Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman

Joseph A. Sisneros,[a,*] Paul M. Forlano,[a] Rosemary Knapp,[b] and Andrew H. Bass[a,c]

[a] *Department of Neurobiology and Behavior, Cornell University, Seeley G. Mudd Hall, Ithaca, NY 14853, USA*
[b] *Department of Zoology, University of Oklahoma, Norman, OK 73069, USA*
[c] *UC Bodega Marine Laboratory, Bodega Bay, CA 94923, USA*

Abstract

This study characterized the seasonal variation of the steroid hormones testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E_2), and cortisol (F) as they relate to the gonadal development and reproductive behavior of the plainfin midshipman fish, *Porichthys notatus*. The plainfin midshipman is a deep-water teleost that seasonally migrates into the shallow intertidal zone where type I, or "singing," males build nests, acoustically court and spawn with females. The gonadosomatic index and plasma steroid levels were measured from adult type I males and females collected over four time periods (non-reproductive, pre-nesting, nesting, and post-nesting) that corresponded to seasonal fluctuations in midshipman reproductive biology and behavior. Among type I males, plasma levels of T and 11-KT were low during the winter non-reproductive period, gradually increased during seasonal recrudescence of the testes in the spring pre-nesting period, and then peaked at the beginning of the summer nesting period. In the latter half of the nesting period and during the fall post-nesting period, plasma levels of T and 11-KT were low or non-detectable. Low, detectable levels of E_2 were also found in the plasma of 50% or more type I males during every seasonal period except the winter non-reproductive period. Among females, plasma levels of T and E_2 were low throughout the year but briefly peaked in April during the spring pre-nesting period when ovaries underwent seasonal recrudescence. Plasma F levels were correlated with collection depth and were lower in males than females when fish were collected deeper than 120 m. The sex-specific peaks of steroid hormone levels in male and female midshipman may serve differential functions related to the physiology, reproductive behavior, and vocal communication of this species.
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1. Introduction

Gonadal steroid hormones can have profound influences on the central nervous system and behavior of vertebrates either through organizational effects during early development or through activational effects in adults (Nelson, 2000). The activational effects of gonadal hormones during reproduction are often associated with seasonal cycles of steroid hormone production and gametogenesis. Such seasonal changes can ultimately influence reproductive behaviors and are necessary for successful reproduction in all vertebrates.

Cyclical changes in the reproductive hormones of teleost fishes are widely known to occur in association with reproductive cycles and have been investigated mainly to understand the mechanisms of reproductive behavior, gametogenesis, and gonadal steroidogenesis (Fostier et al., 1983; Goetz, 1983). Seasonal changes in circulating levels of gonadal steroid hormones during the reproductive cycle are described for a variety of freshwater and marine teleost species (reviews: Fostier et al., 1983; Pankhurst and Carragher, 1991). In marine teleosts, reproductive periodicity of gonadal steroid hormone levels has been documented for wild caught populations of flatfish (Campbell et al., 1976; Harmin et al., 1995; Methven et al., 1992; Wingfield and Grimm, 1977), mullet (Dindo and MacGregor, 1981), salmon

(Ueda et al., 1984), cod (Pankhurst and Conroy, 1987), orange roughy (Pankhurst and Conroy, 1988), rockfish (Nagahama et al., 1991; Takano et al., 1991), sardines (Matsuyama et al., 1994; Murayama et al., 1994), grouper (Johnson et al., 1998), kingfish (Poortenaar et al., 2001), tuna (Stequert et al., 2001), eelpout (Larsson et al., 2002), damselfish (Barnett and Pankhurst, 1994; Pankhurst et al., 1999; Sikkel, 1993), goby (Bonnin, 1979; Pierantoni et al., 1990), and toadfish (Modesto and Canario, 2003a). The focus here is on the plainfin midshipman (*Porichthys notatus*), another member of the same family of teleosts as toadfish (Batrachoididae), that has been the subject of intensive neuroethological investigation.

A series of multidisciplinary investigations has now characterized the vocal-acoustic behaviors and neurobiology of midshipman fish showing, in part, how midshipman serve as a model for identifying the mechanisms of auditory reception, neural encoding, and vocal production shared by all vertebrates (review: Bass and McKibben, 2003). An important component of these studies has been to establish sex differences in vocal-acoustic traits and showing how steroidal and peptidergic hormones and neurohormones can influence the development, maintenance and seasonal changes in the expression of those traits. What has been lacking from these studies, and is now documented in this report, is a portrayal of naturally changing levels of circulating gonadal steroid hormones.

The plainfin midshipman seasonally migrates from deep ocean sites to breed in the intertidal zone where the production and reception of vocal signals are essential to its reproductive success. Midshipman have three adult reproductive morphs, females and two male morphs (types I and II), that can be distinguished by a suite of behavioral, somatic, endocrinological and neurobiological characters (reviews: Bass, 1996, 1998). Type I, "singing" males build nests, acoustically court females, and provide parental care for fertilized eggs, whereas type II males neither build nests nor sing to females, but instead satellite or sneak spawn to steal fertilizations from type Is (Brantley and Bass, 1994). After spawning, females leave the seasonal breeding grounds and return to deep offshore sites (Brantley and Bass, 1994).

Seasonal periodicity of reproduction in the plainfin midshipman is concurrent with changes in vocal behavior and the physiological performance of the peripheral auditory system. Midshipman generate highly stereotyped advertisement and agonistic vocalizations. In particular, during the breeding season (May–August), type I males alone produce a long duration (>1 min), multiharmonic advertisement call or "hum" with a fundamental frequency near 90–100 Hz and several prominent harmonics (Bass et al., 1999). Observations of reproductive behavior together with underwater playback experiments demonstrate the role of the hum in attracting gravid females to a type I male's nest (Brantley and Bass, 1994; Ibara et al., 1983; McKibben and Bass, 1998, 2001). We recently discovered that seasonal changes in type I male vocal behavior (humming during the summer) are accompanied by changes in the frequency sensitivity of the peripheral auditory system such that summer, reproductive females were better suited than winter, non-reproductive females to encode the higher harmonic components of male advertisement calls (Sisneros and Bass, 2003a). This finding suggests that the seasonal enhancement of higher frequency encoding in females may represent an adaptive response to improve the detection of multiharmonic hums. A possible mechanism for the plasticity of frequency sensitivity in midshipman is one that is dependent on seasonal changes in circulating levels of gonadal steroids, like those reported here.

This study reports the seasonal cycle of the gonadal steroids testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E₂) in the plasma of wild-caught populations of plainfin midshipman fish. We relate the plasma levels of gonadal steroids with the gonadal development and reproductive behavior of the midshipman. In addition, we also report plasma cortisol (F) levels, in part, because of the general influence of glucocorticoids on behavioral plasticity across vertebrates (review: Romero, 2002) and on the vocal motor system of batrachoidids (Remage-Healey and Bass, 2002, 2003). This study also complements earlier reports of circulating levels of gonadal and adrenal steroids that only focused on midshipman during the reproductive summer period (Brantley et al., 1993b; Knapp et al., 1999, 2001). The results of the present study have previously been reported in abstract form (Forlano et al., 2003).

2. Materials and methods

2.1. Animals and sample collection

Adult male and female midshipman fish (*Porichthys notatus*) were collected over a 16-month period from March 2001 through July 2002 in northern California (see Brantley et al., 1993a for traits distinguishing adults and juveniles). During the summer spawning period (May–August), parental "type I" males defend nests and court females in the intertidal zone along the Pacific Northwest of the US and Canada. "Type II" males, an alternative male phenotype in this species, do not defend nests or court females but "sneak spawn" by releasing sperm in competition with a spawning type I male and female (Brantley and Bass, 1994). In the past, types I and II males have always been collected from nests during the summer reproductive season; aside from

newly hatched embryos, only sexually mature animals are found in nests. Several criteria have been used over the past 16 years to distinguish the two male morphs during the reproductive season, including body size, sonic muscle coloration, the ratio of sonic muscle mass to body mass, and the ratio of gonad mass to body mass (gonadosomatic index or GSI) (Bass, 1996). Using these criteria, the males collected for this study during the summer were unambiguously of the type I phenotype. However, we recognized that during non-reproductive periods, sonic muscle regression (T.P. Mommsen and K. Nickolichuck, unpublished Observ. in Walsh et al., 1995; Forlano and Bass, 2002) and change in gonadal state (this report) may preclude the use of these traits for distinguishing type I males. Thus, only body size could serve as a reliable criterion for identifying type I males outside of the breeding season. All of the animals included in the present study that were collected outside of the breeding season in offshore sites and designated as type I males or females were well above the previously reported size range of anatomically identified type I males (SL $\geqslant$ 10.5 cm) and females (SL $\geqslant$ 8.5 cm) collected from nest sites during the summer breeding season from this northern California population (Bass et al., 1996; Brantley et al., 1993a; Foran and Bass, 1998; Grober et al., 1994). All of the males used in this study, collected from both nests and trawls, were $\geqslant$ 11.7 cm in standard length (SL). Over the past 16 years, only 2% of type II males ($n = 431$) from this same northern California population have been $\geqslant$ 11.7 cm SL (above references; A.H. Bass, unpublished observation). Thus, we consider there to be a very low probability that we collected type II males during the winter non-reproductive period. Type II males may yet be found in sites close to the intertidal zone.

