and 2009 ($p < 0.05$), whereas no significant difference was found in other comparisons (Table 1).

KSI

No differences in KSI values were found between the sham-operated control group and hemi-castrated control group in 2008 and 2009, but the KSI in the completely castrated control group was significantly lower than those in the sham-operated control group in both 2008-2009 ($p < 0.01$) and 2011 ($p < 0.001$) and also lower than that in the hemi-castrated control group in 2008-2009 ($p < 0.01$) (Fig. 1). In both the hemi- and completely castrated groups, 11KA treatments increased the KSI compared to the empty-implant fish (11KA(H) and 11KA(L), both $p < 0.05$) (Fig. 1). Completely castrated males treated with 2% T had a significantly higher KSI than the castrated controls ($p < 0.05$), but no such effect of 1% T treatment was found ($p > 0.05$) (Fig. 1). In sham-operated fish, the KSI increased after 11KA(H) treatment, but not after 11KA(L), 1% T, or 2% T treatments. Moreover, sham-operated males treated with the AI showed a significantly lower KSI than did the sham-operated controls ($p < 0.05$) (Fig. 1).

Effects of 11KA treatments on androgen levels

In the 2008-2009 samples, both operations and 11KA treatments had significant effects on 11KT levels (two-way ANOVA; $p < 0.001$ for each comparison). Both doses of 11KA significantly increased 11KT plasma levels in completely castrated and hemi-castrated fish ($p < 0.001$

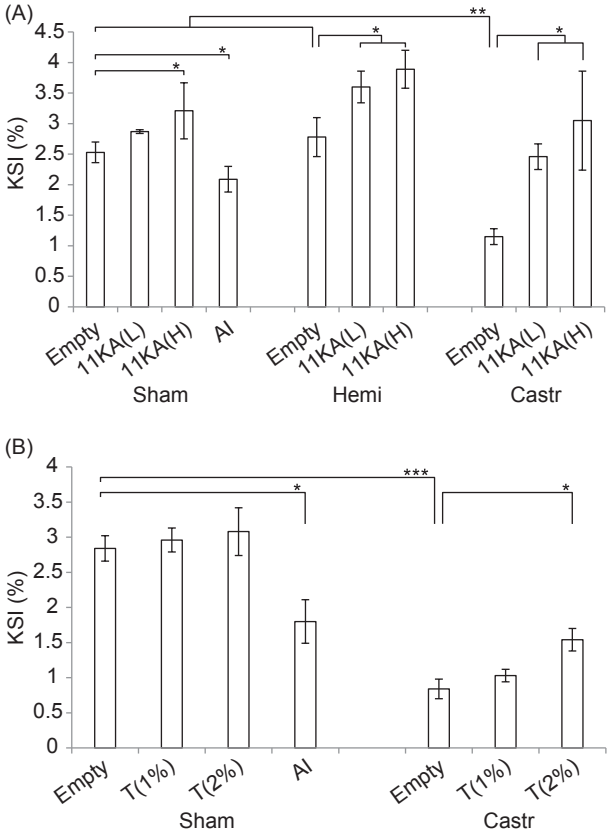

Fig. 1. Kidney-somatic index (KSI) of sham-operated (Sham), hemi-castrated (Hemi), and completely-castrated (Castr) sticklebacks with different androgen and AI treatments. (A) 2008-2009; (B) 2011. Values are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

and < 0.05, respectively) (Fig. 2). In the sham-operated groups, 11KT plasma levels increased in the 11KA(H) treatment group ($p < 0.05$), but not in the 11KA(L) treatment group (Fig. 2). Plasma levels of both 11KT and T in the sham-operated controls were higher than those in hemi-castrated controls ($p < 0.05$), and both were higher than those in the completely castrated control ($p < 0.001$ and < 0.05, respectively). Plasma levels of 11KT and T in sham-operated fish treated with 11KA(H) were higher than those in hemi-castrated fish treated with 11KA(H), and they were higher than in completely castrated fish with similar implants ($p < 0.05$ for each comparison). The operations significantly influenced T levels (two-way ANOVA; $p < 0.001$), but no effect of 11KA treatments was found on plasma T levels (two-way ANOVA; $p > 0.5$).

Effects of T treatments on androgen levels

Sham-operated fish treated with 2% T showed higher T plasma levels than sham-operated fish treated with 1% T or empty implants ($p < 0.05$ for both comparison), but there was no difference in plasma T levels found in fish treated with 1% T and the empty implants ($p > 0.05$) (Fig. 3). Plasma T levels of completely castrated fish treated with 1% T and 2% T implants were far higher than that in the castrated controls ($p < 0.001$), and completely castrated fish treated with 2% T had higher T plasma levels than that of fish treated with 1% T ($p < 0.05$) (Fig. 3).

The plasma T level in the sham-operated controls was higher than that in the completely castrated control ($p < 0.001$). However, T levels in sham-operated fish treated with 1% or 2% T showed no difference from those in completely castrated fish with the same treatments.

