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Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*

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Summary

The swimbladder and associated sonic muscle of the Lusitanian toadfish *Halobatrachus didactylus* increase in size throughout life and are, respectively, 25% and 30% larger in type I (nest-holder) males than females, which may generate sexual differences in sound production. Sexual dimorphism in swimbladder is also evident in the morphological features of sonic muscle fibers. During the breeding season, type I males have smaller myofibril contracting zones surrounded by larger sarcoplasm areas compared with females, possibly an adaptation to speed and fatigue resistance for the production of long mating calls. Type II (floater) males show characteristics that are intermediate, but statistically not significantly different, between type I males and females. Six weeks after castration and androgen (testosterone and 11-ketotestosterone) replacement in type I and type II males there were no alterations either in swimbladder mass or fiber morphology. However, 17β-estradiol induced a significant decrease in swimbladder mass and sarcoplasm area/myofibril area ratio. Six months after castration there was a clear reduction in the seasonal swimbladder hypertrophy in males and induction of sonic fiber morphological characteristics that resemble those occurring in females (low sarcoplasm area/myofibril area ratio). These results suggest that testicular factors are required to initiate sonic muscle hypertrophy and type I sonic fiber phenotype in *H. didactylus*, but a specific involvement of androgens has not been completely clarified.

Key words: muscle hypertrophy, androgen, castration, male dimorphism, toadfish, *Halobatrachus didactylus*.

Introduction

Toadfishes and midshipmen produce sounds associated with courtship and agonistic interactions. During the breeding season, nest-holding toadfish males produce a mating call resembling an intermittent boatwhistle (Breder, 1968; Fine et al., 1977; Fish and Mowbray, 1970; Gray and Winn, 1961), and midshipman nest-holding males emit a continuous humming to attract females (Brantley and Bass, 1994; Ibara et al., 1983). While the long mating calls are only produced by nesting males (Brantley and Bass, 1994; Gray and Winn, 1961; Winn, 1967, 1972), agonistic short duration grunts have been recorded from both male (nesting or not) and female (Brantley and Bass, 1994; Tavolga, 1958) toadfishes and midshipmen. Recordings of Lusitanian toadfish (*Halobatrachus didactylus*) sounds during the breeding season have shown that a variety of sounds are produced – long-duration boatwhistle and short-duration calls, grunts, croaks, mixed grunt/croaks and double croaks – resembling those produced by the other batrachoidids (dos Santos et al., 2000).

The sonic organ of batrachoidids is the swimbladder. Sound production results from the contraction of paired sonic muscles attached to the walls of the swimbladder, which cause a rapid variation in swimbladder volume and internal pressure (Skoglund, 1961; Tower, 1908). Sonic fibers have a number of morphological and biochemical adaptations for rapid contraction speed: (1) an unusual radial morphology [fibers are of polygonal shape and contain a core of sarcoplasm surrounded by a contractile cylinder of alternating slender ribbons of sarcoplasmic reticulum (SR) and myofibrils; Fawcett and Revel, 1961; Fine et al., 1993; Loesser et al., 1997], (2) an extremely well-developed SR forming about a third of the fiber volume (Appelt et al., 1991; Franzini-Armstrong and Nunzi, 1983) and (3) the calcium transient is the fastest ever recorded in a vertebrate muscle (Rome et al., 1996, 1999). The sarcoplasm immediately beneath the cell membrane and inside the core contains glycogen granules and mitochondria, but, unlike most muscle fibers, mitochondria are excluded from the contractile portion of the fiber. The functional design of a radial arrangement of alternative ribbons of SR and myofibrils minimises the distance and thus the transport time of calcium to the contractile proteins and back to the SR (Fine et al., 1993).

The swimbladder and sonic muscles of toadfishes and midshipmen increase in size throughout life and are larger in males than in females; this sexual dimorphism appears to be related to the type of sound produced (Brantley et al., 1993b; Fine, 1975; Fine et al., 1990a). Both sonic muscle growth and

enzyme activity have been shown to be androgen sensitive (Pennypacker et al., 1985; Walsh et al., 1989). The mass of the swimbladder–sonic muscle complex is increased by androgen implants in gonadectomised males and females of the oyster toadfish *Opsanus tau* (Fine and Pennypacker, 1986) and in juvenile males and females and sneaker/satellite males (type II males) of the plainfin midshipman *Porichthys notatus* (Brantley and Bass, 1991; Brantley et al., 1993a). Additionally, androgen implants also influence fiber morphology by increasing the area of sarcoplasm and thereby mitochondria density (Brantley et al., 1993a).

In the Lusitanian toadfish, a significant increase in the sonic muscle mass of type I males occurs during the breeding season, whereas females and type II males do not show this increase (Modesto and Canario, 2003). One hypothesis to explain this seasonal hypertrophy is that elevated levels of 11-ketotestosterone (11KT) at the beginning of the reproductive period modulate the growth of sonic muscle, since, among reproductive males, 11KT but not testosterone (T) is elevated in type I males compared with type II males (Modesto and Canario, 2003). Considering the potency of 11KT at inducing secondary sexual characters in other teleosts (Borg, 1994), this steroid is a strong candidate for generation and/or maintenance of sonic muscle sexual dimorphism.

The present study investigated whether *H. didactylus* sonic muscle size and/or sonic muscle fiber morphology are indeed under androgenic control. Firstly, we have examined the morphological changes in sonic fiber structure underlying the seasonal changes in sonic mass of females, type I and type II males. Secondly, we have evaluated the responsiveness of sonic muscle to steroid treatment. Finally, we have analysed the long-term effect of castration on sonic muscle mass and on fiber structure.

Materials and methods

Animals and seasonal sampling

The swimbladders used in the seasonal analysis of histomorphology were from the same fish reported in a previous study of reproductive and endocrine cycle of *Halobatrachus didactylus* Bloch and Schneider 1801. All the fish were collected in Ria Formosa (South of Portugal, 37°00′ N, 7°65′ W). Fish were divided into two groups based on their relative swimbladder masses: the ‘late spring/summer’ group included fish collected from May to July (breeding season), which had high relative swimbladder masses (females, N=12; type I (nest-holder) males, N=12; type II (floater) males, N=7), and the ‘winter’ group included fish captured from December to February, which had low relative swimbladder masses (females, N=12; type I males, N=12; type II males, N=5). Swimbladders were weighed, fixed and weighed again (total swimbladder mass, M_{TS}) after a period of 3–6 months. Mass loss in fixative was, on average, 22%. Muscles from fixed swimbladders were removed with a scalpel by gently cutting the attachments between the muscles and the swimbladder wall. The muscle mass (sonic muscle mass, M_M) was subtracted from total swimbladder mass to give swimbladder wall mass (M_S).