During the summer reproductive season (May–August), males and females were collected at low tide by hand in the intertidal zone in Tomales Bay, CA by overturning rocks to expose nests made by type I males (Bass, 1996). Females found in nests with a male were either gravid or spent (eggs released), having spawned within the last 24 h (see Brantley and Bass, 1994). Adult midshipman were collected at all other times of the year by otter trawl (R/V John Martin, Moss Landing Marine Laboratories) in Monterey Bay off shore from Moss Landing, CA.

Fish were anesthetized in 0.025% benzocaine in seawater and blood was immediately collected by cardiac puncture with a heparinized 25 g syringe. Blood samples were kept at 4 °C for 6–12 h, centrifuged at approximately 4,000g for 10 min, and plasma removed and stored at −20 or −80 °C until radioimmunoassay was performed. Standard length, body mass, gonad mass, and GSI (defined here as $100 *$ gonad mass/body mass − gonad mass; according to Tomkins and Simmons, 2002) were recorded, and some gonads preserved

for histological examination (see below). For fall, winter and spring collections (offshore), blood samples were taken either within 1 h of being brought to the surface on the boat or within 4 h at the laboratory after capture. Summer fish were collected by hand from nests and blood collected within several hours back at the laboratory. No differences in sex steroid concentrations (T, E_2, and 11-KT) were seen based on time of sampling after capture in either offshore or intertidal collections in both males and females (2-tailed t test, all P values $\geqslant 0.42$). In addition, we found no significant difference in the F levels between animals sampled within 1 and 4 h after capture from approximately the same depths offshore (54–104 m) (t test, $t = -0.51$, df = 37, $P = 0.61$).

2.2. Hormone assays

Circulating steroid levels were measured by radioimmunoassay following chromatographic separation as previously described in other studies on this species (Brantley et al., 1993b; Knapp et al., 1999). Briefly, approximately 2000 cpm of each tritiated steroid were added to each plasma sample (8–100 µl) and incubated overnight at 4 °C. Samples were then extracted twice in 2 ml diethyl ether before being resuspended in 10% ethyl acetate in isooctane and loaded onto diatomaceous earth columns. T, E_2, 11-KT and F were separated from each other using increasing concentrations of ethyl acetate in isooctane. Each fraction was collected, dried down under nitrogen and resuspended in phosphate-buffered saline overnight. Each sample was assayed in duplicate and a charcoal–dextran solution was used to separate unbound steroid from steroid bound to the antibody. The T antibody (Wien Laboratories; now Research Diagnostics, Flanders, New Jersey, USA) cross-reacts with 11-KT and was used to assay this hormone as well as T. The E_2 and F antibodies were purchased from Endocrine Sciences (now Esoterix Endocrinology, Calabasas Hills, California, USA). Final plasma hormone values were corrected for individual losses during extraction and running on the columns.

Samples were run in three sets of assays. Intra-assay variation for the three sets as calculated from steroid standards was as follows: T 10.1, 9.1, and 4.1%, E_2 10.1, 2.3, and 19.2%, 11-KT 16.0, 10.0, and 13.8%, and F 13.0, 14.2, and 5.8%. Inter-assay variation was 9.2, 1.2, 9.2, and 27.2% for T, E_2, 11-KT and F, respectively. A few samples were run in two assays to verify that values were repeatable across assays, which they were. The level of detectability for each assay was identified as the place on the standard curve where two standard deviations around the mean did not overlap. The level of detectability for a given hormone in a given assay was calculated using this percent bound value, the equation that defined that standard curve, the mean plasma volume (68, 77 or 86 µl) and mean individual recovery

value. The level of detectability for T ranged from 0.06 to 0.17 ng/ml, for E_2 from 0.06 to 0.09 ng/ml, for 11-KT from 0.08 to 0.19 ng/ml, and for F from 1.19 to 8.64 ng/ml.

2.3. Tissue histology

Testes were collected from animals (see above) and stored in 4% paraformaldehyde or 10% formalin. Two days prior to sectioning, testes were rinsed in distilled water overnight, cryoprotected in 30% sucrose-phosphate buffer overnight, sectioned on a cryostat at 30 μm and then mounted onto slides. Following staining with hemotoxylin–esoin or cresyl violet, slides were dehydrated in a graded series of alcohols, coverslipped with Permount, and examined under light microscopy. Testes from eight animals (36% of total) collected in the spring (4 with "low" GSI ≤ 0.1, and 4 with "high" GSI ≥ 1.3) and testes from two animals (18% of total) collected in the winter (one "low" GSI, one "high" GSI) were examined. Testis state from summer breeding males was previously reported (Bass and Andersen, 1991), however, one summer male from this study was processed simultaneously for direct comparison with other time periods (above).

2.4. Statistical analyses

The effect of seasonal period (non-reproductive, pre-nesting, nesting, and post-nesting) on gonadal steroids (T, 11-KT, and E_2) and GSI were determined by a one-way ANOVA. When data sets did not meet the ANOVA assumptions of normality or homogeneity of variances, we used the non-parametric Kruskal–Wallis one-way ANOVA to test for differences in sample medians followed by the Dunn's method for pairwise multiple comparisons (Zar, 1999). Although many monthly data samples of steroid concentrations were not normally distributed, we also report means and standard deviations in Tables 1 and 2 to facilitate comparisons with other published studies on this and other species. Associations among levels of gonadal steroids and F concentration and collection depth were determined using Pearson's correlation and linear regression using log-transformed data. The effects of collection depth and sex (type I male vs. female) on F levels were determined by a two-way ANOVA followed by the Newman–Kuels method for pairwise multiple comparisons (Zar, 1999).

For most of the analyses, only a few samples had hormone concentrations that were below the level of detectability in the radioimmunoassay. In the majority of these cases, the non-detectability was because hormone concentrations were indeed very low, not because our plasma sample was too small; the vast majority of samples had plasma volumes greater than 75 μl. We therefore simply used the hormone value estimated from

the standard curve for statistical analysis, as this hormone value was a reasonable estimate of the actual hormone concentration (i.e., the value read off the standard curve was very low). In the figures, some samples are reported as non-detectable despite the reported steroid concentration being greater than the level of detectability reported above, which are calculated for the mean sample volume for each assay. Assignment of a sample as detectable or non-detectable derives from the percent bound hormone read off the standard curve, and so a smaller plasma volume than the mean can result in a non-detectable value that is higher than the mean detectable hormone concentration. Only for E_2 concentrations in males were the majority of values non-detectable (Table 1). Therefore, we analyzed these data for differences in the proportion of detectable values per period using a χ^2 test.

3. Results

3.1. Seasonal periods and collections

The large range of standard lengths and body mass for type I males and females (Tables 1 and 2) suggests that multiple year classes were sampled. Both male and female fish were collected during four time periods that corresponded to seasonal fluctuations in midshipman reproductive biology and migratory behavior. These time periods (see Figs. 1A and B; 3A and B) were defined as: (1) *non-reproductive*, which occurred in the winter when fish were collected at the deepest depths offshore during December 2001 (mean collection depth = 126 ± 34 SD m) and February 2002 (mean collection depth = 122 ± 29 SD m), and had small GSIs with no mature sperm present in the testes (see Section 3.4) and ovaries with only small (<1 mm diameter) unyolked ova; (2) *pre-nesting*, which occurred in the spring when fish were collected at the shallowest depths offshore during March 2001 (mean collection depth = 85 ± 17 SD m) and April 2002 (mean collection depth = 63 ± 8 SD m) and showed the greatest variation in the GSI; (3) *nesting*, which occurred in the late spring and summer (late May–late August; see Brantley and Bass, 1994) when fish were collected from intertidal nests during low tide at depths <0.5 m during June and July 2002; male GSIs were similar to those at the end of the pre-nesting period but their testes were filled with mature sperm (Section 3.4) whereas female GSIs reached their highest values with ovaries containing group-synchronous, large (~5 mm diameter), yolked eggs (see Bass and Andersen, 1991; Brantley and Bass, 1994), and (4) *post-nesting*, which occurred in the fall (September) after the summer nesting period when fish left the intertidal zone and were collected relatively deep offshore (mean = 116 ± 21 SD m) in September 2001, and when low

Table 1
Morphometrics, gonadosomatic index (GSI) and plasma hormone concentrations (ng/ml) for Type I male Midshipman fish

