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Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*

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Abstract

The Lusitanian toadfish has group synchronous oocytes, which grow from November until June–July when they are released probably as a single batch. Blood plasma levels of estradiol-17β (E_2) and testosterone (T) increase during vitellogenesis and drop rapidly during final maturation and ovulation, when 17,20β, 21-trihydroxy-4-pregnen-3-one (17,20β,21-P) levels increase. The male reproductive apparatus is composed of paired testes and multichambered accessory glands, which secrete mucosubstances and are connected to the spermatic duct. Changes in the gonadosomatic index of males paralleled the females but started to drop slightly earlier. The swimbladder and accessory glands also underwent important seasonal changes in weight reaching a maximum at spawning. T, 11-ketotestosterone (11-KT) and 17,20α-dihydroxy-4-pregnen-3-one (17,20α-P) were generally low except for a sharp peak in June. 17,20β,21-P also peaked in June and then declined slowly. 17,20β-dihydroxy-4-pregnen-3-one (17,20α-P) was undetectable in males and females. As with other species of the family two types of males were identified: type I males with smaller testes (ca. 7-fold) and larger accessory glands (ca. 3-fold) and swimbladders than type II. Type I males also had significantly higher (ca. 6-fold) 11-KT levels than type II males. This suggests a role for 11-KT in the development of structures important for reproductive behaviour.

Keywords: Lusitanian toadfish; *Halobatrachus didactylus*; Reproductive cycle; Gametogenesis; Sex steroids; Male dimorphism

1. Introduction

The Batrachoididae family, that includes toadfishes and midshipmen, is considered one of the most highly evolved groups of marine teleosts (Lagler et al., 1977) and has been the subject of many studies of life history, toxicology, ethology, neurophysiology, cardiology, and endocrinology (see Palazón-Fernández et al., 2001, for references). The Lusitanian toadfish, *Halobatrachus didactylus*, is an eastern Atlantic member of the family that inhabits estuaries and coastal lagoons, partly buried or concealed in rock crevices (Roux, 1986). The spawning season extends from March to August with a peak in May–June (Palazón-Fernández et al., 2001). Eggs (227–1233 eggs/female, Palazón-Fernández et al., 2001) are deposited to the roof of a nest where they attach by an adhesive disk and are guarded by a male (Maigret and Ly, 1986). During the breeding season Lusitanian toadfish males produce sounds with the swimbladder, similar to the acoustic emissions used as matting and/or agonistic calls by other batrachoidids (dos Santos et al., 2000). Seasonal ovarian development has been described in detail for the Bay of Cádiz (Southern Spain) and has included histological, histochemical, and biochemical characteristics of the oocytes (Blanco, 1991; González de Canales et al., 1992; Rosety et al., 1992; Muñoz-Cueto et al., 1996). However, no information is available on seasonal testicular development and on the endocrine control of annual reproductive events.

Gametogenesis in teleosts is known to be dependent on several steroid hormones produced by the gonads in

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response to stimulation by pituitary gonadotrophins (GtHs). Among those steroids, estradiol-17β (E_2) is involved in vitellogenesis, whereas 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and/or 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) are required for final oocyte maturation and sperm function (Nagahama, 1994). Recently 17,20β,21-P has been identified as the probable maturation inducing steroid in Lusitanian toadfish (Modesto and Canario, 2002). 17,20α-Dihydroxy-4-pregnen-3-one (17,20α-P) has been associated with male germ cells in a number of species although no specific function has been ascribed to it (e.g., Vermeirssen et al., 2000). Testosterone (T) and 11-ketotestosterone (11-KT) have an important role on male teleost gametogenesis, development of secondary sexual characters and induction of reproductive behaviours (Borg, 1994).

The purpose of the present study was to detail in Lusitanian toadfish the seasonal hormonal cycle, relate gonadal development with plasma levels of sex steroids (E_2, T, 11-KT, 17,20α-P, 17,20β-P, and 17,20β,21-P) and to obtain insights on their roles in gametogenesis. Attention was given to the histological and histochemical characterisation of the male reproductive apparatus and to evidence for the existence of two reproductive types of males based on their morphometric and endocrine characteristics.

2. Materials and methods

2.1. Sampling

Adult Lusitanian toadfishes were collected monthly, except in October because of bad weather, by beam trawler in Ria Formosa (south of Portugal 37°00′N; 7°65′W). After collection, fish were transported alive to the laboratory and immediately processed. Animals were anaesthetised with 2-phenoxyethanol ($0.15\,ml\,l^{-1}$) and a blood sample collected from the caudal vein in heparinised syringes. Plasma was separated by centrifugation (13,000 rpm for 5 min) and stored at −20 °C until analysis. After sacrifice by spinal transection, specimens were measured to the nearest mm in total length (L_T), to the nearest 0.1 g in total (W_T) and eviscerated weights (W_E), and to the nearest 0.01 g in gonad (W_G), accessory testicular organs (W_A), swimbladder (W_S), and liver weights (W_L). Condition factor (K) was calculated as $100W_E(L_T^3)^{-1}$. Hepatosomatic index (I_H), gonadosomatic index (I_G), accessory glands index (I_S), and swimbladder index (I_s) were calculated as $100W_H(W_E)^{-1}$, $100W_G(W_E)^{-1}$, and $1000W_A\ (W_E)^{-1}$, $100W_S\ (W_E)^{-1}$, respectively. I_A was calculated using the weight of fixed material without correction for dehydration. Oocyte diameter (mm) was measured with an ocular micrometer of a stereomicroscope from 15 randomly chosen oocytes of each of 37 ripe females.

2.2. Histology and histochemistry

Fragments of the mid testicular and ovarian region and the whole accessory testicular organs of males were preserved in Bouin's fluid for 48 h and then transferred into ethanol. Paraffin sections (6–10 μm thick) were stained in Harris's haematoxylin and in a staining solution containing light green SF yellowish, orange G, and acid fuchsin (V.O.F., Gutiérrez, 1967). Ovarian development was classified according to Blanco (1991) and González de Canales et al. (1992). The presence of glycogen in paraffin sections was demonstrated by Periodic Acid-Schiff (PAS, McManus and Best's glycogen detection in Cook, 1990). Sulphated and non-sulphated mucosubstances were stained with a combined deamination-Alcian blue-PAS technique at pH 1.0 and pH 2.5 (Cook, 1990). Proteins were stained using the mercury bromophenol blue method (Pearse, 1985).