Animals used in laboratory experiments were collected in Ria Formosa and kept in 1 m^3 tanks in running seawater, at natural water temperature and photoperiod and fed three times a week on frozen squid.

Hormone implants

Synthetic steroids were purchased either from Steraloids (Newport, RI, USA) or Sigma–Aldrich Chemical Co. (Madrid, Spain) and kept at 4°C in ethanol stock solutions (1.5 μmol l^{-1}). Steroids were administered intraperitoneally as a liquid suspension (10%) in warm coconut oil (Leatherland, 1985). As coconut oil solidifies below 25°C, implants formed a single long mass inside the body cavity and acted as a slow-release pellet.

Expt A – in vivo *steroid release by implants*

This experiment was carried out to determine the dynamics of steroid release from the coconut oil implants. Type I males collected in May were anesthetized with 2-phenoxyethanol (0.2 ml l^{-1}; Sigma–Aldrich Chemical Co.), tagged in the operculum with Presadon tags (Chevillot, France), measured to the nearest mm to give total length (L_T) and weighed to the nearest 0.1 g to give total (M_T) and eviscerated mass (M_E). Mean (± S.E.M.) L_T and M_E of the fish used in the experiment were 207±6 mm (range 181–241 mm) and 176.5±16.1 g (range 132.7–262.1 g), respectively. Six fish received implants containing T. The volume of implants was proportional to body mass and was administered intraperitoneally at a steroid dose of 10 μg g^{-1} (group 1) or 100 μg g^{-1} (group 2). Three control fish received implants without hormone (group 0). Blood samples (200 μl) were collected from the caudal vein in heparinised syringes immediately before implantation and 0.5, 1, 2, 4, 7, 14, 21 and 35 days later. Plasma was separated by centrifugation (7500 ***g***, 5 min) and stored at –20°C until analysis.

Expt B – short-term effects of castration and steroid replacement on swimbladder mass and fiber morphometry

H. didactylus type I males were collected in May and acclimated to the laboratory for one week. They were then anesthetized, tagged, measured, weighed and a blood sample collected as described above. Mean values of L_T and M_E of the fish used in the experiment were 228±5 mm (range 172–302 mm) and 204.2±12.1 g (range 94.5–431.4 g), respectively.

Castration was carried out by placing fish on their dorsal side on a table and making an incision in the abdomen of approximately 3 cm. Testes and accessory glands were removed and weighed. Throughout the procedure fish were maintained immobilised by pumping a continuous flow of aerated water carrying anaesthetic *via* the mouth over the gills. The incision was sutured and an antiseptic (betadine; Mundipharma, Zurich, Switzerland) applied to the wound. As controls, some fish received the incision, and testes were

exposed, handled and sutured (SHAM), while others were not operated on (INT). To directly test whether steroids can stimulate muscle hypertrophy or alterations in fiber morphology, castrated fish received coconut oil implants containing 100 μg g^{-1} of T, 11KT or 17β-estradiol (E_2) for a period of six weeks. A control group received steroid-free implants. Hence, the experimental groups were: intact (INT), sham-operated (SHAM), castrated (CAST), castrated plus control implants (CAST + C), castrated plus T implants (CAST + T), castrated plus 11KT implants (CAST + 11KT) and castrated plus E_2 implants (CAST + E_2). The number of fish per group is indicated in Table 1. Fish were allowed to recover from anesthesia in individual receptacles with aerated seawater before being transferred to separate experimental tanks for each group. No food was offered for the first two days after surgery. Two weeks after implantation, blood samples were collected from lightly anesthetized fish for steroid analysis. At the end of the experiment (six weeks after implantation), the fish were measured and weighed and blood collected as described above. The animals were sacrificed by spinal transection and the swimbladders removed, weighed and fixed for histological analysis.

Expt C – long-term effects of castration on swimbladder mass and fiber morphometry

Adult *H. didactylus* were caught in December and surgically castrated as described above. Mean L_T and M_E values for the animals used in the experiment were 251±5 mm (range 197–340 mm) and 282.0±18.5 g (range 105.6–782.0 g), respectively. The experimental groups were: type I males intact, i.e. not operated and not castrated (M_I INT), type I males castrated (M_I CAST), type II males intact (M_{II} INT), type II males castrated (M_{II} CAST) and a group of intact females (F INT). The number of fish per group is indicated in Table 2. Fish were maintained in the experimental tanks for six months after castration. By the time of natural spawning in the field, June, all groups were sacrificed and sampled as described for experiment B. In experiments B and C, at autopsy the body cavity of castrated fish was inspected for testicular and/or accessory testicular gland remnants.

Table 1. *Treatment groups, number of fish (*N*) and adjusted means ± S.E.M. of total swimbladder masses (*M_{TS}*) in experiment B*

Treatment	*N*	M_{TS}
INT	10	4.14±0.15
SHAM	10	3.94±0.14
CAST	8	3.89±0.16
CAST + C	11	3.84±0.13
CAST + T	10	3.84±0.14
CAST + 11KT	7	3.91±0.18
CAST + E_2	7	3.36±0.14*

*Significant differences from respective control (CAST + C) at $P<0.05$.

INT, intact; CAST, castrated; SHAM, sham-operated; C, control (no hormone); T, testosterone; 11KT, 11-ketosterone; E_2, 17β-estradiol.

Table 2. *Treatment groups, number of fish (*N*) and adjusted means ± S.E.M. of total swimbladder masses (*M_{TS}*) in experiment C*

Treatment	*N*	M_{TS}
M_I INT	10	5.41±0.14
M_I CAST	12	4.83±0.10*
M_{II} INT	9	5.23±0.13
M_{II} CAST	9	4.74±0.11*
F INT	12	4.97±0.11

*Significant differences from respective control at $P<0.01$.

INT, intact; CAST, castrated; M_I, type I male; M_{II}, type II male; F, female.

Histology and histomorphometry

Swimbladders were fixed in Bouin's fluid for 48 h and then transferred into 70% ethanol. Sonic muscle fibers extend in a plane roughly perpendicular to the rostrocaudal axis, aligned in concentric layers in a C-shaped course. Fixed muscles were cut with a blade along the rostrocaudal axis in the plane that cross-transected the muscle fibers to define the plane for subsequent sectioning. The muscle slices were embedded in paraffin, sectioned (5–7 μm) and stained with hematoxylin and eosin (H&E).