Month	SL (cm) Mean ± SD (min, med, max) n	Mass (g) Mean ± SD (min, med, max)	GSI Mean ± SD (min, med, max)	Testosterone Mean ± SD (min, med, max) % Detectable	11-Ketotestosterone Mean ± SD (min, med, max) % Detectable	Estradiol Mean ± SD (min, med, max) % Detectable	Cortisol Mean ± SD (min, med, max) % Detectable
Non-reproductive							
December 2001	16.6 ± 5.4 (11.9, 13.6, 22.7) 8	72.1 ± 67.7 (18.1, 28.3, 146.0)	0.55 ± 0.98 (0.03, 0.10, 2.81)	0.16 ± 0.10 (ND, 0.12, 0.32) 75.0	0.93 ± 1.28 (0.07, 0.32, 3.54) 100.0	ND (ND, ND, 0.34) 37.5	265.48 ± 133.63 (105.23, 246.65, 489.93) 100.0
February 2002	13.5 ± 2.8 (11.7, 12.1, 16.8) 3	33.0 ± 21.4 (19.3, 22.0, 57.6)	0.25 ± 0.19 (0.09, 0.21, 0.46)	0.13 ± 0.04 (0.09, 0.13, 0.18) 66.7	0.27 ± 0.33 (ND, 0.15, 0.64) 66.7	ND (ND, ND, 0.08) 33.3	193.12 ± 92.12 (97.39, 200.41, 281.54) 100.0
December and February	**15.8 ± 4.9** (11.7, **13.2**, 22.7 **11**	**62.0 ± 61.2** (19.3, **28.2**, 146.0)	**0.47 ± 0.84** (0.03, **0.14**, 2.81)	**0.15 ± 0.09** (ND, **0.13**, 0.32) 72.7	**0.75 ± 1.12** (ND, **0.23**, 3.54) 90.9	**ND** (ND, **ND**, 0.34) 36.4	**245.74 ± 123.88** (97.39, **236.88**, 489.93) 100.0
Pre-nesting							
March 2001	18.0 ± 1.8 (15.2, 18.4, 21.0) 10	79.2 ± 27.0 (41.4, 84.4, 126.7)	1.91 ± 1.21 (0.09, 1.86, 3.89)	0.26 ± 0.11 (ND, 0.27, 0.40) 80.0	2,58 ± 1.10 (0.29, 2.92, 3.92) 100.0	0.11 ± 0.07 (ND, 0.10, 0.26) 80.0	159.96 ± 141.52 (36.72, 98.80, 458.97) 100.0
April 2002	18.0 ± 3.3 (13.4, 17.4, 23.3) 12	88.8 ± 48.1 (32.0, 73.7, 174.7)	0.71 ± 0.65 (0.05, 0.59, 1.43)	0.42 ± 0.47 (ND, 0.22, 1.53) 75.0	7.14 ± 10.21 (0.13, 2.99, 34.82) 100.0	0.20 ± 0.22 (ND, 0.13, 0.69) 58.3	132.15 ± 100.45 (41.35, 104.90, 347.99) 100.0
March and April	**18.0 ± 2.7** (13.4, **17.9**, 23.3) **22**	**84.5 ± 39.3** (32.0, **81.2**, 174.7)	**1.26 ± 1.11** (0.05, **1.37**, 3.89)	**0.35 ± 0.35** (ND, **0.25**, 1.53) 77.3	**5.07 ± 7.78** (0.13, **2.93**, 34.82) 100.0	**0.16 ± 0.17** (ND, **0.10**, 0.69) 72.7	**144.79 ± 118.62** (36.72, **98.82**, 458.97) 100.0
Nesting							
June 2002	19.9 ± 2.6 (16.7, 19.8, 23.1) 6	111.1 ± 41.9 (63.4, 106.8, 161.7)	0.72 ± 0.48 (0.25, 0.56, 1.59)	0.55 ± 0.28 (0.13, 0.55, 0.90) 100.0	3.58 ± 1.91 (1.77, 3.19, 6.98) 100.0	0.13 ± 0.06 (0.06, 0.12, 0.20) 100.0	119.60 ± 84.19 (40.37, 102.10, 282.94) 100.0
July 2002	16.7 ± 4.4 (12.0, 16.7, 21.4) 4	73.2 ± 55.9 (18.2, 67.1, 140.4)	0.98 ± 0.47 (0.53, 0.88, 1.65)	ND (ND, ND, 0.12) 25.0	0.93 ± 1.52 (ND, 0.24, 3.21) 75.0	ND (ND, ND, 0,09) 25.0	129.94 ± 112.47 (35.82, 95.69, 292.53) 100.0
June and July	**18.6 ± 3.6** (12.0, **18.7**, 23.1) **10**	**95.9 ± 49.0** (18.2, **89.1**, 161.7)	**0.83 ± 0.47** (0.25, **0.71**, 1.65)	**0.36 ± 0.33** (ND, **0.26**, 0.90) 70.0	**2.52 ± 2.16** (ND, **2.50**, 6.98) 90.0	**0.09 ± 0.07** (ND, **0.08**, 0.20) 70.0	**123.73 ± 90.46** (35.82, **101.74**, 292.53) 100.0
Post-nesting							
September 2001	**19.9 ± 2.7** (17.1, **19.1**, 24.7) **7**	**105.9 ± 54.7** (55.8, **89.0**, 214.9)	**0.67 ± 0.56** (0.07, **0.63**, 1.40)	**0.19 ± 0.08** (0.09, **0.22**, 0.26) 100.0	**0.72 ± 0.34** (0.46, **0.63**, 1.43) 100.0	**0.10 ± 0.07** (ND, **0.08**, 0.22) 57.1	**305.89 ± 119.40** (145.04, **338.10**, 469.28) 100.0

Table 2
Morphometrics, gonadal somatic index (GSI) and plasma hormone concentrations (ng/ml) for female Midshipman fish

