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Individual, temporal, and population-level variations in circulating 11-ketotestosterone and 17β-estradiol concentrations in the oyster toadfish *Opsanus tau*

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ABSTRACT

Sex steroid hormones are important for reproduction in all vertebrates, but few studies examine inter-individual, temporal, and population-level variations, as well as environmental influences on circulating steroid levels within the same species. In this study we analyzed plasma 11-ketotoestosterone (11-KT) and 17β-estradiol (E_2) levels in the oyster toadfish to test for 1) individual and temporal variations by serially sampling the same individuals during the reproductive and post-reproductive period, 2) variations in steroid levels among toadfish obtained from different sources or maintained under different holding conditions, and 3) correlations with environmental parameters. Results from serial sampling showed marked inter-individual variations in male 11-KT levels in two separate groups of toadfish, but no temporal differences from June to September. Females also showed inter-individual variations in E_2 concentrations, but most had elevated levels late in the reproductive season coincident with oocyte growth prior to winter quiescence. E_2 concentration, but not 11-KT, was positively correlated with water temperature, and negatively correlated with daylength and lunar phase. Maricultured toadfish held under constant conditions had elevated levels of E_2 and 11-KT that should be considered when using these fish for experimentation. This study provides important comparative information on the relationship between individual variations in steroid levels, and how they relate to physiological and environmental correlates in a model marine teleost.

1. Introduction

Successful reproduction in seasonally breeding vertebrates depends on temporal variations in gametogenesis, reproductive behaviors, and steroid hormone production. Previous studies in phylogenetically diverse fishes show distinct population-level seasonal cycles of sex steroid levels that are related to gametogenesis and reproductive behaviors (Tricas et al., 2000; Modesto and Canario, 2003; Sisneros et al., 2004a; Fine et al., 2004; Orlando et al., 2007). These studies are performed by taking blood samples from ~5–10 individuals within the population, and then comparing monthly means (or medians) to examine seasonal or yearly fluctuations. However, relatively few studies examine variations in circulating steroid levels among serially sampled individuals or on finer time scales within the seasonal cycle. These experiments are critical for understanding why large variations in sex steroid levels routinely exist within the sampled population. As the biological significance of these inter-individual variations are not well understood, and individual variation is the essence of evolutionary change (Kempenaers et al., 2008; Williams, 2008), finer resolution of individual steroid levels is imperative to understand the evolution of reproductive physiology and behavior.

Inter-individual variation in circulating sex steroid levels, such as 17β-estradiol (E_2) in females and androgens in males, are important to examine both proximate and ultimate aspects of phenotypic change (e.g. Williams, 2008). In female teleost fishes, E_2 functions to stimulate hepatic synthesis and secretion of vitellogenin (major precursor of yolk proteins) (Nagahama, 1994), which is packaged into yolk proteins in developing oocytes such that oocyte growth, gonadosomatic index (GSI), and plasma levels of E_2 increase in parallel with vitellogenesis (e.g. Nagahama, 1994; Barcellos et al., 2001; Corriero et al., 2004). Thus circulating E_2 is related to seasonal cycles of reproduction and ovarian recrudescence in many fishes (Scott et al., 1980; Nagahama, 1994; Barcellos et al., 2001; Modesto and Canario, 2003; Corriero et al., 2004; Sisneros et al., 2004a). In male teleost fish, 11-ketotestosterone (11-KT) is a potent androgen that often circulates in higher concentrations than testosterone (T) (e.g. Kime, 1993; Borg, 1994), and was shown to induce all stages of spermatogenesis (see Nagahama, 1994). Variations in 11-KT levels are also associated with aggressive, territorial, and vocalization behaviors in many fishes (e.g. Oliveira et al., 1996, 2001a,b, 2002; Hay and Pankhurst, 2005; Remage-Healey and Bass, 2005; Parikh et al., 2006; Desjardins et al., 2006). Therefore, examination of temporal and inter-individual variations in circulating steroid levels of both males and

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females is important to interpret seasonal reproduction and corresponding behaviors within a population.