2.3. Hormone assays

Sex steroids were measured by radioimmunoassays (RIAs). RIA methodology and cross reactions for 17,20β-P and 17,20β,21-P assays were described in Canario et al. (1989); cross reactions for T, 11-KT, E_2, and 17,20α-P were described, respectively, in Scott et al. (1984), Kime and Manning (1982), and Moore et al. (2000). Intra-assay and inter-assay precision (coefficient of variation) were 4.6 and 15.5% for 17,20β-P, 5.0 and 12.0% for 17,20α-P, 1.5 and 10.4% for 17,20β,21-P, 7.5 and 12.4% for T, 8.2 and 11.6% for 11-KT, 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, $160\,pg\,ml^{-1}$ for 11-KT, 17,20αP, and 17,20βP, and $320\,pg\,ml^{-1}$ for 17,20β,21-P.

Individual plasma samples (50 μl) of 10 females and of 10 males taken in January, March, May, June, July, September, and November were diluted 1:20 in 0.1 M phosphate BSA buffer, pH 7.6, and heat-treated for 1 h at 80 °C. After cooling, samples were stored at −20 °C until hormone assay. A preliminary study comparing heat-treated and diethyl ether extracted samples showed no significant differences in the levels of steroids quantified by the two methodologies.

2.4. Statistical analysis

Deviation from 1:1 sex ratio monthly and at each of 20 mm total length intervals were tested by Chi-square. All data were expressed as the means ± SEM (standard error of the mean). Normality and homogeneity of variance was obtained by transforming data before statistical tests were applied: inverse sine square root transformation for percentages and log transformation

for steroid concentrations and fish or tissue weights. Significant differences between monthly mean values of the various indices and plasma steroid levels were tested by analysis of variance (ANOVA) followed by Tukey's honestly significant difference test. Analysis of covariance (ANCOVA) was used for comparison of morphometric parameters. Plots in figures are based on untransformed data. Statistical significance was considered at the 5% level.

3. Results

3.1. Morphometric changes

Of a total of 312 specimens examined, 139 were females and 173 were males. Sex ratio did not deviate significantly from 1:1 when calculated for each month or for all the sampled individuals ($\chi^2 = 3.705$; $p > 0.05$). The range of the length distribution of females and males was 128–266 mm and 130–372 mm, respectively. There were no statistical differences in the number of females and males at length classes < 220 mm (mean = 224.3 g W_E) ($p > 0.05$) but males dominated in number above 220 mm ($p < 0.05$).

Mean K for females and males showed a similar seasonal pattern. Mean K was significantly higher during the period from February to April and decreased thereafter until June–July ($p < 0.001$; Fig. 1a). The mean I_H exhibited two peaks for each sex, one in April (females) or June (males) and the other in September (females and males). Sex differences in I_H during ovarian growth was significantly higher than at post-spawning ($p < 0.05$; Fig. 1b).

During May to September the I_S in males was significantly elevated compared to the remainder of the year ($p < 0.001$), with a peak in June. In females, however, no significant changes in I_S were observed ($P = 0.187$) throughout the year. I_S values were significantly lower in females compared to males at every month except February, April, and December (Fig. 1c).

3.2. Ovarian development

In females the I_G was significantly elevated from April to June, when it reached its maximum ($p < 0.001$; Fig. 2a). Spawning occurred in June and July and the period of resting and endogenous vitellogenesis was for most females from July to January. In February, oocytes in exogenous vitellogenesis were dominant, increased dramatically in size throughout May, and in June most of the females showed large hyaline oocytes (5.54 ± 0.24 mm). A second, very distinct, batch of oocytes arrested at endogenous vitellogenesis was also present which probably represented those that would be recruited in the next spawning season.

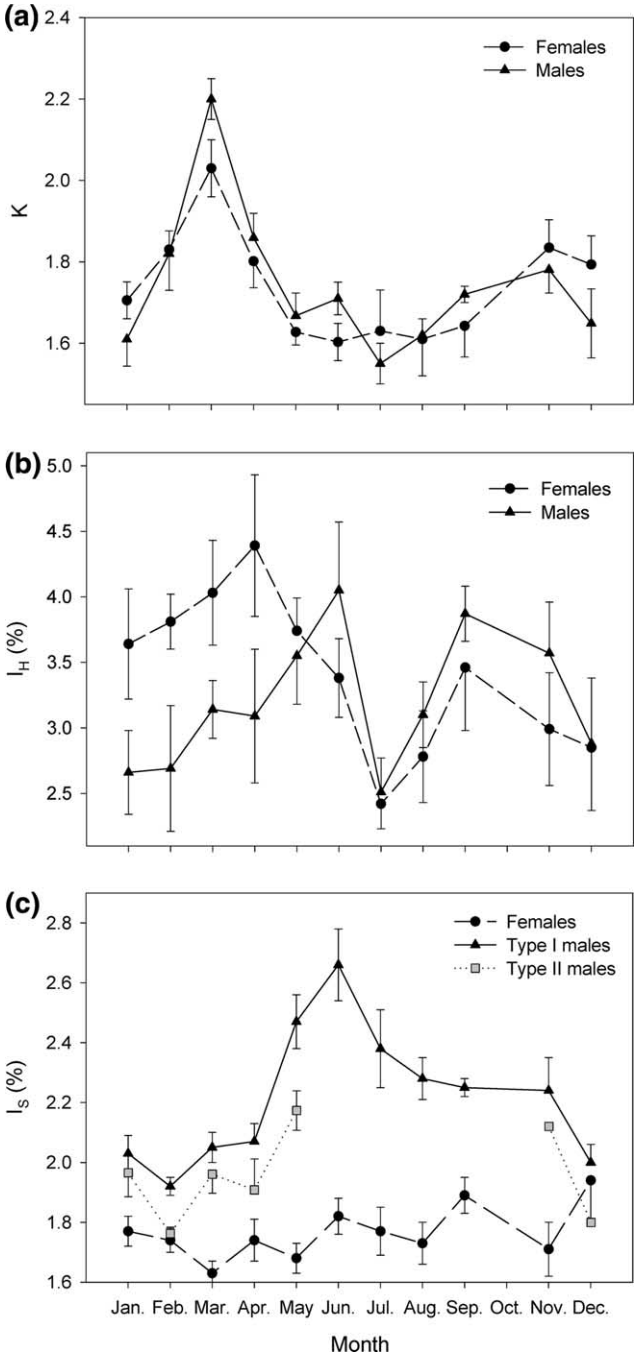

Fig. 1. Monthly changes in (a) condition factor (K), in (b) hepatosomatic index (I_H) and in (c) swimbladder index (I_S) of females and males of *H. didactylus*. Type I males are those with low gonadosomatic index (I_G) and large accessory glands index (I_A) and type II males is the reverse (see details in text). Vertical bars represent the SEM.