In 2011, 11KT plasma levels were close to or below the detection level in all castrated groups. Also, there was no difference in 11KT levels among the control, 1% T, and 2% T treatments in the sham-operated groups ($p > 0.1$ for all comparisons). Both operations and dosages of T treatments had highly significant effects on T plasma levels (two-way ANOVA; $p < 0.001$ for each

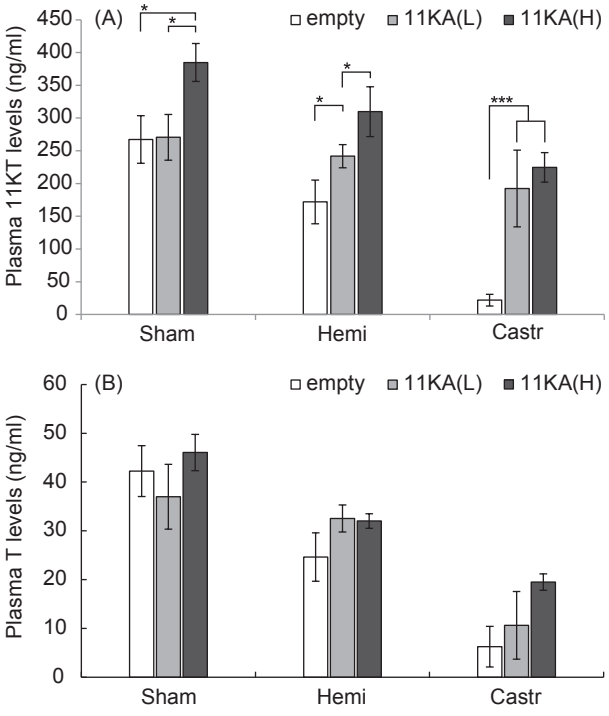

Fig. 2. Plasma androgen levels in sham-operated (Sham), hemi-castrated (Hemi), and completely-castrated (Castr) sticklebacks treated with different dosages of 11-ketoandrostenedione (11KA). (A) 11-ketotestosterone (11KT) levels; (B) testosterone (T) levels. Values are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

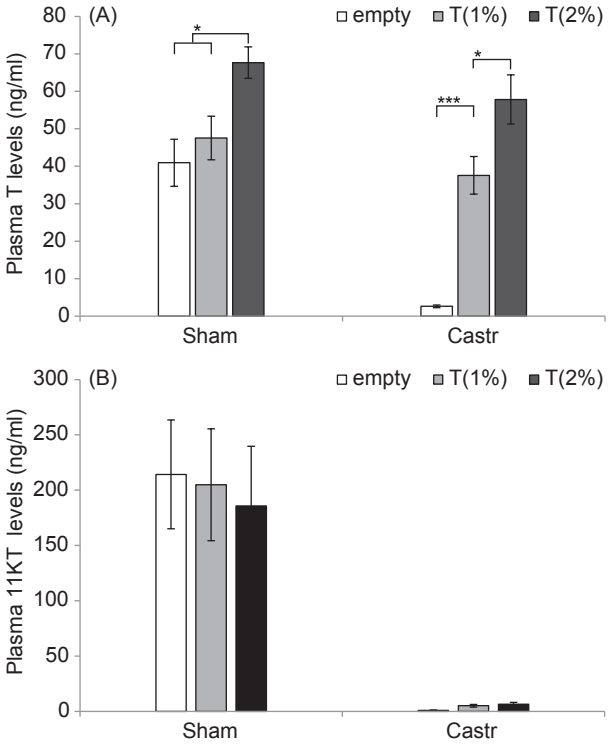

Fig. 3. Plasma androgen levels in sham-operated (Sham), hemi-castrated (Hemi), and completely-castrated (Castr) sticklebacks treated with different dosages of testosterone (T). (A) T levels; (B) 11-ketotestosterone (11KT) levels. Values are shown as the mean ± SEM. *$p < 0.05$; ***$p < 0.001$.

comparison). Furthermore, there was a significant interaction between operations and T dosages on T plasma levels (two-way ANOVA; $p < 0.05$).

AI treatment

Both in the 2008-2009 and 2011 samples, fish treated with the AI had similar plasma 11KT (2008-2009, $p = 0.084$; 2011, $p = 0.139$) and T (2008-2009, $p = 0.259$; 2011, $p = 0.141$) levels as the sham-operated controls (Fig. 4).

DISCUSSION

Although the low-T treatment increased T levels in castrated fish, it had no effect on levels in sham-operated fish. Similarly, 11KT levels in both hemi- and completely castrated fish treated with 11KA(L) rose significantly, which was not the case in sham-operated fish. This indicates that there is homeostatic control of androgen levels in the 3-spined stickleback. High doses of T and 11KA, on the other hand, were able to increase respective levels of T and 11KT in all groups of operated fish. Also, 11KT levels in sham-operated males treated with a high dose of 11KA were higher than those in similarly treated castrated fish, suggesting that levels in the former were due to both endogenous and exogenous androgens. This indicates that even though there is homeostatic control, its capacity is limited, as also suggested by reduced androgen levels found in the hemi-castrated controls. Kurtz et al. (2007) implanted high and low doses of 11KA in intact breeding stickleback males. In contrast to our results, plasma 11KT rose significantly after treatment with either dose. A reason for this discrepancy may have been that they used larger capsules and smaller fish, thus their low dose was probably effectively higher than the one we used.