Many external or environmental cues can have profound effects on circulating hormone levels. For example, water temperature, photoperiod, lunar and tidal cycles, and food availability are known to influence spawning behavior, gametogenesis, and circulating steroid levels in fishes (e.g. Taylor, 1984; Greeley et al., 1988; Tyler and Stanton, 1995; Takemura et al., 2004a; Wang et al., 2008). This link between external cues and internal reproductive physiology highlights the importance of examining temporal or seasonal variations in circulating steroid concentrations with an ecological perspective. Further, reproductive cycles and hormone levels often differ among wild versus captive or cultured populations of the same species, which is commonly due to dysfunction in gametogenesis under conditions of captivity (reviewed in Zohar, 1989; Lee and Yang, 2002; Garcia-Lopez et al., 2006). As the trend in commercial fisheries targets maricultured populations, it is imperative to understand the effect of holding conditions on growth and reproduction. Indoor and outdoor pens can have dramatically different nutrient, temperature, and lighting conditions, and subtle changes could affect hormonal levels that may lead to different growth rates and economic consequences. Studies that examine differences in sex steroid levels among cultured, wild-caught, and captive fishes of the same species are rare.

The oyster toadfish *Opsanus tau* (Linneaus) has been an important research commodity of the Marine Biological Laboratory (MBL) since the 1880s (Ryder, 1886). It is one of the few marine species stocked and raised exclusively for research purposes. As these fish are used in neurobiological, biomedical, and behavioral experiments where results could be influenced by circulating steroid levels, investigators have always desired freshly caught fish each spring when toadfish mating patterns make them amenable to capture. Unfortunately, high experimental demand has resulted in a decline in availability of wild *O. tau* from the waters around Woods Hole (MA, USA). In order to meet the demand, fish have been imported from southern locations (New Jersey), overwintered (i.e. maintained for more than one season) at the MBL, and/or maricultured (Mensinger et al., 2001, 2003). Thus, several distinct subpopulations are maintained at the laboratory and provide an exceptional opportunity to examine differences in reproductive-endocrine systems.

In the spring, male oyster toadfish establish territories in shallow estuarine waters where they begin boatwhistle advertisement calls to attract females for spawning. Females deposit a clutch of hundreds of ~5 mm eggs on the wall or ceiling of the male's spawning habitat where he fertilizes them. The male then remains to guard the embryos and may continue to vocalize and attract additional females for spawning. In the MA area, nests with guarding males and boatwhistle vocalizations are present from about late-May to mid-July, but this varies among years depending on water temperature (Mensinger, personal observation; Edds-Walton et al., 2002). Despite the popular use of this species in biomedical research, almost nothing is known about key aspects of basic natural history at the northern extent of the range (i.e. MA) such as fish distribution during the fall and winter, age at sexual maturity, natural growth rates, and reproductive physiology.

The main goal of this study was to analyze sex steroid levels from serially sampled oyster toadfish throughout the reproductive and post-reproductive period to examine inter-individual and temporal variations. This type of serial sampling is rare in teleost fishes, but can provide critical data that can be used to interpret phenotypic traits. In addition, we tested whether sex steroid levels differed among holding conditions or geographic location, and whether they were correlated with environmental parameters such as temperature, daylength, and the lunar cycle.

2. Materials and methods

2.1. Animals

All toadfish used in this study were obtained from the Marine Resource Center (MRC) of the MBL, Woods Hole, MA, USA. The MRC maintained several distinct populations of toadfish during the summers of 2006 and 2007: 1) Wild fish: these fish were obtained from commercial fisherman on Cape Cod, MA or southern NJ in the spring of each year and maintained segregated by origin. [All *O. tau* found north of Cape Hatteras, North Carolina (USA) have similar genotypes (Avise et al., 1987) and thus MA and NJ fish were assumed to be genetically similar for these experiments]. 2) Overwintered fish: these were wild fish from MA and NJ captured in the spring of 2005, which were then combined after the summer season and maintained indoors until sampling in the summer of 2006. 3) Maricultured fish: These fish were spawned in captivity in the summer of 2004 and raised in the MRC under constant conditions to promote growth until sampling in 2006.