3.3. Testicular development

In the Lusitanian toadfish testes are paired elongated and well-separated organs. The anterior edges of two testes diverge and extend along the ventral–lateral sides of the swimbladder. On the medial–ventral side, testicular ducts and blood vessels are prominent (Fig. 3).

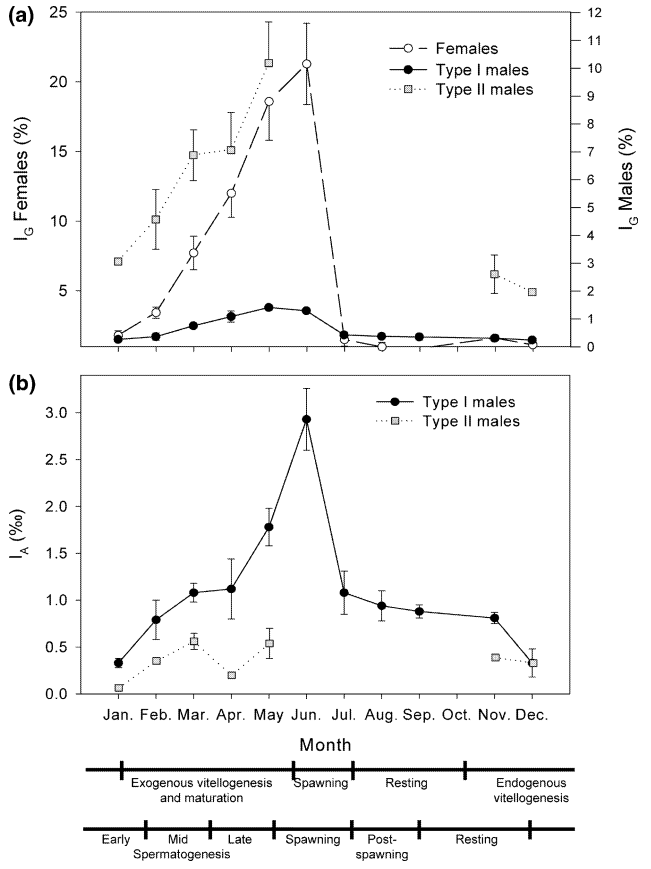

Fig. 2. Monthly changes in (a) gonadosomatic index (I_G) and in (b) accessory gland index (I_A) of *H. didactylus*. At the bottom is the progress of gametogenesis during the annual cycle for females and males. For definitions of type I and type II males see Fig. 1 and the text. Vertical bars represent SEM.

The I_G of males began to increase in January and peaked in May (Fig. 2a). From April to June the I_G was significantly elevated compared to the remaining months. Spawning occurred during May and June and at this time the testes were large and white structures soft to the touch with sperm running out of the genital pore upon slight pressure to the flanks. From July to December the testicular volume decreased considerably and the testes became very thin. On the basis of the histological changes, the annual testicular development could be divided as follows:

(1) Immature (virgin)—*spermatogonia proliferation* (Fig. 4a)—small transverse section of lobules compared to resting males. Many groups of spermatogonia in the periphery of seminal lobules.
(2) Maturation:

Early spermatogenesis (Fig. 4b)—the number of spermatogonia declined, while seminal lobules were filled with cysts containing primary and secondary spermatocytes. A small population of spermatids present.

Mid-spermatogenesis (Fig. 4c)—the number of cysts with spermatids increased and small populations of spermatozoa were present in the central part of lobules.

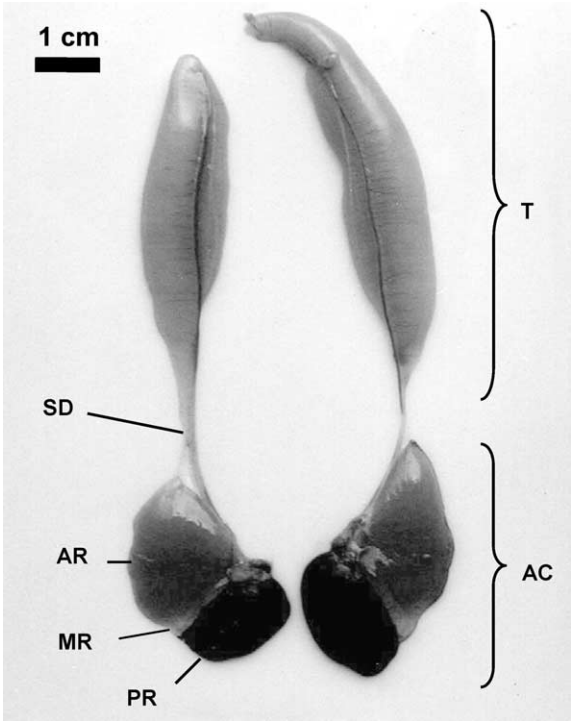

Fig. 3. Aspect of the male reproductive apparatus of *H. didactylus*. T, testes; AC, accessory glands; SD, spermatic duct; AR, MR, and PR, anterior, middle, and posterior regions of accessory glands.

Late spermatogenesis (Fig. 4d)—cysts with spermatocytes showed a considerable decrease while the number of cysts with spermatids and spermatozoa increased enormously in relative abundance.

(3) Spawning (Fig. 4e)—ripe spermatozoa in large quantities filled the lumen of all the lobules.
(4) Post-spawning (Fig. 4f)—Residual spermatozoa remained in the lobules. The lobule walls become folded.
(5) Resting (Fig. 4g)—Some residual spermatozoa, though less than in post-spawning stage. There is an increased presence of connective tissue in the lobule walls and apparent cellular disorganisation of lobule periphery. Sertoli cells and spermatogonia are visible.