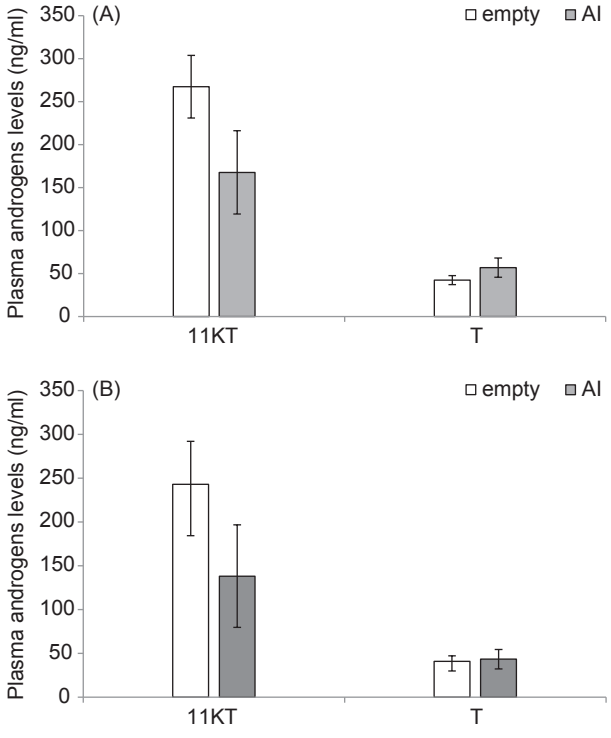

Fig. 4. Plasma androgen levels of the sham-operated stickleback treated with an androgen inhibitor (AI). (A) 2008-2009; (B) 2011. Values are shown as the mean ± SEM.

Sex steroid secretion is controlled by gonadotropic hormones (GtHs) from the pituitary. The release of GtHs is controlled by the hypothalamus and also by feedback effects on the BPG axis. Several studies showed that circulating GtH levels increase after castration in breeding fish, indicating negative feedback (Billard et al. 1977, de Leeuw et al. 1986, Larsen and Swanson 1997, Schulz et al. 2012). However, both negative and positive feedback mechanisms on the BPG axis are known from fishes, including the stickleback (Hellqvist et al. 2003 2008, Shao et al. 2013). Borg et al. (1985) treated breeding stickleback males with a high dose of methyltestosterone via the water, which suppressed steroidogenesis (3β-hydroxysteroid-dehydrogenase activity) by the testes. In the present study, it is doubtful if androgen treatments led to a dramatic decrease in steroid production, since 11KT levels were not changed by T treatments or T levels by 11KA treatments.

Circulating androgen levels decreased after hemi-castration, and plasma 11KT and T levels of hemi-castrated fish were only a little more than 1/2 of those found in sham-operated fish. This is in agreement with results of Hellqvist et al. (2002) on sticklebacks and largely also on salmon. However, in hemi-castrated African catfish, circulating T and 11KT levels showed only minor and non-significant reductions, whereas plasma luteinizing hormone levels significantly increased (Schulz et al. 2012). Furthermore, androgen production in tissues of the remaining testis after hemi-castration was 2-3-fold higher than that in sham-operated fish (Schulz et al. 2012). The lack of androgen compensation in hemi-castrated male sticklebacks, which contrasts with homeostatic compensation after low-dose androgen administration, is difficult to explain, although the results are clear. Apparently there is no compensatory increase in androgen production;

but why not? The remaining testis not possessing the capacity to do this is a possibility, though perhaps farfetched. However, hemi-castration did not suppress the KSI. Thus, it seems that the lower androgen levels were sufficient to support secondary sexual characters over this limited time period.

Unlike the non-aromatizable 11KA, T can be converted to estrogen by aromatase, and there is the possibility that the homeostatic effect of T was mediated (at least partly) via estrogen receptors. However, our results did not support this hypothesis, as no effects on plasma 11KT or T levels were found in sham-operated fish treated with the AI. AI treatment suppressed the KSI in both experiments. The mechanism behind this is not clear. There was a non-significant tendency for lower 11KT levels after AI treatment, but significantly reduced 11KT levels following hemi-castration did not result in lower KSI values.

Although androgen levels were lower in hemi-castrated than in sham-operated fish, our results showed that both 11KA and T treatments increased plasma levels much less in sham-operated fish than in castrated ones, indicating that there was nevertheless homeostatic control of both 11KT and T, reducing the risk that excessively high plasma levels are attained.

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