2.2. Experimental design

All *wild-new 2006*, *wild-overwintered 2006*, *wild-MA*, and *wild-NJ* fish (described below) were placed under the same initial holding

Table 1
Summary of toadfish used in this study

Experiment	Fish source	Identification	Year sampled	Sex	*N*
1. Geographic variation	MA wild-caught	*wild-MA*	2007	M	14
				F	7
	NJ wild-caught	*wild-NJ*	2007	M	8
				F	9
2. Inter-individual variation, temporal variation, and environmental correlations	MA wild-caught	*outdoor*	2007	M	7
				F	6
	MA wild-caught	*indoor*	2007	M	9
				F	1
3. Subpopulation variation	MA and NJ new wild-caught	*wild-new 2006*	2006	M	8
				F	9
	MA and NJ wild-caught overwintered	*wild-overwintered 2006*	2006	M	29
				F	14
	Maricultured	*maricultured*	2006	M	8
				F	20
4. Population-level temporal variation	Combined *wild-MA, wild-NJ, outdoor, indoor, wild-new 2006 and wild-overwintered 2006*	June	2006 and 2007	M	29
				F	21
		July	2006 and 2007	M	22
				F	9
		Aug–Sept	2006 and 2007	M	12
				F	10

N is the total number of animals sampled. F, female; M, male; MA, Massachusetts; NJ, New Jersey.

conditions in May of both 2006 and 2007. They were maintained in large fiberglass tanks in the MRC, provided with ambient flow through sea water, and fed thawed baitfish or squid three times per week. Fish were maintained on ambient photoperiods by indirect sunlight, which was often supplemented during the day with overhead florescent lights. Water temperature and light levels were recorded every 15 min from May 23 to September 21, 2007 by a HOBO® Pendant Temperature/Light Data Logger (#UA-002-08; Onset Computer Corp.; resolution=0.10 °C) submerged in the tanks (5 cm off the substrate). In contrast, *maricultured* fish were maintained in the MRC under constant conditions designed to promote rapid growth (16:8 photoperiod; 25 °C; fed silver cup trout pellets 3× per week).

Fish were individually identified by Passive Integrator Transponder (PIT) tags (Biomark, Inc., ID, USA) or unique markings, and sex was verified for all fish at the end of the study by dissection and physical examination of gonads. All experimental procedures for fishes used in this study were approved by the Marine Biological Laboratory Institutional Animal Care and Use Committee.

Circulating E_2 and 11-KT levels were examined as described below for 1) geographic variation (2007 *wild-MA* vs *wild-NJ*); 2) inter-individual variation, temporal variation, and environmental correlations in serially sampled toadfish (2007 *outdoor* and *indoor*); 3) subpopulation variation (*wild-new 2006*, *wild-overwintered 2006* and *maricultured*); and 4) population-level temporal variation (2006 and 2007 fish combined). See Table 1 for a summary of toadfish used in each of the experiments.

2.2.1. Experiment 1 — Geographic variation

Steroid levels from *wild-MA* fish were compared to *wild-NJ* fish in 2007. All fish were acclimated in the MRC for one week prior to sampling and therefore subject to identical light, water temperature, and feeding regimes, which mimicked the normal MBL holding conditions for research fish. Both groups were sampled at a single time point in June (E_2 females: n=7 MA; 9 NJ; 11-KT males: n=14 MA; 8 NJ).

2.2.2. Experiment 2 — Inter-individual variation, temporal variation, and environmental correlations in serially sampled toadfish

Following a one week acclimation in the MRC, two separate groups of toadfish were established for serial blood sampling in 2007. These groups were maintained at ambient photoperiod and water temperatures, and used to examine both inter-individual and temporal variations in circulating steroid levels, as well as environmental correlations. Thirteen wild-MA fish (7 males; 6 females) were transferred to an outdoor habitat (concrete rectangular raceway tank; 1230 long×120 wide×150 high cm; water depth=70 cm) at the Quissett campus of WHOI in May 2007. These toadfish were designated as 2007 *outdoor* fish (x standard length [SL]=26.7±4.3 SD cm; range=22–33 cm) (see Maruska and Mensinger, in press for additional details on tank setup, feeding schedule, and holding conditions for 2007 *outdoor* fish).

A second group (9 males, 1 female; x SL=26.2±4.2 SD cm), were selected from the same cohort as the 2007 *outdoor* fish (wild MA) and maintained inside the MRC in a rectangular tank (133 long×77 wide×40 high cm; water depth=31 cm). These toadfish were designated as 2007 *indoor* fish.