3.4. Accessory testicular organs

Connected to the posterior part of each of the main testicular ducts there was a well-developed accessory structure of a glandular nature (Fig. 3), which could be macroscopically divided into a fan shaped anterior yellow coloured region (AR) and a rounded dark brown to black posterior region (PR). Connecting these two regions there was a thin pale yellow middle region (MR). The accessory gland was connected laterally through the three regions with the spermatic duct, which opened to the exterior through the genital pore placed at the

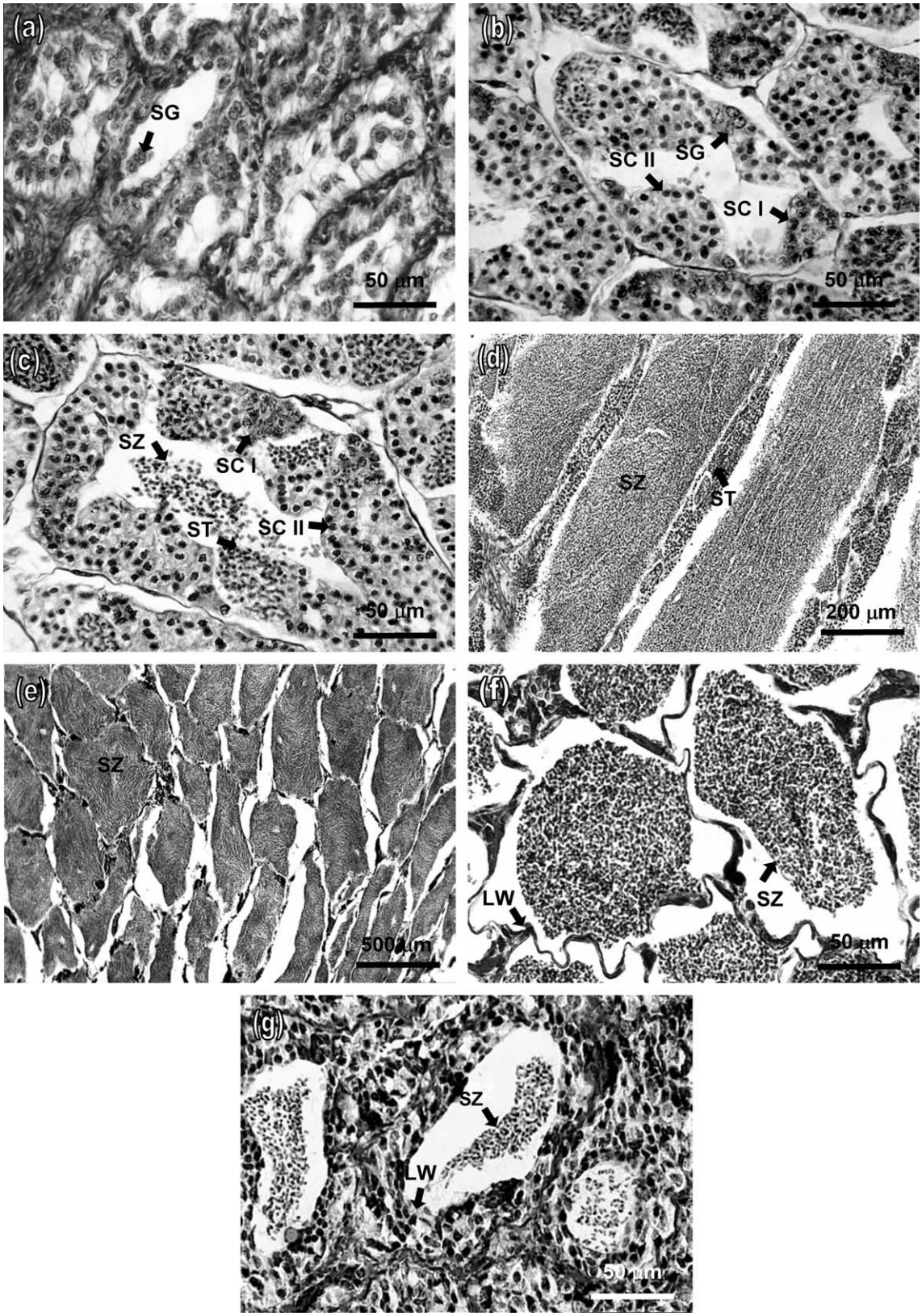

Fig. 4. Histological sections showing different phases in testicular development of *H. didactylus* (haematoxylin-V.O.F. stain). (a) Immature. (b) Early spermatogenesis. (c) Mid-spermatogenesis. (d) Late spermatogenesis. (e) Spawning (f) Post-spawning. and (g) Resting. SG, spermatogonia; SC I, primary spermatocytes; SC II, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; and LW, lobule wall.

extremity of an elongated papilla. The accessory glands were well defined during testicular development and had numerous septa forming chambers. During the reproductive season these glands produced an abundant fluid, which could be expressed through the genital pore by gentle pressure applied to the surrounding area. In the more developed glands this fluid was mostly dark, while in the less developed glands the fluid was yellowish.

Chambers of the AR were elongated and their walls were thicker when compared to the PR. Septa were specially folded in the region adjacent to the PR. The AR epithelium consists of columnar cells, which probably secretes a homogeneous fluid which can be visualised inside the chambers. In chambers with a large amount of fluid the height of epithelial cells declined. Various degrees of glandular activity, depending on the amount of stored secretory material, could usually be found in this part of the accessory glands. Positive reaction to histochemical stains indicated the presence of weakly sulphated mucosubstances and proteins inside the chambers. No glycogen was detected (Figs. 5a and c). In the most apical zone of the AR, columnar cells were taller and the peripheral end of the cells were pinched. The coarse secretion of vesiclous non-homo-