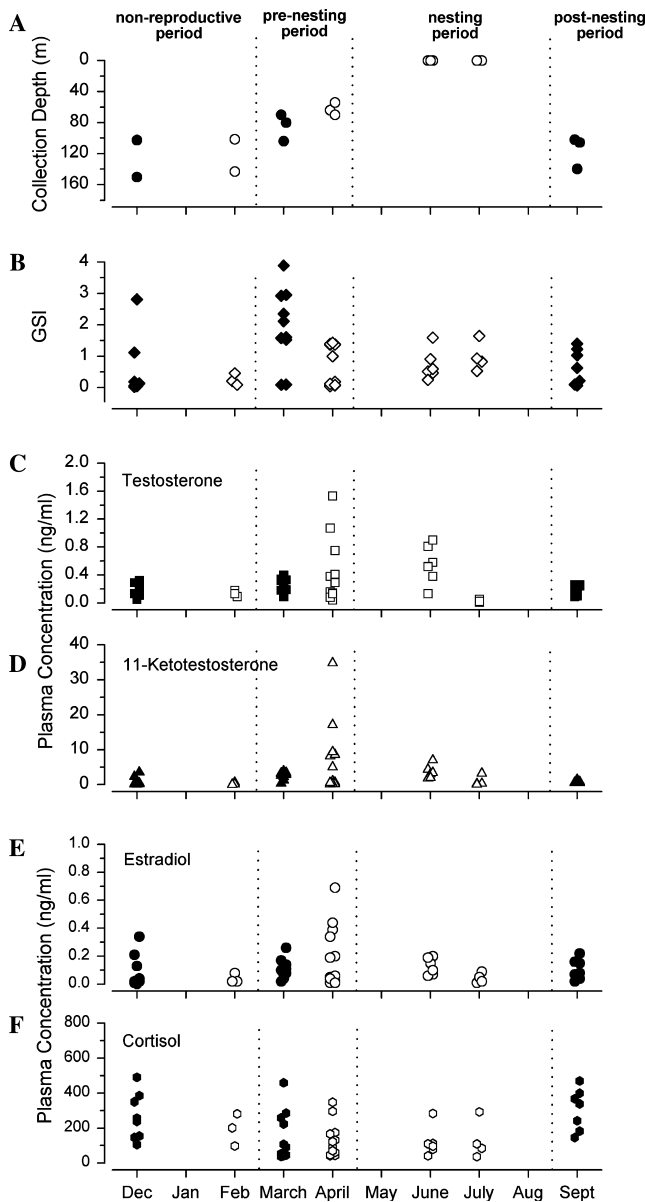| Month	SL (cm) Mean ± SD (min, med, max) n	Mass (g) Mean ± SD (min, med, max)	GSI Mean ± SD (min, med, max)	Testosterone Mean ± SD (min, med, max) % Detectable	Estradiol Mean ± SD (min, med, max) % Detectable	11-Ketotestosterone Mean ± SD (min, med, max) % Detectable	Cortisol Mean ± SD (min, med, max) % Detectable
Non-reproductive							
December 2001	10.1 ± 0.3 (9.7, 10.2, 10.6) 6	11.6 ± 1.1 (9.8, 11.6, 12.8)	0.86 ± 0.50 (0.01, 0.88, 1.54)	0.09 ± 0.03 (ND, 0.09, 0.20) 50.0	0.16 ± 0.04 (ND, 0.17, 0.30) 66.7	ND (ND, ND, ND) 0	975.76 ± 821.61 (320.26, 572.08, 2448.31) 100.0
February 2002	10.6 ± 0.3 (10.2, 10.6, 10.9) 5	13.4 ± 1.1 (12,6, 13.7, 14.4)	0.61 ± 0.34 (0.01, 0.70, 0.84)	0.18 ± 0.05 (0.05, 0.17, 0.35) 80.0	0.09 ± 0.02 (0.02, 0.09, 0.16) 80.0	ND (ND, ND, ND) 0	1067.15 ± 1438.10 (111.45, 268.57, 3525.36) 100.0
December and February	**10.3 ± 0.3** (9.7, **10.2**, 10.9) **11**	**12.4 ± 1.4** (9.8, **12.5**, 14.4)	**0.74 ± 0.43** (0.01, **0.80**, 1.56)	**0.13 ± 0.10** (ND, **0.10**, 0.35) 63.6	**0.13 ± 0.08** (ND, **0.13**, 0.30) 72.7	ND (ND, **ND**, ND) 0	**1017.30 ± 1080.30** (111.45, **516.79**, 3525.36) 100.0
Pre-nesting							
March 2001	13.2 ± 2.8 (9.9, 12.4, 17.5) 6	27.8 ± 19.3 (10.4, 20.5, 58.1)	5.33 ± 5.57 (0.01, 4.31, 12.24)	0.30 ± 0.14 (ND, 0.20, 0.89) 50.0	0.89 ± 0.46 (ND, 0.51, 3.01) 83.3	0.08 ± 0.03 (0.01, 0.07, 0.17) 50.0	207.77 ± 46.53 (155.18, 193.47, 277.51) 100.0
April 2002	14.6 ± 2.1 (11.9, 14.9, 17.1) 11	43.8 ± 21.4 (20.5, 44.6, 82.4)	12.95 ± 9.24 (0.46, 12.36, 27.13)	3.36 ± 0.95 (0.18, 2.11, 7.96) 100.0	5.04 ± 1.44 (0.02, 4.32, 13.88) 90.9	0.05 ± 0.01 (ND, 0.05, 0.14) 18.2	272.32 ± 130.70 (135.54, 219.24, 479.37) 100.0
March and April	**14.1 ± 2.4** (9.9, **13.2**, 17.5) **17**	**38.2 ± 21.6** (10.4, **29.1**, 82.4)	**10.26 ± 8.78** (0.01, **10.55**, 27.13)	**2.28 ± 2.91** (ND, **0.58**, 7.96) 82.4	**3.57 ± 4.33** (ND, **1.12**, 13.88) 88.2	**ND** (ND, **ND**, 0.17) 29.4	**249.54 ± 111.20** (135.54, **211.24**, 479.37) 100.0
Nesting							
June 2002	14.6 ± 1.5 (11.2, 14.9, 15.7) 8	33.5 ± 6.5 (25.3, 32.8, 43.3)	37.46 ± 6.52 (26.38, 38.29, 45.60)	0.09 ± 0.02 (ND, 0.10, 0.15) 50.0	0.19 ± 0.03 (0.06, 0.20, 0.32) 100.0	ND (ND, ND, ND) 0	266.56 ± 222.19 (62.68, 197.20, 724.36) 100.0
July 2002	13.2 ± 1.6 (11.5, 12.9, 15.5) 7	25.8 ± 10.3 (16.4, 20.5, 42.9)	24.49 ± 17.90 (5.59, 32.54, 45.79)	0.08 ± 0.02 (ND, 0.08, 0.17) 42.9	0.23 ± 0.05 (ND, 0.25, 0.39) 71.4	ND (ND, ND, ND) 0	178.48 ± 80.22 (111.44, 130.80, 308.19) 100.0
June and July	**14.0 ± 1.7** (11.2, **14.5**, 15.7) **15**	**29.9 ± 9.1** (16.4, **32.1**, 43.3)	**31.41 ± 14.26** (5.59, **36.60**, 45.79)	**0.09 ± 0.05** (ND, **0.08**, 0.17) 46.7	**0.21 ± 0.11** (ND, **0.22**, 0.39) 86.7	**ND** (ND, **ND**, ND) 0	**225.45 ± 171.79** (62.68, **180.60**, 724.36) 100.0
Post-nesting							
September 2001	**17.3 ± 2.4** (14.2, **17.1**, 21.8) 8	**60.2 ± 26.4** (33.0, **55.3**, 116.5)	**2.67 ± 0.37** (2.13, **2.67**, 3.09)	**0.11 ± 0.02** (0.07, **0.10**, 0.19) 87.5	**0.44 ± 0.08** (0.21, **0.41**, 0.82) 100.0	ND (ND, **ND**, ND) 0	**564.71 ± 423.38** (142.48, **463.86**, 1435.51) 100.0

Fig. 1. Plasma steroid concentrations for wild-caught type I male plainfin midshipman, *Porichthys notatus*, collected from Monterey Bay and Tomales Bay, CA during the non-reproductive, pre-nesting, nesting, and post-nesting periods between March 2001 and July 2002 (also basis for data shown in Figs. 2–7). (A) Collection depth and (B) Gonadosomatic index (GSI) profiles of males collected across the sampling period. (C) Testosterone, (D) 11-ketotestosterone, (E) estradiol, and (F) cortisol concentrations in the sampled population of type I males across the sampling period. Note that both detectable and estimated non-detectable steroid concentrations are reported. Data collected during the years 2001 (solid symbols) and 2002 (open symbols) are noted separately but are presented together to show a single annual cycle.

GSIs reflected the regressed state of the gonads in females. Detailed descriptions of the summer nesting period are available elsewhere (Bass, 1996 and references therein). Previous studies using light and electron microscopy described the gonads of juveniles and adults

(Bass and Andersen, 1991; also see Section 3.4). Although the samples were collected over 16 months, we present the data as a single "annual cycle" because the timing of the breeding season has been very consistent across years for the past 16 years (A. H. Bass, personal observations).

3.2. Male plasma steroid levels

Type I male midshipman showed seasonal changes in plasma levels of T that varied with season and reproductive behavior (Fig. 1C, Table 1). A maximum T concentration among all males was 1.53 ng/ml for a 20.2 cm male collected during the pre-nesting period in April. The lowest detectable T concentration was 0.09 ng/ml recorded for two males, a 16.8 cm male collected during the non-reproductive period in February and the other a 18.8 cm male collected during the post-nesting period in September. Levels of T were lowest during the non-reproductive period and during the latter half of the nesting period in July. Following the non-reproductive period, T levels increased during the pre-nesting period and peaked in June during the nesting period (Kruskal–Wallis one-way ANOVA on December–June, Dunn's test, $H = 10.95$, df $= 4$, $P < 0.05$). After peaking in June, T levels then decreased to non-detectable values during July, followed later by a small but significant increase in the post-nesting period (Kruskal–Wallis one-way ANOVA on June–September, Dunn's test, $H = 10.86$, df $= 2$, $P < 0.005$). Thus, T levels were highest and then lowest in the first and second halves, respectively, of the nesting period.

Plasma levels of 11-KT followed a similar pattern of seasonal variation (Fig. 1D, Table 1). The maximum 11-KT concentration among all males was 34.82 ng/ml for a 23.3 cm male collected during the pre-nesting period in April; the lowest was 0.07 ng/ml for a 12.4 cm male collected during the non-reproductive period in December. Levels of 11-KT were lowest during the non-reproductive period, the latter half of the nesting period, and in the post nesting period. The winter 11-KT minima were followed by markedly higher 11-KT levels during the pre-nesting period that subsequently peaked in June during the nesting period (Kruskal–Wallis one-way ANOVA on December–June, Dunn's test, $H = 13.06$, df $= 4$, $P < 0.05$). Plasma 11-KT levels were dramatically lower, down to one-thirteenth of their peak, in July followed by slightly higher values during September (Kruskal–Wallis one-way ANOVA on June–September, Dunn's test, $H = 9.49$, df $= 2$, $P < 0.01$). Males collected in June had nests that contained either no eggs or only newly fertilized eggs, while males collected in July had both eggs and embryos (M.A. Marchaterre, personal communication). June males had higher plasma 11-KT and T levels than July males (Figs. 1C and D; Table 1) (*t* tests, *P* values < 0.05).

A significant relationship between 11-KT and T levels was identified when either both detectable and estimated non-detectable (Ho: $\beta = 0$; $t = 9.43$; $P < 0.001$) or only detectable (Ho: $\beta = 0$; $t = 8.71$; $P < 0.001$) concentrations were used in regression analyses. 11-KT and T levels were positively correlated (Fig. 2A).

Estradiol was detected at very low levels in the blood of males during every seasonal period. However, detectable levels of E_2 were only recorded in 50% or more of the males during the pre-nesting period, during June

of the nesting period, and in September during the subsequent post-nesting period (Table 1). There was no significant difference in the proportion of males with detectable E_2 levels across the four seasonal periods ($\chi^2 = 5.54$, df = 3, $P = 0.21$). A maximum E_2 concentration of 0.69 ng/ml was recorded from the same male collected in April that also had the highest measured 11-KT. The lowest detectable E_2 concentration of 0.06 ng/ml was recorded for two males, a 16.6 cm male collected during the pre-nesting period in April and the other a 21.5 cm male collected during the nesting period in June. A significant relationship between E_2 and T levels was identified when either detectable and estimated non-detectable (Ho: $\beta = 0$; $t = 6.66$; $P < 0.001$) or only detectable (Ho: $\beta = 0$; $t = 3.98$; $P < 0.001$) values were used for regression analyses. E_2 and T levels were positively correlated (Fig. 2B).