Muscle structure was analysed using the OPTIMAS 5.2 computerized image analysis system (BioScan, Inc., Edmonds, WA, USA). Mean areas of fiber components were derived from measurements of 50 randomly chosen cells in each swimbladder. Video images of the cells were digitized to generate cross-sectional areas of the muscle fibers (total fiber area), the myofibril-containing zone (myofibril area) and the peripheral sarcoplasm (sarcoplasm area) (see Fig. 1). In *H. didactylus*, the sarcoplasmic central core of myofibrils, which is present in adult fibers of other toadfishes, is nonexistent or barely visible by light microscopy and is therefore not measurable.

Steroid assays

Sex steroids in plasma were measured by radioimmunoassays (RIAs) following the methodology described in Scott and Canario (1992); the origin and cross-reactions for the T, 11KT and E_2 antisera have been previously reported by Scott et al. (1984), Kime and Manning (1982) and Guerreiro et al. (2002), respectively. Intra-assay and inter-assay precision (coefficient of variation) were 7.5% and 12.4% for T, 8.2% and 11.6% for 11KT, and 8.0% and 8.8% for E_2, respectively. The limit of detection of assays was 100 pg ml^{-1} for E_2 and T and 160 pg ml^{-1} for 11KT.

Statistical analysis

All data are expressed as means ± S.E.M. Data (masses, section areas and hormone concentrations) were first log-

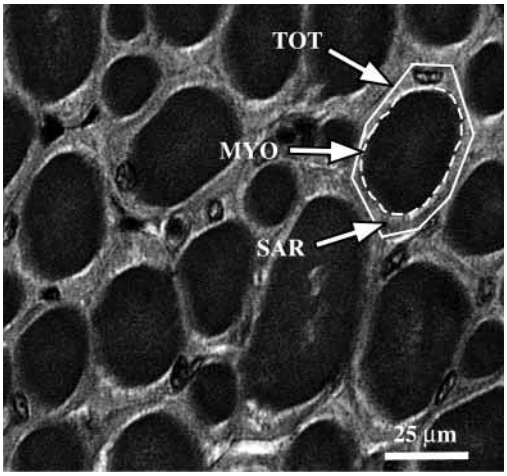

Fig. 1. Transverse section of sonic muscle fibers of *H. didactylus*. TOT, perimeter of fiber, continuous line; MYO, perimeter of myofibril, broken line; SAR, peripheral sarcoplasm. H&E stain.

transformed before statistical treatment to obtain normality and homogeneity of variance. Swimbladder and sonic muscle masses and morphometric measurements of sonic muscle fibers were compared between groups using analysis of covariance (ANCOVA), with eviscerated body mass as covariate. Least squares means and their standard errors adjusted for eviscerated body mass are reported. Plasma steroid concentrations were compared by two-way analysis of variance (ANOVA). Tukey's honest significant difference test was used for *post-hoc* comparisons among means. Statistical significance for all tests was considered at the 5% level. Pairwise comparisons of fiber characteristics between the intact females and each of the experimental male groups in experiment C were made using linear contrasts in ANCOVA. The software SPSS (version 9.0, SPSS Inc., Chicago, IL, USA) was used to carry out the statistical analysis.

Results

Seasonal changes in the swimbladder and morphometry of muscle fibers

Total swimbladder mass (M_{TS}), sonic muscle mass (M_M) and swimbladder wall mass (M_S) were linearly related to eviscerated fish mass (M_E) for both type I males and females: $M_{TS}=0.2790+0.0202M_E$, $r^2=0.922$; $M_{TS}=0.0744+0.0168M_E$, $r^2=0.895$; $M_M=0.5340+0.0127M_E$, $r^2=0.839$; $M_M=-0.0701+0.0126M_E$, $r^2=0.902$; $M_S=0.1290+0.0035M_E$, $r^2=0.673$; $M_S=0.0796+0.0033M_E$, $r^2=0.597$, respectively, for males and females ($P<0.001$ in all cases). During the reproductive months, type I males had swimbladders and sonic muscles 25% and 30% heavier than females, respectively (M_{TS}, 3.24±0.05 g and 2.59±0.05 g; M_M, 2.67±0.09 g and 2.05 g; adjusted means for type I males and females, respectively; $P<0.0001$), but swimbladder wall masses were not significantly different between sexes (0.71±0.03 g and 0.63±0.05 g for type I males and females, respectively; $P=0.136$).

Sonic muscle fibers exhibited seasonal changes in morphology (Fig. 2). In winter, total fiber area of sonic muscle was significantly smaller in type I males than in females or type II males, but no significant differences were found during late spring/summer. Total fiber area in type I males was also significantly larger in late spring/summer compared with winter. In winter, the myofibril area was significantly smaller in type I males than in females or type II males. In late spring/summer, type I males still had a myofibril area significantly smaller than females but not than type II males. In winter, the sarcoplasm area was similar between females and the two types of males, but in spring/summer it showed a significant increase only in type I males. If mean sarcoplasm area is scaled to mean myofibril area, the resulting ratio (sarcoplasm area/myofibril area) for type I males in spring/summer far exceeds those of females but not of type II males. Altogether, morphometric measurements of sonic fibers showed that during the breeding season type I males had smaller myofibril contracting zones surrounded by larger areas of peripheral sarcoplasm compared with females, while type II males showed intermediate (but not significantly different) values of sarcoplasm area/myofibril area ratio between type I males and females.

Experiment A – in vivo *steroid release by implants*

Plasma T levels in the implanted animals showed a sharp increase within 0.5 days of implantation (Fig. 3). In group 1 (10 μg g^{-1} implant), mean T plasma levels reached 89.02±6.40 ng ml^{-1}, while group 2 (100 μg g^{-1} implant) reached significantly higher levels of 175.05±8.66 ng ml^{-1}. In both groups, a hyperbolic decay of T plasma levels followed, and after a further two weeks T levels in group 1 and group 2 were 5.40±3.47 ng ml^{-1} and 24.00±8.76 ng ml^{-1}, respectively. At this time, plasma T levels in group 1 no longer differed significantly from the control group ($P=0.674$). Five weeks after implantation, group 2 still had significantly higher plasma T levels than groups 1 and 0 (6.24±0.17 ng ml^{-1} *vs* 2.12±0.72 ng ml^{-1} and 2.64±1.13 ng ml^{-1}, respectively; $P<0.05$). Plasma T levels of control animals did not change significantly over the duration of the experiment ($P=0.250$).