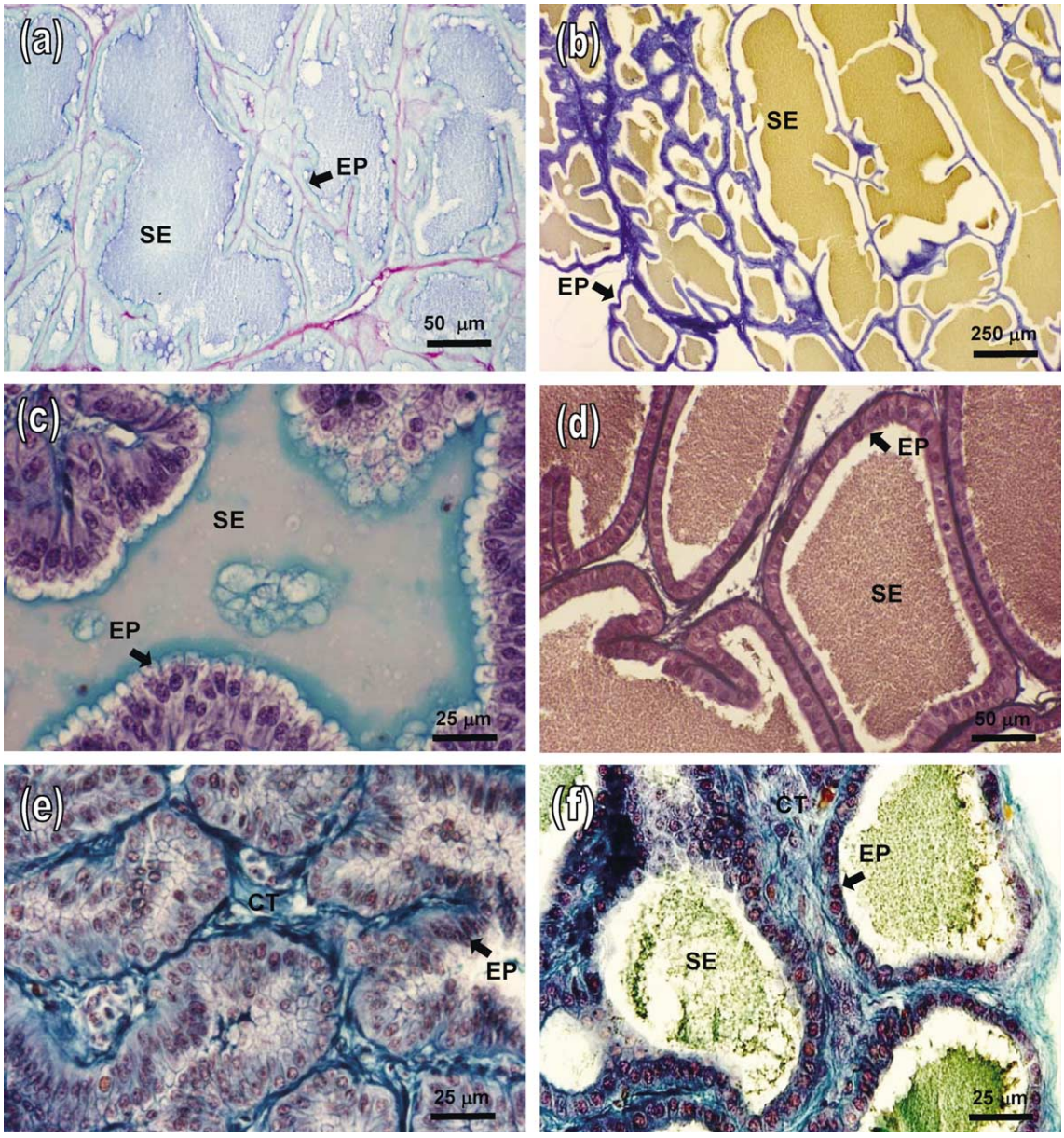

Fig. 5. Histological appearance of accessory testicular organs. (a) Chambers of anterior region filled with secretion. Combined deamination-Alcian blue, pH 2.5 – PAS. (b) Chambers of the posterior region. Mercury bromophenol blue stain. (c) Detail of anterior region. (d) Detail of posterior region. (e) Anterior region during the non-reproductive period. (f) Posterior region during the non-reproductive period. Haematoxylin-V.O.F. stain. EP, epithelium; SE, secretion; CT, connective tissue.

geneous material that could be identified with bromophenol blue but reacted weakly to PAS or alcian blue at pH 2.5 was probably produced by the epithelium.

The MR is histologically and histochemically similar to AR but with larger chambers filled with fluid.

The dark colour of the PR of the accessory organs was given by a pigment accumulated in rounded lobules. Microscopically, the septa had cuboid cells. As chambers became full of a dark extremely fine granular secretion, cells became progressively smaller and flat. Epithelial cells reacted strongly to mercury bromophenol blue and moderately to PAS and alcian blue at pH 2.5 (no reaction was detected at pH 1.0). Secretory material in the chambers reacted weakly to PAS and alcian blue at pH 2.5 and did not react with mercury bromophenol blue. No positive reaction to glycogen was detected in any structure (Figs. 5b and d). The I_A followed a similar profile to that of the I_G and reached their maximum size in June (Fig. 2b) corresponding also to the maximum enlargement of the chambers and thinning of the septa. After the spawning season a reduction in the total size of the accessory glands took place with loss of fluid secretion and an increase of connective tissue in the septa. In the AR, chambers became quite folded with no visible secretion. In the PR, secretion of the dark fluid was minimal and chambers became round and small (Figs. 5e and f). In January, the secretory material reappeared in the chambers of both regions and fluid could be expressed from the genital pore when the glands were pressurised. From February to June, the septa became progressively thinner, the chambers filled with secretory material and fluid could be expressed.

3.5. Male dimorphism

A number of males (14% of the total number of captured males), noticeable from November onwards had the I_G approximately seven times greater that of the remaining males (ANCOVA, $p < 0.001$; Figs. 2a and 6a). During the spawning season testes from the high I_G males could be so enlarged that, they could be easily mistaken with mature females, because of the similar distended abdomen. In contrast, high I_G males had smaller accessory glands with a mean I_A ca. three times smaller than that of low I_G males (ANCOVA, $p < 0.001$; Figs. 2b and 6b). The accessory glands of high I_G males had always less developed chambers than the low I_G males, with little fluid secretion and thicker septa containing a larger proportion of connective tissue. In addition, the swimbladder of high I_G males was significantly larger than that of females (ANCOVA, $p = 0.002$) but smaller than that of low I_G males (ANCOVA, $p = 0.013$; Figs. 1c and 6c). Above 213 mm (206.5 g W_E) only low I_G males were found, while below that size the two types of males were present (Figs. 6a–c).