3.3. Female plasma steroid levels

Female midshipman also showed seasonal variation in plasma T levels, with a peak in the pre-nesting period (Fig. 3C, Table 2). The maximum T concentration was 7.96 ng/ml for a 17.1 cm female collected during the pre-nesting period in April. The lowest detectable T concentration was 0.05 ng/ml recorded for a 10.9 cm female collected during the non-reproductive period in February. In general, females had relatively low, median T concentrations that did not exceed 0.17 ng/ml throughout the seasons except during the pre-nesting period, which is when the ovaries underwent recrudescence before the spawning season. Females showed a brief spike in plasma T during the pre-nesting period in April. Detectable 11-KT concentrations were observed among 29% of females during the pre-nesting period (Table 2; not shown in Fig. 3 because concentrations were generally very low).

Females also showed a seasonal increase in plasma E_2 levels during the pre-nesting period just prior to the summer spawning season (Fig. 3D, Table 2). Females had relatively low E_2 levels throughout the year except during the pre-nesting period, when E_2 levels peaked in April. The maximum E_2 concentration among all females was 13.88 ng/ml recorded for a 13.2 cm individual collected during the pre-nesting period in April. The lowest detectable E_2 concentration was 0.02 ng/ml recorded for two females, a 10.9 cm female collected during the non-reproductive period in February and the other a 12.1 cm female during the pre-nesting period in April. Similar to T, plasma E_2 levels in females showed a brief spike that occurred during the pre-nesting period in April. A significant relationship between E_2 and T levels was identified when either both detectable and estimated non-detectable (Ho: $\beta = 0$; $t = 9.90$; $P < 0.001$) or only detectable (Ho: $\beta = 0$; $t = 9.08$; $P < 0.001$) values were considered. E_2 and T levels were highly correlated

A

log 11-KT = 1.53 $*$ log T + 1.08
$r = 0.80$
$n = 51$

Legend:
● 11-KT & T detectable
■ T non-detectable
□ 11-KT non-detectable
◇ 11-KT & T non-detectable

Y-axis: 11-Ketotestosterone (ng/ml)
X-axis: Testosterone (ng/ml)

B

log E_2 = 0.92 $*$ log T - 0.50
$r = 0.70$
$n = 51$

Legend:
● E & T detectable
▲ E non-detectable
○ E & T non-detectable

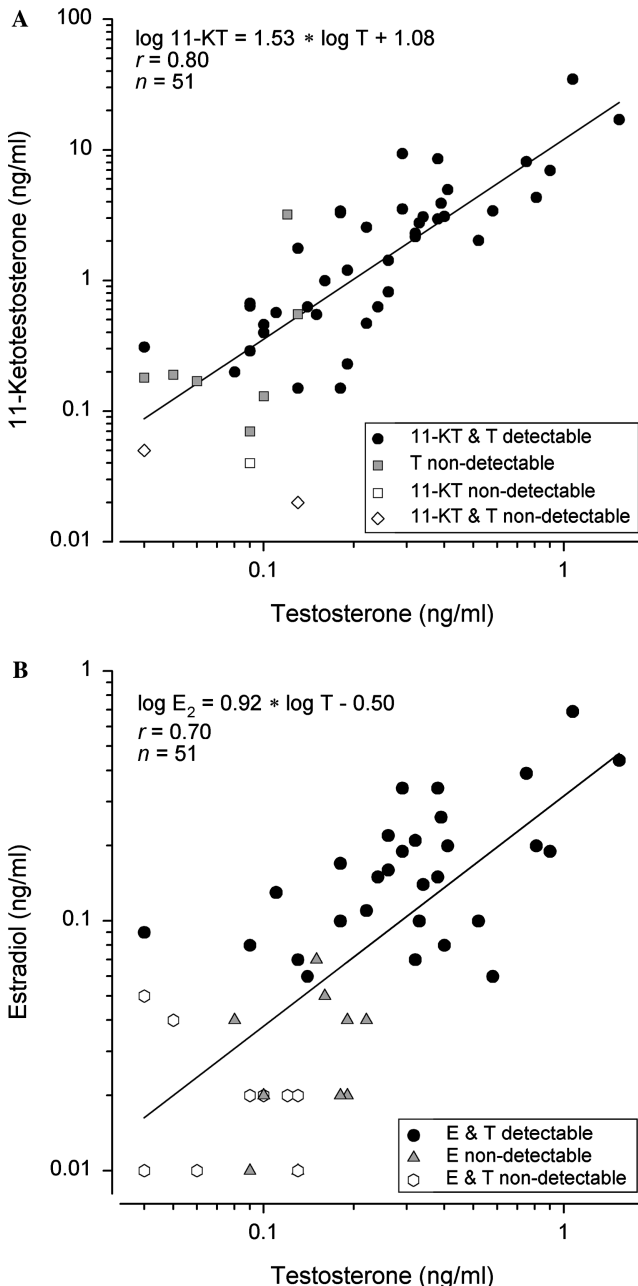Y-axis: Estradiol (ng/ml)
X-axis: Testosterone (ng/ml)

Fig. 2. The co-variation between the plasma concentrations of: (A) 11-ketotestosterone and (B) estradiol, with testosterone for type I male midshipman. Values identified as non-detectable are plotted as the estimate from the radioimmunoassay (see Section 2).
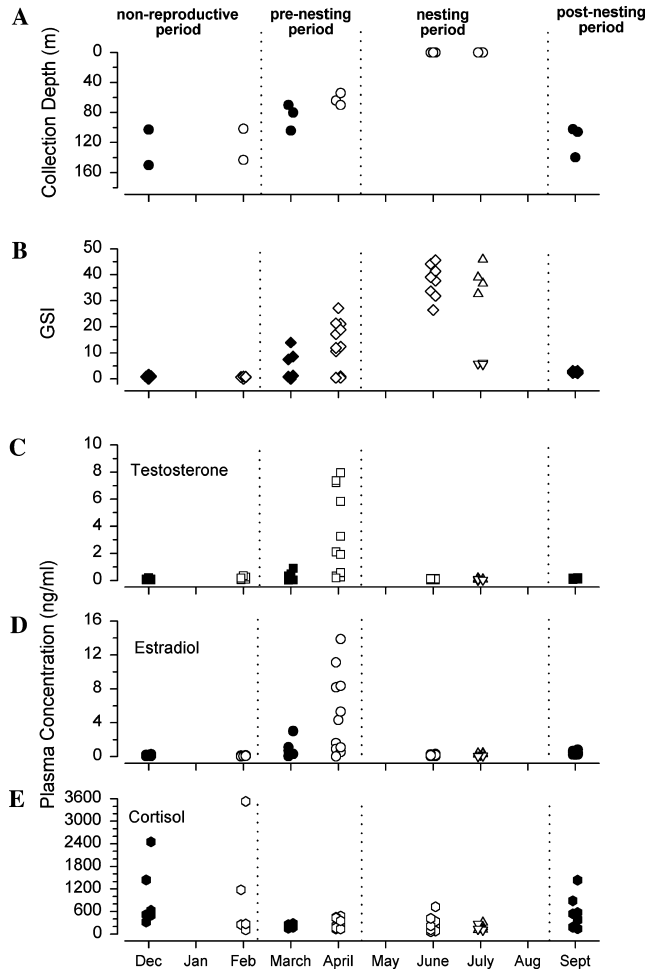
Fig. 3. Plasma steroid concentrations for wild-caught female midshipman. (A) Collection depth and (B) gonadosomatic index (GSI) profiles of females collected across the sampling period. (C) Testosterone, (D) estradiol, and (E) cortisol concentrations in the sampled population of females across the sampling period. Note that both detectable and estimated non-detectable steroid concentrations are reported and that the y-axis scales for hormone concentrations differ from those shown for males in Fig. 1. Gravid (Δ) and spent (∇) females are shown separately for the month of July and data collected during the years 2001 (solid symbols) and 2002 (open symbols) are noted separately but are presented together to show a single annual cycle.
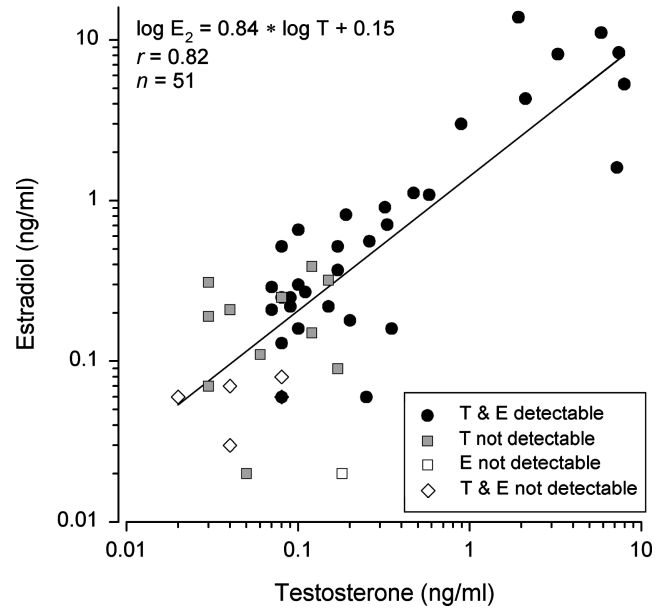

Fig. 4. The association between plasma concentrations of testosterone and estradiol for female midshipman. Values identified as non-detectable are plotted as the estimate from the radioimmunoassay (see Section 2).