Experiment B – short-term effects of castration and steroid replacement on swimbladder mass and fiber morphometry

Castrated animals showed a reduction in T, 11KT and E_2 levels after six weeks compared with initial values and with levels of intact (INT) and sham-operated (SHAM) animals ($P<0.05$; Fig. 4). Two weeks after implantation, T, 11KT and E_2 levels were significantly higher in the respective hormone-implanted groups ($P<0.05$). In addition, E_2 levels were elevated in the CAST + T group after 6 weeks ($P<0.05$), indicating some degree of extra-gonadal aromatization.

Adjusted swimbladder masses of SHAM animals were not significantly different from those of INT animals, showing that surgery itself had no effect ($P=0.410$; Table·1). Additionally,

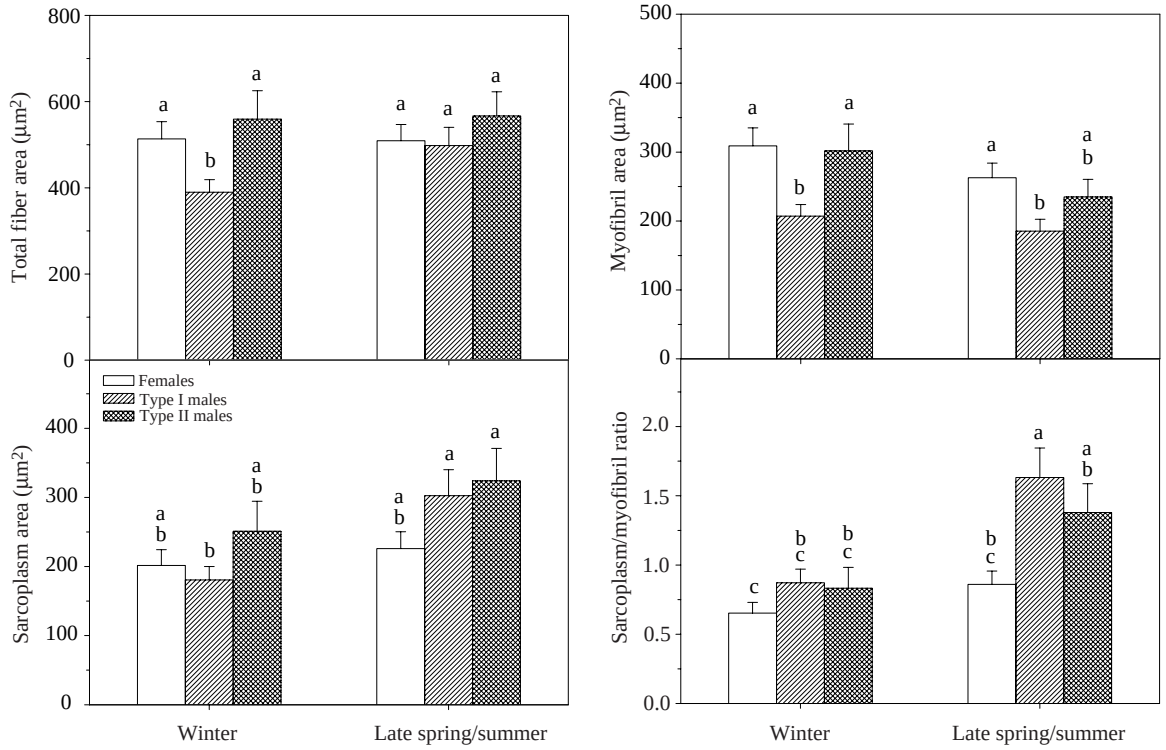

Fig. 2. Comparison of sonic muscle fiber components (adjusted mean areas) of females, type I males and type II males between winter season and late spring/summer season (breeding season). Different letters in the same graph indicate statistically significant differences among groups and seasons (P<0.05). Vertical bars represent S.E.M.

CAST animals had an M_{TS} similar to INT animals (P=0.200). Only E_2-implanted castrated animals (CAST + E_2) showed a significant reduction in M_{TS} compared with control animals (CAST + C; P<0.05; Table 1).

Neither the incisions nor the castration had significant effects on any of the structural features of sonic fibers (P>0.100; not shown). The hormone treatment had no significant effect on total fiber area (P=0.471), myofibril area (P=0.627) or sarcoplasm area (P=0.059) but did have a significant effect on sarcoplasm area/myofibril area ratio (P<0.01; Fig. 5): E_2-treated animals exhibited a sarcoplasm area/myofibril area ratio significantly lower than that of the control (CAST + C; P<0.05).

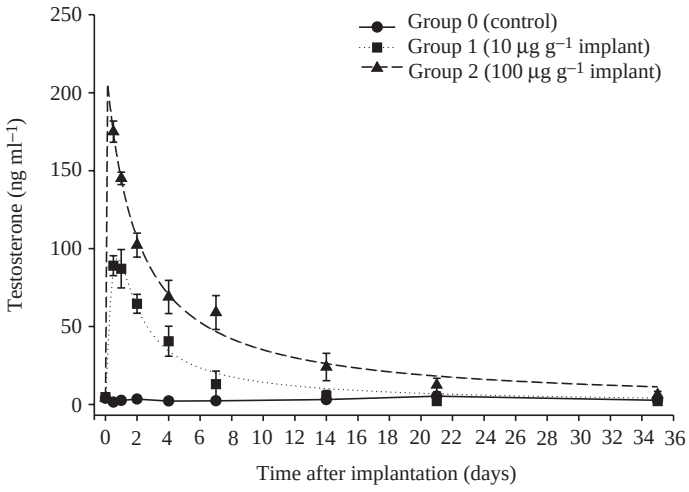

Fig. 3. Plasma levels of testosterone after intraperitoneal testosterone implantation in type I males (experiment A). Vertical bars represent S.E.M.

Experiment C – long-term effects of castration on swimbladder mass and fiber morphometry

In control groups of both male morphs, plasma levels of androgens increased significantly from the beginning of the experiment, reflecting normal testicular recrudescence. By contrast, plasma levels of castrated groups decreased significantly during the experimental period (P<0.05; Fig. 6).

Six months after castration, there were significant differences in M_{TS} between the control and castrated groups for both types of males (P<0.01; Table 2). Castrated fish had a lower M_{TS} than non-castrated fish. The swimbladders of both morphs responded similarly to castration, both in mass (morph × treatment, P=0.115) and in morphology of sonic muscle fibers (morph × treatment, P>0.9 for any sonic fiber characteristics).