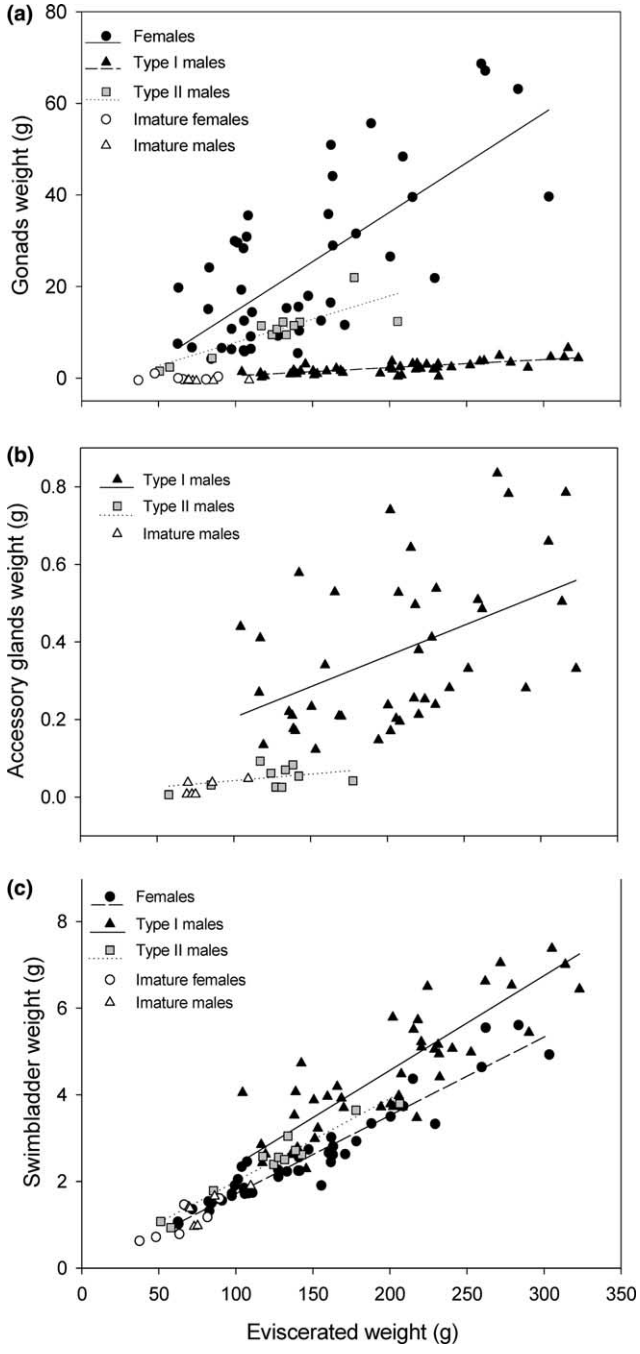

Fig. 6. Relationship between the weight of (a) gonads, (b) accessory glands, and (c) swimbladder to eviscerated weight for females, type I males and type II males of *H. didactylus* between March and June. For definitions of type I and type II males see Fig. 1 and the text. For each relationship covariance analysis showed significant differences between all groups.

3.6. Seasonal plasma steroid profiles

In females, E_2 plasma levels increased from January to May, coinciding with period of gonadal growth (vitellogenesis), and reached a maximum ($4.00 \pm 0.52\,\mathrm{ng\,ml^{-1}}$) a month before the start of spawning (Fig. 7a). From June to November plasma levels of E_2 were

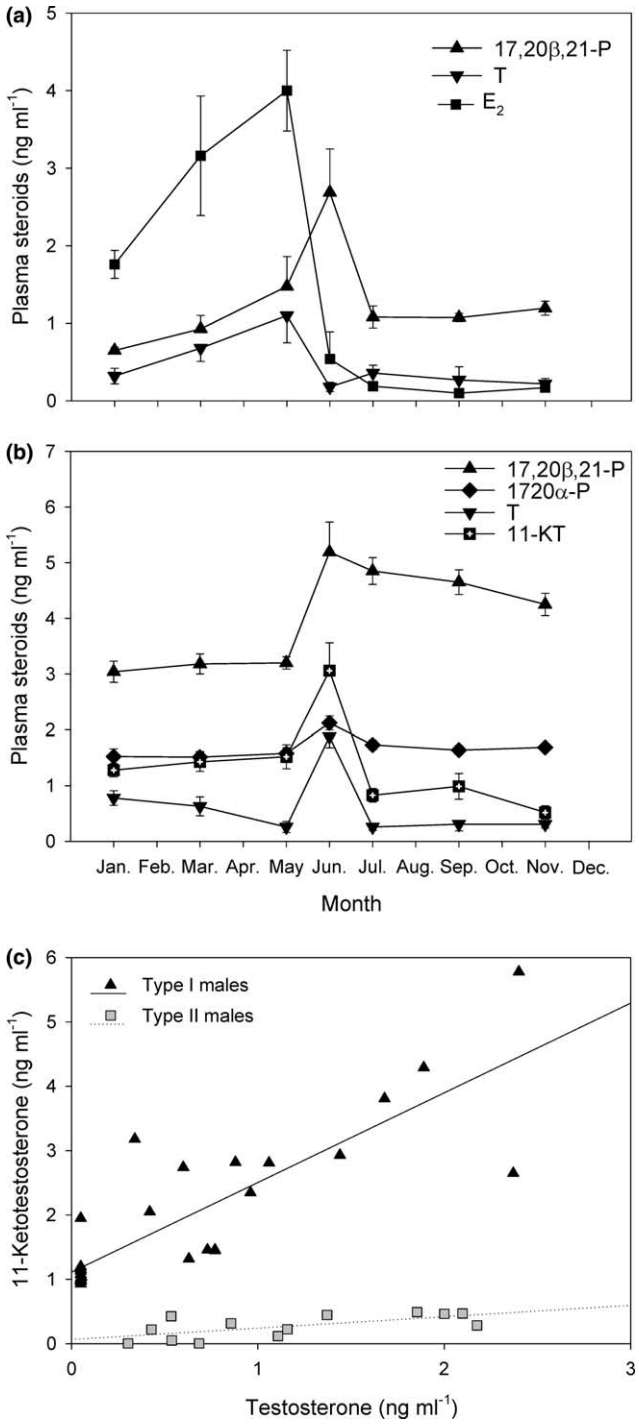

Fig. 7. Monthly changes of plasma sex stroids in (a) females and (b) type I males of *H. didactylus*. (c) Relationship between plasma concentrations of 11-KT and T in type I and type II males. For definitions of type I and type II males see Fig. 1 and the text. Vertical bars represent the SEM.

significantly lower than during the period of gonad growth ($p < 0.001$). Plasma T levels in the females were always very low ($\leqslant 1\,\mathrm{ng\,ml^{-1}}$), although there was a significant increase from January to May ($P < 0.05$). Plasma 17,20β,21-P levels increased significantly from their lowest concentrations in January ($0.65 \pm 0.04\,\mathrm{ng\,ml^{-1}}$), to peak levels in June ($2.69 \pm 0.56\,\mathrm{ng\,ml^{-1}}$) and then declined quickly during the next month to low levels ($1.08 \pm 0.14\,\mathrm{ng\,ml^{-1}}, P < 0.001$). 17,20β-P was undetectable throughout the year.