(Fig. 4), and the slope of the relationship was very similar to that for type I males (Fig. 2B).

3.4. Steroid levels and GSI

Among type I males, gonadal steroid hormone levels increased most during the pre-nesting period, coincident with the greatest increase in GSI (Table 1). Variation in male GSI was greatest in the pre-nesting period (Fig. 1B, Table 1), although mean GSI did not differ significantly among the four seasonal periods (one-way ANOVA, $F = 2.46$, df = 3, 47, $P = 0.07$). Despite no significant seasonal difference in mean GSI, histological examination

of representative examples from each time period revealed that non-reproductive testes contained no sperm, prenesting testes had an increased incidence of mature sperm with increasing GSI, and nesting testes were full of mature sperm (Fig. 5). Three-dimensional plots of steroid levels vs. gonadal mass vs. body mass during the pre-nesting period showed that the largest males (body mass > 116 g), in general, had the largest testes and the highest levels of T, 11-KT, and E_2 (Figs. 6A–C). Note that the largest males appear to be the first to undergo gonadal recrudescence in the spring pre-nesting period.

Among females, T and E_2 levels were also highest during the pre-nesting period when ovaries were undergoing recrudescence (Table 2, Fig. 3). Female GSI steadily increased from the non-reproductive period to the pre-nesting period, and then peaked in June during the nesting period (Kruskal–Wallis one-way ANOVA, Dunn's test, $H = 22.20$, df = 2, $P < 0.001$). Median GSI in July was somewhat less because the sample contained both gravid females and spent females (females that had released most of their eggs). The GSI of gravid females was dramatically higher (mean = 38.49, $n = 4$) than that of spent females (mean = 5.82, $n = 3$; t test, $t = 9.93$, df = 5, $P < 0.001$) (Fig. 3; July females are separated into gravid, Δ, and spent, ∇, groups). Three-dimensional plots of steroid levels vs. gonadal mass vs. body mass during the pre-nesting period showed that the largest females (body mass > 58.0 g), in general, had the largest ovaries and the highest levels of T and E_2 (Figs. 6D and E). Similar to males, the largest females also appear to be the first to undergo gonadal recrudescence in the spring pre-nesting period.
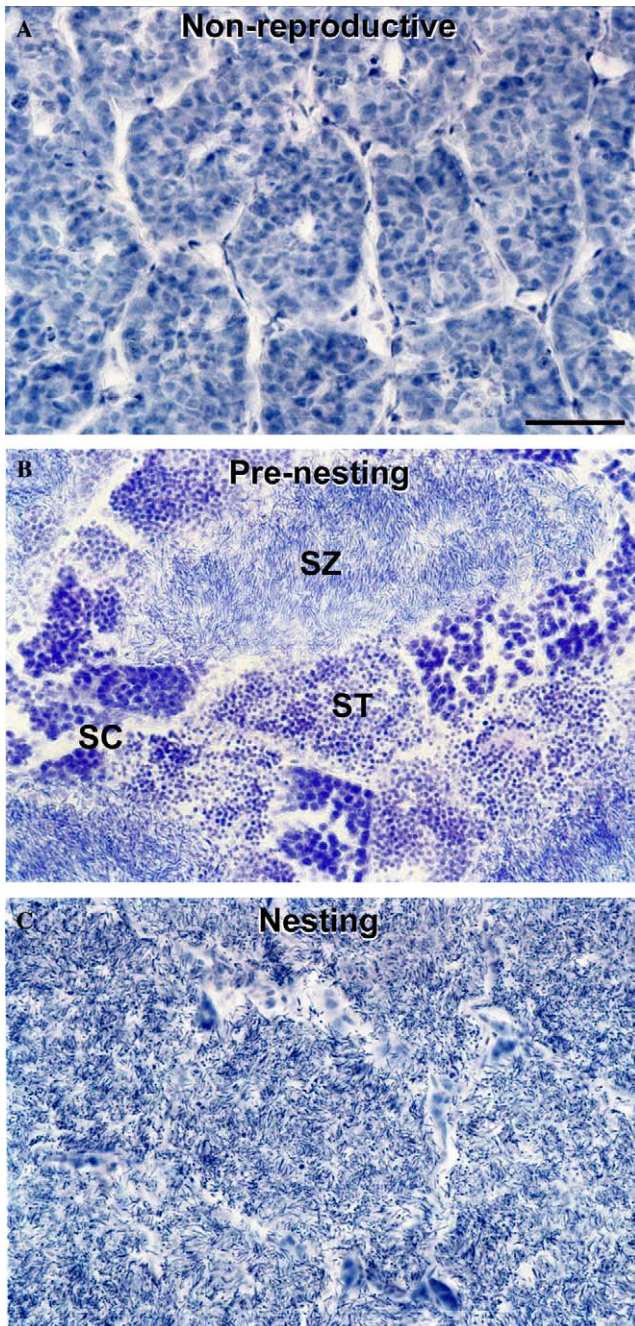
Fig. 5. Representative stages of testis development during the annual reproductive cycle of midshipman. (A) Regressed testis from the non-reproductive period (December–February); only spermatocytes/spermatogonia present. (B) Testis from the pre-nesting period (March–April); multiple types of germ cells surround packets of mature sperm. (C) Testis from the nesting period (May–August) contains a homogenous population of mature sperm (SZ). SC, spermatocytes; ST, spermatids; SZ, spermatozoa. Scale bar, 40 μm.

3.5. Cortisol profiles and collection depths

We assayed F levels to determine whether there were any associations between F and gonadal steroid levels and to assess the relative magnitude of the handling stressor associated with trawling, which we expected a priori.

Trawling is the only means by which non-nesting animals can be collected outside of the breeding season and so a concern was whether high F levels would inhibit sex steroid levels. Although F concentrations were very high in some animals, there was no relationship among males between concentrations of either F and T, 11-KT, or E_2 during each of the time periods (Ho: $\beta = 0$; P values $\geqslant 0.26$). This was also the case when only detectable levels were considered (Ho: $\beta = 0$; P values $\geqslant 0.08$). Among females, there was no relationship between levels of F and either T or E_2 during the pre-nesting, nesting and post-nesting periods (Ho: $\beta = 0$; P values $\geqslant 0.35$); this was also the case when only detectable values were considered (Ho: $\beta = 0$; P values $\geqslant 0.18$). During the non-reproductive period, there was no relationship between the levels of F and T (Ho: $\beta = 0$; $P = 0.08$), but there was a significant relationship between F and E_2 (Ho: $\beta = 0$; $t = 2.42$; $P < 0.05$). Plasma F and E_2 concentrations were positively correlated ($r = 0.63$) and a linear relationship was identified (log F (ng/ml) $= 0.81 * \log E_2$ (ng/ml) $+ 3.60$). However, this relationship was not significant when the estimated non-detectable values for E_2 were removed from the regression analysis (Ho: $\beta = 0$; $P = 0.37$).

Plasma F levels were positively correlated with collection depth in both type I males ($r = 0.84$; Ho: $\beta = 0$; $t = 3.45$; $P < 0.05$) and females ($r = 0.77$; Ho: $\beta = 0$; $t = 2.72$; $P < 0.05$) (Figs. 1A and F; 3A and E). A linear relationship was identified as log F (ng/ml) $= 1.5 * \log$ collection depth (m) $+ 100.1$ for type I males and log F (ng/ml) $= 5.3 * \log$ collection depth (m) $+ 116.1$ for females. Mean F levels did not differ between males and females at collection depths $\leqslant 115$ m. However, during the non-reproductive period (Figs. 1F and 3E), the mean F levels of females were approximately 3.0 and 3.7 times higher than those of males at 122 and 126 m (the deepest collection depths), respectively (two-way ANOVA and the Newman–Kuels method; interaction of sex and collection depth; $F = 25.72$; df $= 2, 5$; $P < 0.05$).