Castration increased total fiber area (marginally significant, P=0.048) and myofibril area (P<0.001) in both morphs but had no effect on sarcoplasm area (P=0.836). This was reflected in the ratio of sarcoplasm area/myofibril area, which showed a significant decrease in castrated groups (P<0.001; Fig. 7).

Castrated type I males were similar to females in sarcoplasm area (P=0.989) and sarcoplasm area/myofibril area ratio

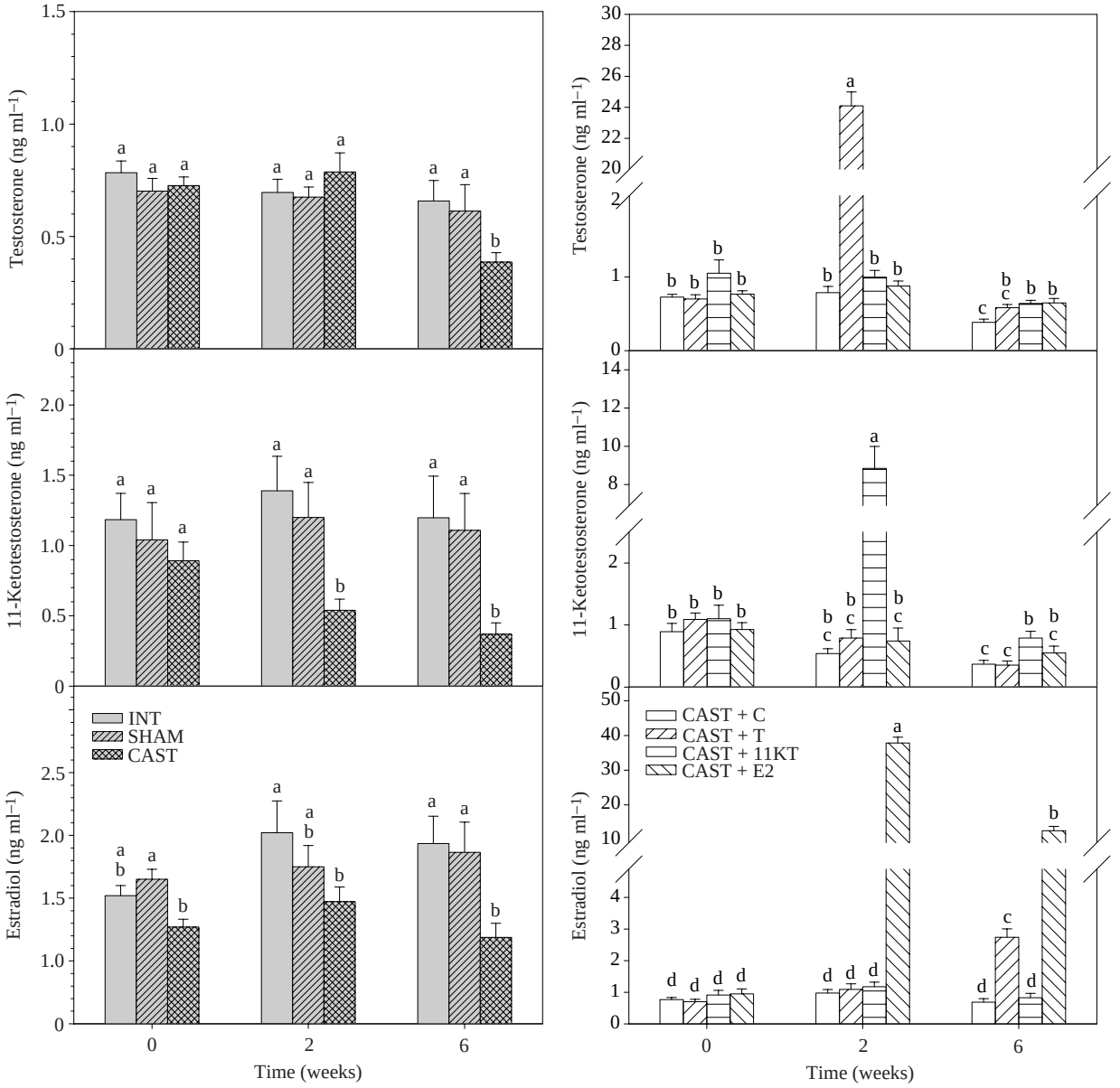

Fig. 4. Change in plasma levels of sex steroids according to treatment in experiment B immediately before surgery and 2 weeks and 6 weeks after surgery. Treatment groups are: intact (INT), sham-operated (SHAM), castrated (CAST), castrated plus control implants (CAST + C), castrated plus testosterone implants (CAST + T), castrated plus 11-ketotestesterone implants (CAST + 11KT), castrated plus 17β-estradiol implants (CAST + E_2). Different letters in the same graph indicate statistically significant differences among groups and sampling time (P<0.05). Vertical bars represent S.E.M.

(P=0.477), while castrated type II males were similar to females in all of the fiber characteristics (total fiber area, P=0.964; myofibril area, P=0.666; sarcoplasm area, P=0.649; sarcoplasm area/myofibril area ratio, P=0.975).

Discussion

In *H. didactylus*, the swimbladder grew heavier in males than in females and most of the difference was a consequence of sonic muscle development, since the swimbladder wall mass did not differ among sexes.

During the breeding season, the general hypertrophy of sonic muscle mass in *H. didactylus* males was accompanied by structural modifications in muscle fibers. Males had thinner myofibrils and more sarcoplasm and, therefore, a bigger sarcoplasm area/myofibril area ratio than females, which is similar to what has been observed in male weakfish (*Cynoscion regalis*; Connaughton et al., 1997) and the toadfish *O. tau* (Fine et al., 1990a). For the toadfish, Fine et al. (1990a) hypothesized that the thinner myofibers in males are an adaptation to the increased speed and fatigue resistance necessary for production of the boatwhistle mating call, since the small size and