In low I_G males, 11-KT and T had a similar seasonal profile with highest levels in June ($P < 0.001$), $3.06 \pm 0.50\,\mathrm{ng\,ml^{-1}}$ and $1.88 \pm 0.20\,\mathrm{ng\,ml^{-1}}$, respectively (Fig. 7b). During the spawning period, low I_G males had significantly higher levels of 11-KT ($2.02 \pm 1.22\,\mathrm{ng\,ml^{-1}}$) than high I_G males ($0.33 \pm 0.29\,\mathrm{ng\,ml^{-1}}$; $P < 0.001$). However, both types of males had similar levels of testosterone ($P = 0.161$; Fig. 7c). Plasma 17,20β,21-P in low I_G males increased immediately before spawning and remained significantly elevated until November ($P < 0.001$), while 17,20β-P was undetectable. 17,20α-P plasma concentrations remained stable throughout the year at around $1.5\,\mathrm{ng\,ml^{-1}}$ with the exception of a slight but significant peak in June ($P < 0.05; 2.12 \pm 0.12\,\mathrm{ng\,ml^{-1}}$; Fig. 7b).

4. Discussion

Two morphological male types have been previously described for the batrachoidid *Porichthys notatus* (Girard) on the basis of I_G and hormonal profile (Brantley and Bass, 1994; Brantley et al., 1993c), type I males with a I_G sevenfold smaller and higher levels of 11-KT than type II males. In the Lusitanian toadfish the two male morphotypes could also be distinguished on the basis of these parameters with type I males having sevenfold lower I_G and sixfold higher 11-KT levels. In addition, type II males had less developed accessory glands with smaller chambers suggesting less secretory activity compared to type I males.

In the Lusitanian toadfish size differences were observed between sexes with type I males predominating at larger sizes (>220 mm and 224.3 g W_E). This, together with the fact that type I males had a lower I_G, suggests that they allocate more energy towards growth than females and type II males, both of which preferentially allocate energy towards gamete production. What is not clear is whether type II males can change into type I males (and vice-versa) since the two types of males coexist below 220 mm. The studies on the plainfin midshipman *P. notatus*, that have type II males significantly smaller in body sizes than type I males, indicate a distinct, non-sequential, ontogenetic trajectory for type I and type II males rather than a developmental sequence (Brantley et al., 1993b). Swimbladder sexual dimorphism in Lusitanian toadfish and in other batrachoidids (Brantley et al., 1993b; Fine, 1975; Fine et al., 1990) is related to the acoustic emissions used as matting and/or agonistic calls during the reproductive period (Brantley and Bass, 1994; dos Santos et al., 2000). Like in the

plainfin midshipman, the swimbladder (I_S) is less developed in type II males than in type I males. In *P. notatus* the two male morphotypes maintain alternative reproductive tactics. Type I males are territorial and use their muscular swimbladders to make specific "humming" sounds to attract females into depositing eggs in nests guarded by these males until the young become free swimming. In contrast, type II males mimic females behaviourally and morphologically and sneak or satellite-spawn at the nests of type I males and only produce short duration grunts that are similar in spectral, temporal and amplitude characteristics to the grunts of females (Brantley and Bass, 1994). Altogether evidence points towards territoriality and sound communication having evolved together in the family. Whether the swimbladder muscle organisation and microscopic appearance, such as area of mitochondria-filled sarcoplasm, and myofiber number of the sonic muscles, which enable the types of sound observed in *P. notatus* (Brantley et al., 1993b), exist in the two types of Lusitanian toadfish males still requires to be investigated. Also, although no behavioural observations of the Lusitanian toadfish have been made, the morphological evidence suggests that alternative reproductive tactics may be present in Lusitanian toadfish. Like *P. notatus*, Lusitanian toadfish females deposit eggs in nests, which are fertilised and guarded by territorial males, possibly of type I, which have smaller testis and heavier swimbladders. The larger testis and lighter swimbladders in type II Lusitanian toadfish are consistent with a male (sneaker/satellite) which would emit opportunistically large amounts of sperm to fertilise eggs from a distance without requiring development of heavy sound producing muscular structures to attract females.

The spawning season of Lusitanian toadfish in Ria Formosa (June–July) lasts little more than a month. From the group synchronous distribution pattern of the oocytes and the short spawning period there is strong indication that each female spawns only once. In Ria Formosa spawning is also a month delayed compared to the population in Cádiz, southern Spain (Blanco, 1991; Palazón-Fernández et al., 2001; Rosety et al., 1992), possibly related to the lower yearly average temperature in Ria Formosa. The differences in spawning time between the two locations are also apparent in the I_G and I_H, suggesting that alterations in the two indices are related. This is also indicated by the fact that total lipids and phospholipids in the ovary are correlated to I_H during ovarian growth (Blanco, 1991; Rosety et al., 1992; Muñoz-Cueto et al., 1996). The liver is the organ of synthesis of precursors of egg components (Wallace et al., 1987) and its mass started to decline when vitellogenesis became more intense in April, with a minimum in July at the end of spawning. In agreement with the role of the liver in oogenesis, the I_H in males, although showing important monthly changes, was generally smaller than in females and continued to grow until June when it underwent a marked reduction to its minimum in July, at the end of spawning. The decrease of I_H in males is probably related to the metabolic needs of territorial defence, partner calling and nest guarding which lasts at least 30 days until fry become free living (Modesto, unpublished observations). Both in Cádiz and in Ria Formosa males mature early than females; this apparent asynchronism between sexes has also been shown in other teleost species (e.g., Htun-Han, 1978).

The testicular organisation of the Lusitanian toadfish is typical of most teleosts and corresponds to the "unrestricted" or "lobular" type (Billard, 1986; Grier, 1981). Spermatogenesis occurs within cysts where germ cells undergo synchronous differentiation. The cysts break down at the end of spermatogenesis and spermatozoa are released into the lumen of lobules. The testicular accessory glands have many of the morphological and histological features of those of the toadfish *Opsanus tau* (L.) and *P. notatus* (Barni et al., 2001; Hoffman, 1963a,b). In all cases they are paired extensions of the posterior portions of main testicular ducts differentiated in well-defined portions, which are organised in chambers filled with secretory material. However, the dark brown coloured anterior region of the accessory gland of *O. tau* (Hoffman, 1963a) corresponds to the most posterior region of the accessory gland of *H. didactylus*. Mature spermatozoa tend to concentrate inside the cysts of the testes and do not need to cross these secretory structures to reach the spermatic duct. Prior to release, however, spermatozoa receive in the final portion of the paired spermatic ducts the secretion from the different regions of the accessory glands. These accessory glands do not appear to have a nutritional function for the sperm, since in *H. didactylus* and in any of the other batrachoidids studied so far there is no evidence for glycogen production (Barni et al., 2001). Also, mixing sperm with the accessory organ secretions does not change its mobility or behaviour (Hoffman, 1963a). The AR of the accessory glands of *H. didactylus* can produce mucosubstances and proteins while in the PR the production of these substances is not so intense. The exocrine production of mucosubstances by the accessory glands seems to be a common feature of batrachoidids and of many other teleost species: some blennies produce sialomucins (Lahnsteiner et al., 1990), while gobies produce sialomucins and sulphomucins (Cinquetti, 1997; Lahnsteiner et al., 1992). These mucosubstances have been suggested to increase the viscosity of the seminal fluid and help to agglutinate the sperm (Cinquetti, 1997; Lahnsteiner et al., 1990; Mann, 1964). Recently, Barni et al. (2001) suggested that the mucosubstances released by nesting *P. notatus* type I males, which have active large accessory glands, may embed sperm and help produce sperm trails or layers that slowly dilute in water reducing sperm dispersion