4. Discussion

The aim of this study was to document the seasonal cycle of plasma levels of the gonadal steroids T, 11-KT, and E_2 as they relate to gonadal development and reproductive behavior in wild populations of plainfin midshipman fish. Fig. 7 presents a graphic summary of this cyclicity. Among type I males, T and 11-KT were at minima during the winter non-reproductive period followed by pronounced increases during the spring pre-nesting period. 11-Ketotestosterone remained elevated into the summer nesting period. Low, detectable concentrations of E_2 were also found in the plasma of 50% or more type I males during every seasonal period except the winter non-reproductive period. Among females, T and E_2 were relatively low (<1 ng/ml) throughout the
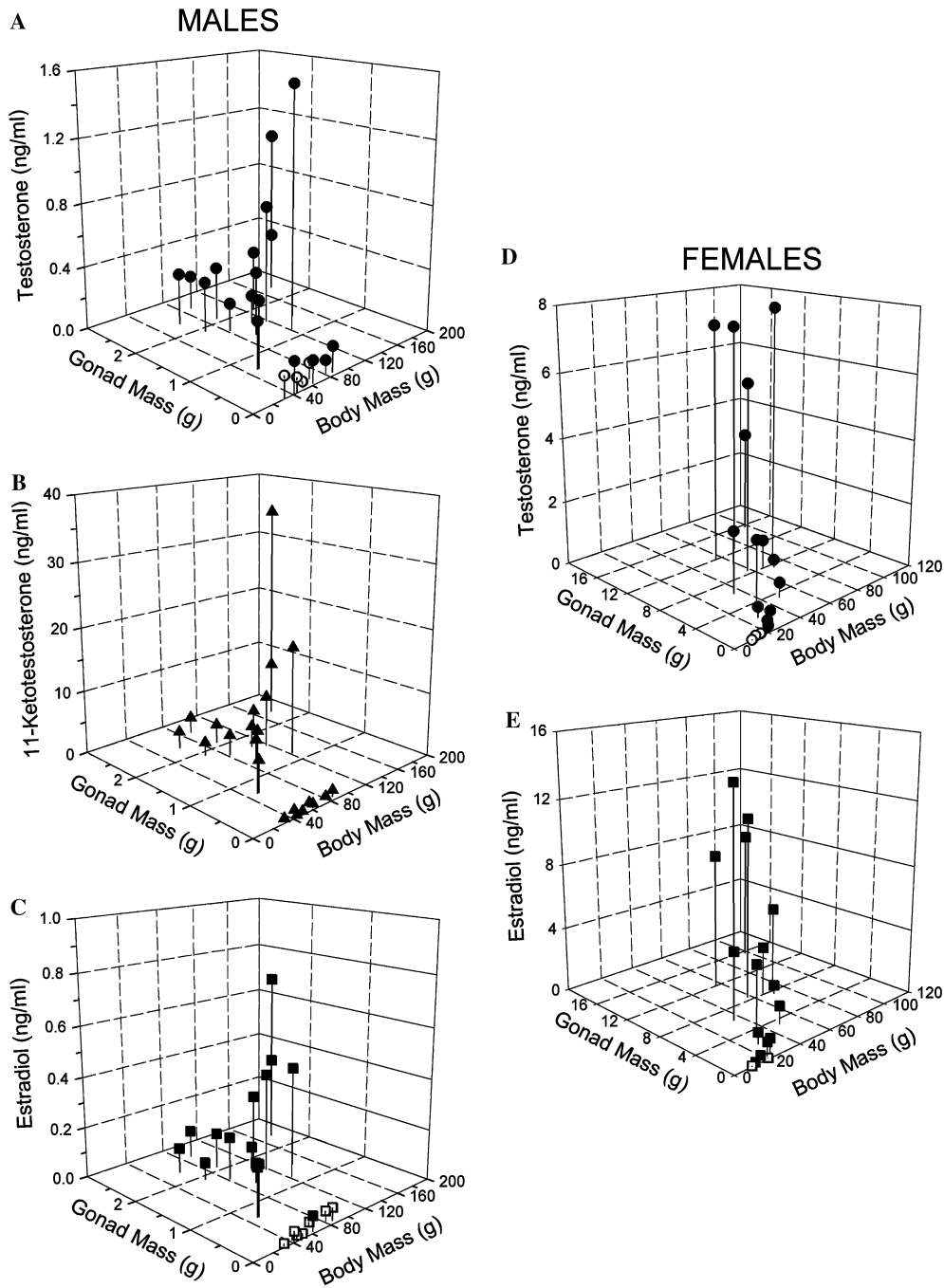
Fig. 6. Three-dimensional plots of body mass vs. gonad mass vs. gonadal steroid levels for type I male (A–C) and female (D and E) midshipman during the pre-nesting period (March–April). Note that all steroid scales differ. Closed symbols represent detectable steroid concentrations while open symbols represent non-detectable steroid concentrations estimated from the radioimmunoassay (see Section 2 for details).

year except during the pre-nesting period when they peaked in April, approximately one month before the beginning of the summer spawning season.

4.1. Male gonadal steroid levels, GSI, and associated behaviors

This study demonstrates clear seasonal patterns of circulating androgen and estrogen levels associated with

gonadal development, reproductive season and behavior of type I males. Type I males show distinct seasonal increases and then decreases across the pre-nesting/ nesting periods in plasma T and 11-KT levels. Although we did not sample during the last month of the nesting period in August, we expect levels would resemble those in July and September. The initial rise in T and 11-KT levels during the pre-nesting period occurs when testes recrudesce, exhibit the largest seasonal variation in size,
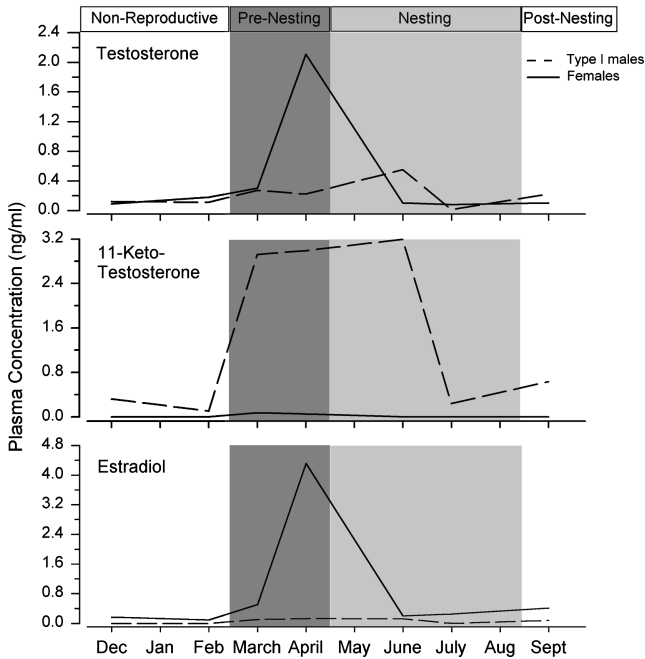
Fig. 7. Summary diagram of the plasma sex steroid levels for wild-caught type I male and female plainfin midshipman collected from Monterey Bay and Tomales Bay, CA during the non-reproductive, pre-nesting, nesting, and post-nesting periods between March 2001 and July 2002. Median steroid concentrations are plotted for both type I males and females. Note that the temporal difference in seasonal changes in major circulating steroid levels between males and females parallels sex differences in reproductive behavior. The maintenance of elevated levels of androgens in males into the spawning period reflects their continued courtship and spawning activity throughout the summer, while a single peak of E_2 and T prior to spawning in females reflects the behavior of a single spawning event.

and show an increased incidence of mature sperm with increasing GSI (Fig. 5). Highest median concentrations of T and 11-KT occur during the first half of the nesting period, and are then much lower or non-detectable in the second half. The temporal pattern of androgen levels is consistent with the majority of type I males shifting from courtship to parental care over the course of the breeding season and is similar to that reported during the period of parental care in other vertebrates including rodents (Brown et al., 1995), birds (Beletsky et al., 1995; Silverin, 1990; Wingfield et al., 1990), amphibians (Townsend and Moger, 1987) and other teleost fishes (Kindler et al., 1989; Pankhurst and Barnett, 1993; Páll et al., 2002; Sikkel, 1993).

Androgens likely play an important role in mediating a suite of seasonal reproductive behaviors in type I male midshipman including courtship and parental care, as well as aggression and territoriality in the establishment of nest sites. A previous study showed that T and 11-KT levels were significantly higher in type I males that had no eggs or eggs only in their nests compared to nesting males that had developmentally advanced embryos (Knapp et al., 1999). Similarly, in this study we report higher levels of T and 11-KT for type I males sampled in

June that had either no eggs or only newly fertilized eggs in their nests, compared to males sampled in July that had both eggs and embryos. The observed decline in T and 11-KT levels may reflect a compromise between the continued expression of male secondary sexual characters for courtship and territoriality versus the suppressive effects of androgens on the immune system (e.g., Casto et al., 2001; Folstad and Karter, 1992), and/or investment in parental care behavior (Knapp et al., 1999). Knapp et al. (1999) proposed several mechanisms to explain the rather slow pattern of declining androgen levels associated with the expression of parental care including pheromonal cues produced by embryos to suppress androgen production, physiological changes linked to the cessation of sperm production and maturation, and stimuli directly or indirectly associated with conspecific interactions that may influence androgen levels in parental midshipman.