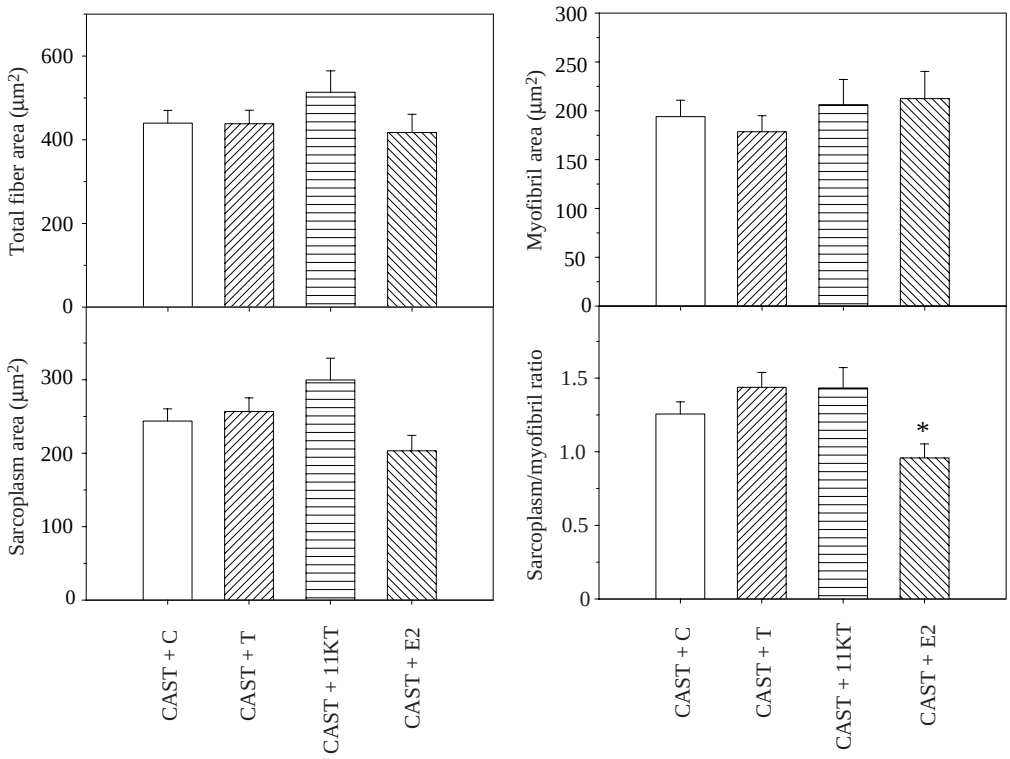

Fig. 5. Sonic muscle fiber components (adjusted mean areas) according to treatment in experiment B six weeks after surgery. Treatment groups are: castrated plus control implants (CAST + C), castrated plus testosterone implants (CAST + T), castrated plus 11-ketotestesterone implants (CAST + 11KT), castrated plus 17β-estradiol implants (CAST + E_2). *Significant differences from control group (CAST + C) at $P<0.05$. Vertical bars represent S.E.M.

concomitant large surface-to-volume ratio would facilitate rapid fluxes of glucose, oxygen, lactic acid and CO_2. Additionally, enlargement of the sarcoplasm area permits larger mitochondria content around the myofibril zone, essential to the energetic demands of boatwhistles. Appelt et al. (1991) found that sonic muscles in males have approximately three times as many mitochondria as those in females. Electron microscopy has shown that in *O. tau* the larger fibers develop fragments of a contractile cylinder separated by channels of expanded sarcoplasmic reticulum (containing glycogen granules and mitochondria) that will form new, smaller and, therefore, more energy efficient fibers (Fine et al., 1993). In *H. didactylus*, although we did not measure the number of muscle fibers, the enlargement of the sonic mass in type I males during the breeding season suggests that hyperplasia of sonic fibers occurs and, together with alterations of sonic fiber morphology, may explain the ability to produce boatwhistle calls (dos Santos et al., 2000). In winter, when long calls are not used in mating behavior, sarcoplasm areas of fibers from females and type I male were not significantly different, possibly reflecting similar energetic and

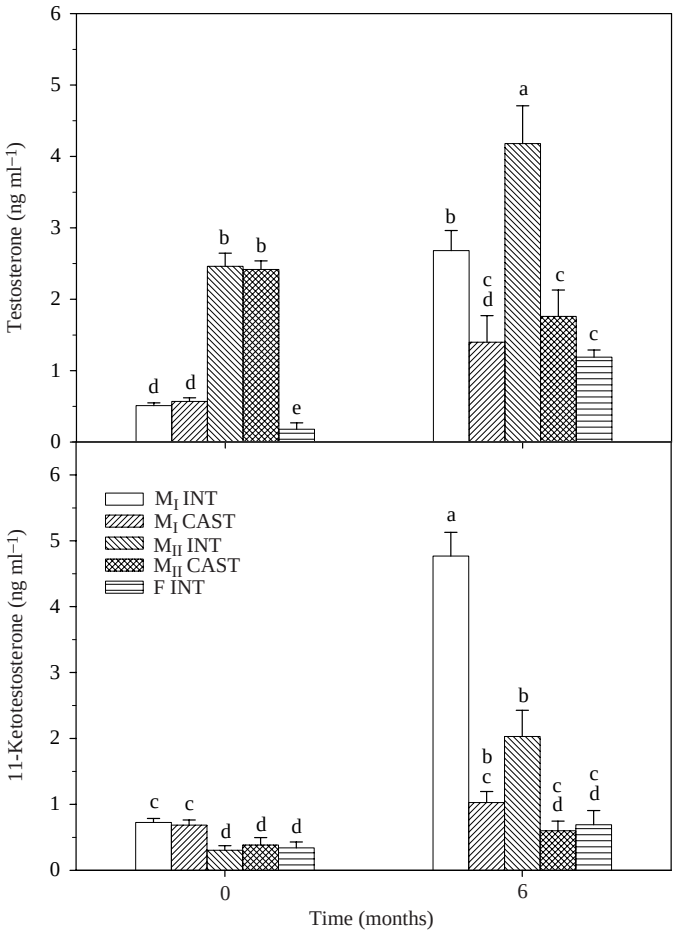

Fig. 6. Plasma levels of testosterone and 11-ketosterone in experimental groups in experiment C. Experimental groups are: intact type I males (M_I INT), castrated type I males (M_I CAST), intact type II males (M_{II} INT), castrated type II males (M_{II} CAST), intact females, (F INT). Different letters indicate statistically significant differences between groups and sampling time ($P<0.05$). Vertical bars represent S.E.M.