and allowing the presence of sperm in water more constantly over time. This would increase the efficacy of fertilisation of the sticky benthic eggs, and allow parental males to remain guarding nests. Type II males, however, having less developed accessory glands (poor in mucosubstances) and large testes, would need and are adapted to release large quantities of free moving sperm quickly outside the type I male nest entrance or sneaking inside to try to reach the eggs inside the nest.

E_2 plasma concentrations increased with I_G as expected, considering the group synchronous pattern of oocyte development. However, E_2 levels dropped markedly a month before the peak I_G, when T and 17,20β,21-P levels surged. This pattern of hormone changes in females $E_2 \rightarrow T \rightarrow$ progestagen is common to most teleosts and is the result of a GtH-mediated shift in steroidogenesis, in which there is a progressive decrease in aromatase activity and concomitant activation of 20β-hydroxysteroid dehydrogenase (20β-HSD) to produce the maturation-inducing steroid (MIS, Kobayashi et al., 1987; Nagahama et al., 1995; Scott et al., 1983). In Lusitanian toadfish 17,20β,21-P, has been identified as the probable MIS by in vitro studies (Modesto and Canario, 2002) and that is supported in the current study by the elevated 17,20β,21-P during spawning and the simultaneous absence of 17,20β-P.

Circulating levels of androgens in *H. didactylus* type I males underwent marked seasonal changes and peaked during the spawning period. Similar observations have been made for other species specially those that display conspicuous reproductive behaviour: e.g., garibaldi, *Hypsypops rubinculus* (Girard) (Sikkel, 1993), bluegill, *Lepomis macrochirus* (Rafinesque) (Kindler et al., 1989), and demoiselle *Chromis dispilus* (Griffin) (Barnett and Pankhurst, 1994). For many other teleosts (rainbow trout, *Salmo gairdneri* (Richardson), Scott et al., 1980; white sucker, *Catostomus commersoni* (Lacepède), Scott et al., 1984; Artic charr, *Salvelinus alpinus* (L.), Mayer et al., 1992) 11-KT and T peak during the pre-spawning period possibly related to the role of 11-KT in spermatogenesis (Miura et al., 1991a). Furthermore, type I males had significantly higher plasma levels of 11-KT than type II males. This seems to be a common pattern of teleosts that have male dimorphism and alternative reproductive tactics in which elevated 11-KT characterise the territorial courting morph relative to its non-courting counterpart (see Brantley et al., 1993c; Oliveira et al., 2001a,b). For example, in *Salaria pavo* (Risso) nest-holders had a higher 11-KT metabolisation indices (11-KT relative to total 11-KT plus T) than floaters both in the testis and in the testicular gland suggesting that nest holding promotes the conversion of T into 11-KT (Oliveira et al., 2001a). The higher levels of 11-KT, but not T, in *H. didactylus* type I males suggests also that 11-KT must be the androgen responsible for the increase in size of sonic muscle. Swimbladder muscle hypertrophy during the matting season has been identified in three sound-generating species, the haddock *Melanogrammus aeglefinus* (L.) (Templemen and Hodder, 1958), the midshipman *Porichthys* sp (Mommsen & Nickolichuck in Walsh et al., 1995) and the weakfish *Cynoscion regalis* (Bloch and Schneider) (Connaughton et al., 1997; Connaughton and Taylor, 1995b). In *C. regalis* T implants induced sonic muscle growth but 11-KT was not tested (Connaughton and Taylor, 1995a). In *P. notatus* and in toadfish *O. tau* implants of both T and 11-KT androgens were effective in inducing sonic muscle growth (Brantley et al., 1993a; Fine and Pennypacker, 1986). Both studies are consistent with a role for 11-KT in the process of sonic muscle growth.

Accessory gland development also appears to be dependent on 11-KT, both its growth and secretory activity. The fact that 11-KT levels in type II males were relatively low, even though their relative testes size was larger than that of type I males suggests however that spermatogenesis may not require very high levels of that androgen. Whether the extraordinary growth of the testis has per se any direct influence on accessory gland growth is not known. However, it can be hypothesised that the lower 11-KT levels in type II males may release a feedback to the hypophysis to increase the secretion of a hormone (possibly prolactin, Bartke et al., 1975) responsible for stimulating testis hypertrophy.

Therefore, it would appear most likely that elevated serum levels of 11-KT in type I males could be associated with the development of reproductive behaviour and related swimbladder growth and mate calls, female courtship, development of accessory testicular organs for controlled release of sperm, while similarities between the plasma levels of testosterone among the type I and type II males seem to indicate that this hormone may be associated with an aspect common to both types of males, such as gonadal development and/or spermiation.

In males, 17,20β,21-P but not 17,20β-P or 17,20α-P, was elevated during the spawning and post-spawning period. While until now 17,20β-P has been shown to be involved in sperm function in teleosts (Baynes and Scott, 1985; Miura et al., 1991b, 1992), for those species in which this hormone is lacking a similar role may be taken by 17,20β,21-P (Thomas et al., 1997) or 17,20α-P (Vermeirssen et al., 2000). However, in Lusitanian toadfish 17,20β,21-P stays elevated for a long period and follows the pattern of swimbladder hypertrophy. Whether it has any significance in this process requires further studies.

In conclusion, two male morphotypes (type I and type II) could be distinguished in Lusitanian toadfish on the basis of differences in relative size of testis, testicular accessory glands and swimbladder as well as androgen levels. Although confirmation is required, it is probable that type II males correspond to a sneaker/satellite

morph with alternative reproductive tactics. Thus, this species which combines alternative reproductive tactics, parental care and a diversified repertoire of mating vocalisations can be a useful model in the investigation of endocrine mechanisms that control reproductive behaviour and in the understanding of the evolution of reproductive systems within teleosts.

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