The seasonal increase in plasma T levels in type I males is similar to that reported for male Lusitanian toadfish (*Halobatrachus didactylus*) (Modesto and Canario, 2003a). Testosterone levels peak in both male toadfish and type I male midshipman during the early to mid spawning period. In male toadfish, 11-KT levels peak during the spawning period and are coincident with peak T levels. In contrast, type I male midshipman show pronounced increases in 11-KT levels at the beginning of the pre-nesting period which then remain elevated until the peak of the nesting period in July (Fig. 1). As discussed below, the prolonged elevation of 11-KT levels in midshipman males may be related to the induction of exaggerated secondary sex characteristics required for acoustic courtship (sonic muscles are greatly hypertrophied compared to those of toadfish; Bass and Marchaterre, 1989; Modesto and Canario, 2003b; Walsh et al., 1995).

A number of studies support the role of 11-KT in the induction of secondary sex characteristics that are involved in display behaviors by courting male midshipman (review: Brantley et al., 1993b). Rising 11-KT levels during the pre-nesting period could promote sonic muscle growth in preparation for prolonged humming during summer courtship activity. Such an anabolic role for 11-KT is supported by previous demonstrations that androgens induce sonic muscle growth and that 11-KT has a greater efficacy than other androgens in such a process (Brantley et al., 1993a,b). Sustained, elevated 11-KT levels during the summer may contribute to either the maintenance of sonic muscle size and/or the sonic behavior itself. Elevated 11-KT levels are associated with humming behavior (Knapp et al., 2001) and can induce changes in the temporal attributes of the rhythmic motor volley generated by the brain's vocal pacemaker; these changes are consistent with the transition from a non-humming to a humming state (Bass, 1995; Remage-Healey and Bass, 2002).

We also found low, detectable concentrations of E_2 in 50% or more type I males during every seasonal period except during the winter non-reproductive period. In comparison, E_2 concentrations were not detected during any period of the male Lusitanian toadfish's reproductive cycle (Modesto and Canario, 2003a). In type I male midshipman, the estrogen-producing enzyme aromatase is found at low or non-detectable concentrations in the testis compared to the relatively high aromatase concentrations found in the brain (Forlano et al., 2001; Schlinger et al., 1999). Thus, the circulating levels of E_2 found in the blood plasma of type I male midshipman may be the result of extra-gonadal secretion of E_2 by the brain.

4.2. Female gonadal steroid levels, GSI, and associated reproductive behavior

Estradiol and T levels show brief seasonal increases across the females sampled. Median E_2 levels are very low throughout most of the year, except for the brief peak in April that is coincident with the peak median T level. The increases in E_2 and T levels occur during the pre-nesting period in April, a month before the summer spawning season, and is similar to the increase in the Lusitanian toadfish which also occurs a month before the start of the toadfish spawning season (Modesto and Canario, 2003a). Increases in plasma E_2 and T levels in female midshipman are associated with ovarian recrudescence as reflected by the increase in GSI (Fig. 3). Estradiol is known to promote synthesis of hepatic yolk precursors (vitellogenin) in a variety of teleost fish species; a number of previous studies have reported a good correlation of E_2 levels with GSI and oocyte size during the period of vitellogenesis (Crim and Idler, 1978; Lambert et al., 1978; Schulz, 1984; Wingfield and Grimm, 1977).

Testosterone levels are higher in females than type I males. This pattern has been observed in other teleosts, especially during vitellogenesis (Campbell et al., 1976; Scott et al., 1980; Stuart-Kregor et al., 1981). The role of T in female teleosts still remains unclear. T may have vitellogenic action of its own at high concentrations (Fostier et al., 1983), and may play a role in maintaining oocytes once vitellogenesis is complete (Kime, 1993). However, T does not seem to be important in final oocyte maturation (Goetz, 1983; Kime, 1993; Nagahama et al., 1986, 1993). Clearly, similar studies that examine the production of T and E_2 during gonadal recrudescence in midshipman will be needed to determine the function and roles of T and E_2 during gametogenesis in this species.

Recent work on the same wild population of midshipman fish shows that the frequency sensitivity of the peripheral auditory system changes with female reproductive state, such that reproductive females collected during the nesting period are better suited than females collected during the non-reproductive period to encode the higher harmonic components of type I male advertisement calls (Sisneros and Bass, 2003a). The harmonics of the hum likely increase signal (hum) detection in shallow water environments, such as the shallow breeding grounds of the intertidal zone, where higher harmonic frequencies are transmitted over a greater distance than the lower fundamental frequency of the hum due to the inverse relationship between water depth and the cut-off frequency of sound transmission (Bass and Clark, 2003; Fine and Lenhardt, 1983). In addition, McKibben and Bass (2001) showed that the encoding of hum-like tones by the peripheral auditory system is enhanced when harmonics are added to tonal stimuli. Thus, the female's increased sensitivity to the hum's upper harmonics during the nesting period may both increase the detection of the advertisement call as well as the detection and encoding of the hum's fundamental frequency at close range, thereby enhancing the female's probability of mate detection and localization. Furthermore, these seasonal changes in the female's auditory response properties can be induced by T and E_2 (Sisneros and Bass, 2003b), which are present in females at peak concentrations approximately one month before the nesting period in April during ovarian recrudescence (Fig. 3). The current results are consistent with the hypothesis that the seasonal increases in T and E_2 can lead to the observed shift in auditory frequency sensitivity of female midshipman.

4.3. Plasma estrogen levels and brain aromatase

Estradiol levels in type I males and females are elevated seasonally and concurrently with T levels. One likely source for such seasonal increases in E_2 may be the conversion of T into E_2 by the enzyme aromatase (Campbell et al., 1976). Teleost fish have the highest brain levels of the estrogen producing enzyme aromatase among vertebrate classes (Pasmanik and Callard, 1985). In goldfish (*Carassius auratus*), toadfish (*Opsanus beta*), and midshipman, aromatase levels in testis are non-detectable or relatively low in comparison to aromatase activity in the brain (Pasmanik and Callard, 1985; also see Callard et al., 1990; Schlinger et al., 1999). In addition, whereas aromatase mRNA is abundant in ovarian tissue, transcripts are not found in midshipman testis by in situ hybridization (Forlano et al., 2001).

Because other tissues outside the brain in male teleosts essentially show either low or non-detectable aromatase activity, we hypothesize that E_2 circulating in male midshipman may be secreted by the brain. This case would be similar to that in songbirds, another group with relatively high brain aromatase levels, where Schlinger and Arnold (1992) demonstrated that the brain was the primary source of E_2 detected in

circulation in male zebra finches. Aromatase activity and mRNA are highest in the brain of teleost fishes during periods associated with high circulating levels of steroids, including androgens (Forlano and Bass, 2003; Gelinas et al., 1998; Pasmanik and Callard, 1988). As predicted, type I male midshipman express high aromatase during the pre-nesting and nesting periods (Forlano and Bass, 2003) when T and E_2 levels are at or near their peak. Since aromatase mRNA transcripts cannot be found in testis via in situ hybridization (Forlano et al., 2001), the mostly likely source of E_2 synthesis from T in type I male midshipman is brain aromatase. In addition to ovarian aromatase, elevated levels of brain aromatase in female midshipman during the pre-nesting period (Forlano and Bass, 2003) may also contribute to increased plasma levels of E_2 prior to spawning. In other teleosts such as the female catfish, *Ictalurus punctatus*, circulating E_2 levels are maintained in the summer despite the absence of aromatase transcripts in the ovary, which also provides evidence for extra-gonadal secretion of E_2, possibly from the brain (Trant et al., 1997).

4.4. Male and female cortisol profiles

In general, the cortisol levels in our samples are correlated with collection depth. We believe that the F levels correlate with collection depth rather than collection date because the very high F levels we measured clearly reflect the handling stressor of trawling and/or transport back to the laboratory. There may be, and in fact is likely to be, monthly and/or yearly variation in physiological processes supported by F, but our study was not designed to address this question. Although our F levels reflect differing degrees of a handling stressor, this handling was not sufficient to mask a seasonal pattern of plasma sex steroid levels. In general, low steroid levels correlated well with gonadal state rather than F levels.

We report a significant difference in plasma F levels between type I males and females collected from depths greater than 120 m that may reflect sex-specific differences in seasonal exposure to environmental stressors during the nesting period. During the spawning season, type I males maintain nests in the shallow intertidal zone and are exposed daily at low tide to stressful hypoxic conditions (see Bass, 1996). In addition, type I males may also undergo starvation stress since nesting males usually do not eat during the nesting period (Arora, 1948). In contrast, females do not spend prolonged periods in the intertidal zone, but apparently move into nests on a single night, spawn within a 24 h period, and then leave nests to return to offshore sites (Arora, 1948; Brantley and Bass, 1994; Hubbs, 1920). Thus, type I males, which maintain nests in habitats that are environmentally stressful, may have higher thresholds for producing F under conditions of acute stress. For a more extended discussion of F levels among teleosts, see Knapp et al. (1999). Current studies are investigating the possible sexually dimorphic effects of F on the vocal physiology and behavior of midshipman and the closely related toadfish (e.g., Remage-Healey and Bass, 2002, 2003).

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