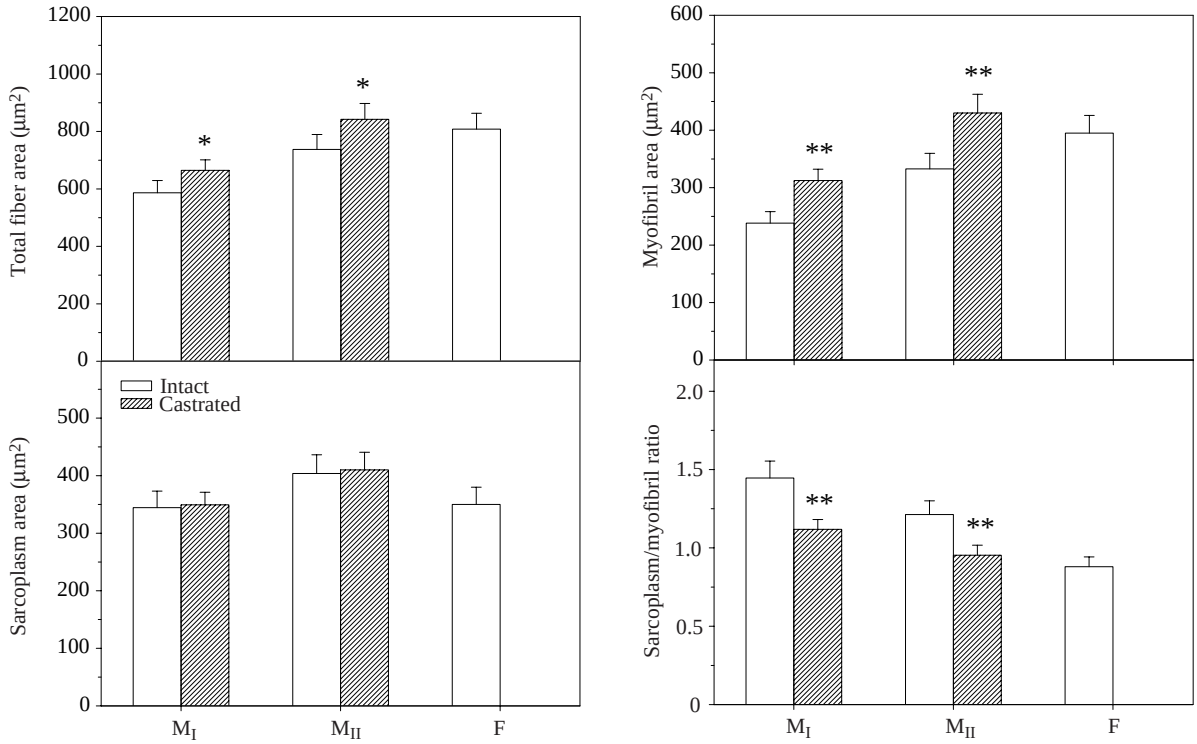

Fig. 7. Sonic muscle fiber components (adjusted mean areas) according to treatment in experiment C six months after surgery. Treatment groups are: type I males (M_I), type II males (M_{II}), intact females (F). * and ** indicate significant differences from corresponding control (intact group of same morph) at $P<0.05$ and $P<0.001$, respectively. Vertical bars represent S.E.M.

mechanical demands to produce mainly agonistic short calls by both sexes.

Comparison of the characteristics of the swimbladder and sonic fibers among batrachoidids shows that the midshipman *P. notatus* exhibits a pronounced swimbladder dimorphism compared with the two toadfishes (Table 3). Nesting midshipman (type I) males have a much bigger sonic muscle mass, which is related to the elevated number of larger fibers, with larger myofibrils and larger peripheral and central zones of sarcoplasm than females (or sneaker/satellite type II males).

Additionally, inter- and intrasexual dimorphism of the peripheral sonic organs of *P. notatus* is also evident in the morphophysiological characteristics of the neuronal pathway controlling sonic muscle: type I males have larger cell bodies, dendrites, axons and terminal junctions between nerves and muscles, and pacemaker neurons fire at higher frequencies than in females or type II males (Bass and Marchaterre, 1989; Bass, 1996; Bass and Andersen, 1991; Fluet and Bass, 1990). In the toadfish *O. tau*, although no morphological differences in body size and sonic muscle characteristics are evident among males,

Table 3. *Characteristics of swimbladder and sonic fibers of three species of the order Batrachoidiformes*

	Opsanus tau[a]	*Porichthys notatus*[b]	*Halobatrachus didactylus*[c]
Swimbladder			
Swimbladder total mass	M 33%>F	M 600%>F	M 25%>F
Swimbladder wall mass	M 20%>F	–	M 13%>F
Sonic muscle mass	M 44%>F	–	M 30%>F
Sonic fibers			
Number of fibers per muscle	M 47%>F	M 500%>F	–
Total fiber area	M 15%<F	M 400%>F	M≅F
Myofibril area	M 21%<F M 13%<F	M 60%>F	M 42%<F
Sarcoplasm area	–	M 650%>F	M 34%>F

Relationships were established from adjusted means to body mass. M, nest-holder males; F, females.
[a]Data taken from Fine et al., 1990a, 1993; Appelt et al., 1991.
[b]Data taken from Brantley et al., 1993b.
[c]Data taken from present study.

as occurs in *P. notatus*, sonic motoneuron histology has showed that males can be separated into two groups – one with large motoneurons and the other with small motoneurons – but both morphs have similar dendrite diameter and neuron number. Females show morphological neuron characteristics similar to those of males with small motoneurons (Fine, 1997).

In contrast to *P. notatus*, where type II males are much smaller than type I males, *H. didactylus* type II males are not markedly different in size from type I males or females. In *H. didactylus*, there was a partial overlap in body sizes of females, type I males and type II males, although only type I males were found larger than 220 mm (Modesto and Canario, 2003). The moderate inter- and intrasexual dimorphism in body size of *H. didactylus* also applies to swimbladder masses and to features of sonic fibers and resembles that of the toadfish *O. tau*. During the breeding season (late spring/summer), type II male swimbladders were significantly heavier than those of females but were lighter than those of type I males, while both myofibril area and the ratio of sarcoplasm area/myofibril area of type II male fibers was intermediate, although not significantly different, between those of type I males and females. How these differences correlate with distinct characteristics of the neuronal circuitry, capacities in sound production and reproductive behavior still needs to be investigated.

In *H. didactylus*, six months but not six weeks of castration reduced swimbladder masses and decreased sarcoplasm area/myofibril area ratio. Furthermore, males castrated for six months showed morphological characteristics of fibers similar to those of intact females. Since castration of *H. didactylus* was performed in December, before the period of natural increase of sonic mass, results obtained after six months (experiment C) may indicate that testicular factors are required to initiate seasonal sonic muscle hypertrophy. However, these factors do not seem to be necessary to maintain sonic hypertrophy for short periods, since six weeks after castration (experiment B), during the period of sonic hypertrophy, no alterations in sonic mass or fiber morphology were observed. Similar negative results for swimbladder masses of short-term (3–4 weeks) castration have been obtained for *O. tau* (Fine and Pennypacker, 1986). Results obtained for short-term castration are corroborated by the fact that swimbladder masses of type I males of *H. didactylus* in nature peaked in June but stayed significantly higher until November, when gonadosomatic index and plasma androgen levels fell to the lowest levels (Modesto and Canario, 2003). So, it is possible that seasonal hypertrophy experienced by the sonic muscles may be triggered by an increase in plasma androgen levels in spring and by the progressive workload of sonic muscles to produce mating calls. By contrast, the slight decrease in sonic mass during the months after spawning (mid- to late summer) may be the result of a slow decrease in the use of sonic muscles and by the low levels of androgens.

Androgen implants (T and 11KT) did not affect sonic muscle mass in *H. didactylus*. However, E_2 caused a significant decrease in swimbladder mass and a reduction in the sarcoplasm area/myofibril area ratio, which possibly resulted from a slight simultaneous decrease in sarcoplasm area and an increase in myofibril area, both features characteristic of females. These effects of E_2 implantation were similar to those induced by the long-term castration and resemble the inhibitory effect in the development of type IIA skeletal fibers (characteristic of swimbladder muscles; Fine and Pennypacker, 1988) induced by E_2 in rats (Koboni and Yamamuro, 1989; Suzuki and Yamamuro, 1985). Walsh et al. (1995) cited unpublished results by Mommsen and Bass in which treatment of adult type I males of *P. notatus* with E_2 for a few weeks resulted in significant decreases in the overall mass of sonic muscle.

The effects of androgens either on sonic mass or on muscle fiber structure of *H. didactylus* were not as pronounced as reported in other teleosts. For example, in *P. notatus*, androgen (T and 11KT) implantation for nine weeks markedly increased the relative sonic muscle size in juvenile males, juvenile females and type II males, whereas E_2 and cholesterol had no effect. The principal androgen effect on fiber structure was an increase in the area of mitochondria-filled sarcoplasm, and thus the sarcoplasm area/myofibril area ratio increased by 1.4–2-fold in the androgen-treated groups (Brantley et al., 1993a). In the toadfish *O. tau*, although no seasonal pattern was found for changes in total swimbladder mass, sonic muscle mass or sonic motor nucleus neuron size (Johnson et al., 2000), sonic muscle was stimulated to grow by androgen (T and dihydrotestosterone) implants for 3–4 weeks both in males and in females, while E_2 implants only caused a significant increase in females but not in males (Fine and Pennypacker, 1986). Sonic muscle enzyme activity was also increased by androgens (Pennypacker et al., 1985) but had no effect on sonic motor nucleus neuron size, and these neurons did not concentrate androgens (T and dihydrotestosterone) or E_2 (Fine et al., 1982, 1990b, 1996). In *C. regalis*, a sciaenid species in which males have extrinsic sonic muscles, sonic muscle mass of T-implanted groups increased 2.5-fold over a period of three weeks compared with sham-implanted groups. In T-implanted groups, the myofibril-contracting zone of sonic fibers was significantly greater than those of the sham-implanted and time-zero groups, but little increase in sarcoplasmic area was noted (Connaughton and Taylor, 1995).

The failure of the sonic muscle in *H. didactylus* to respond to androgen could have been a consequence of several factors: (1) an inadequate dose of androgen and/or duration of the experiment, (2) the muscle already being at its maximum stimulation when implants were applied or (3) in this species other factors rather than androgens being responsible for sonic muscle hypertrophy and hyperplasia.

In the present study, we have tested the effect of sex steroids over a period of six weeks after a single implantation of 100 $\mu g\ g^{-1}$. This dose was similar to that used in *O. tau* (Fine and Pennypacker, 1986) and in *C. regalis* (Connaughton and Taylor, 1995) over a period of 3–4 weeks, which induced significant alterations in sonic muscle mass and fiber morphology. Additionally, during our study, plasma levels of

T and 11KT were always higher (experiment A) than those found in animals collected in nature during the same period (Modesto and Canario, 2003). So, it seems unlikely that dose/duration factors by themselves could explain the inefficiency of androgens to induce alterations in sonic muscles.

In nature, swimbladder masses of type I males start to increase in March and peak in June when plasma T and 11KT reach their highest values (Modesto and Canario, 2003). It is therefore possible that when steroid implants were applied (mid-May), the sonic muscle was already fully stimulated due to a period (from January) of continuous relatively elevated levels of androgens (particularly 11KT; Modesto and Canario, 2003) and of a progressive workload of sonic muscles to produce mating calls. In mammals, the intake of androgens and concomitant strength-training exercise have a synergetic effect and can induce an increase in muscle protein synthesis (Lamb, 1975), which is reflected in an increase in cross-sectional area of muscle fibers and formation of new muscle fibers (Alen et al., 1984; Kadi et al., 1999).

In *P. notatus*, androgen implants markedly induced the increase of sonic muscle size and the muscle cell phenotype typical of type I males in juvenile males but not in a similar way in juvenile females or type II males. This suggests that sexually immature males have a unique sensitivity to androgen-induced changes in sonic muscle mass and architecture, but type II males and females have either lost this sensitivity at some point in their life history or, in fact, never possessed it (Brantley and Bass, 1991; Brantley et al., 1993a). This difference in responsiveness of type I and type II males to androgens emphasizes the existence of different developmental trajectories between males (Bass, 1996). In some teleosts, a critical period exists during which organizational changes in morphology can be elicited by steroid treatment or gonadectomy. Following this period, full reversal cannot be totally accomplished through either method (Adkins-Regan, 1981). Since we have used adult mature type I males for steroid treatment, it is possible that if a critical period (age- and/or season-dependent hormone effects) exists in *H. didactylus* for the expression of sonic muscles it had already passed for these specimens prior to the time of experiments.

Moreover, muscle growth in vertebrates is often attributed to other factors such as growth hormone and insulin-like growth factors (Florini, 1987; Florini et al., 1996), and it cannot be ruled out that non-steroidal mechanisms (or other non-androgen steroids) could be involved in the development of sonic muscles in *H. didactylus*. Future studies to detect androgen/estrogen receptors in sonic muscles of *H. didactylus* would be important, since the presence of such receptors should reflect tissue hormone sensitivity.

In conclusion, although factors derived from the testes appear to be required for sonic muscle seasonal hypertrophy and hyperplasia, the involvement of androgens, as demonstrated for *O. tau* and *P. notatus*, was not found in *H. didactylus*, possibly because of a previous endogenous stimulation of adult type I males. Further studies testing specific sensitivity to inductive effects of androgens during winter and with juveniles and females are required to clarify this aspect and to understand swimbladder sonic muscle ontogeny.

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