The 2007 *outdoor* and *indoor* fish were both serially sampled from June to September 2007. Blood sampling was performed at the same time of day (12:30 pm±1 h) across all sampling periods. *Outdoor* fish were sampled on June 3 and 16, July 2 and 27, August 19, and September 13, 2007, while *indoor* fish were sampled on June 9 and 24, July 14, August 11, and September 19, 2007. Male and female hormone levels within the 2007 *outdoor* group were also compared at each sample time.

Steroid levels from both the 2007 *indoor* and *outdoor* fish were also tested for correlations with daylength (hours between sunrise and sunset), water temperature (measured by temperature probes in the tanks as described above), and previously entrained tidal amplitudes (maximum high tide minus minimum low tide) associated with the lunar cycle. Daily sunrise and sunset times, lunar phase, and high and low tide heights in feet were obtained from Woods Hole, MA (N 41° 31.4′ W 70° 40.3′) tide tables.

2.2.3. Experiment 3 —Subpopulation variation

This experiment compared steroid levels among *wild-new 2006* (pooled NJ and MA fish, n=8 males; 9 females), *wild-overwintered 2006* (n=29 males; 14 females), and *maricultured* (n=8 males; 20 females) toadfish populations sampled in June 2006.

2.2.4. Experiment 4 — Population-level temporal variation

All fish with similar hormone profiles in 2006 and 2007 were combined to test for population-level temporal differences (e.g. June, July and Aug–Sept sampling times compared) in median circulating steroid levels. *Maricultured* fish were excluded due to statistically different concentrations (see results).

2.3. Blood sampling

Toadfish were removed from their holding areas with a dip-net, anesthetized by immersion in benzocaine (~0.020%), and 200–500 µL of blood collected from the caudal vein at the base of the tail with a heparinized 23-gauge needle attached to a 3-mL syringe. Blood was sampled within 3 min of capture and fish were immediately returned to their holding areas. Blood was stored on ice until all individuals within a session were sampled, centrifuged at 2000 g for 15 min, and the upper plasma layer was removed and stored at −80 °C.

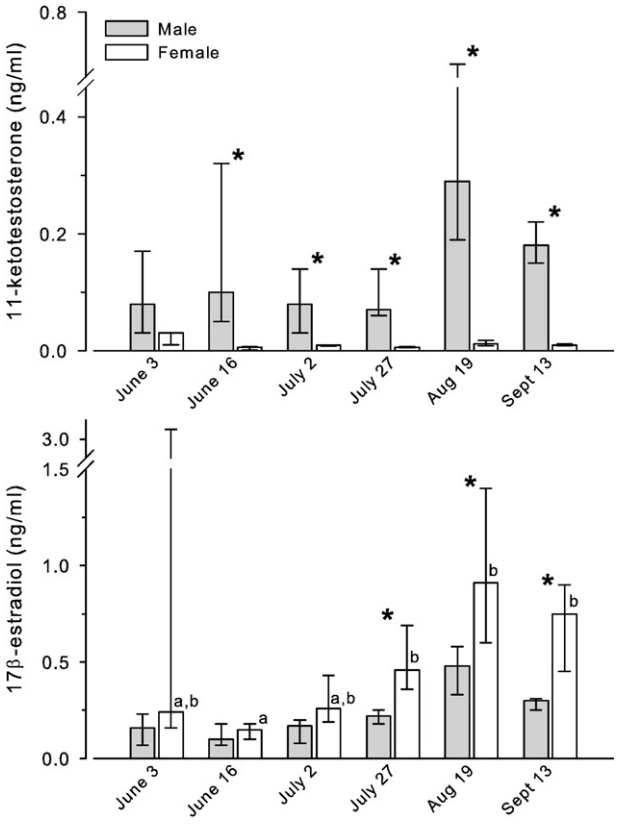

Fig. 1. Temporal and sex differences in circulating 11-KT and E_2 concentrations in serially sampled 2007 *outdoor* oyster toadfish *Opsanus tau*. Data are plotted as medians (bar) and 25th–75th quartiles (error bars). Asterisks indicate sex differences within a sampling time (Mann–Whitney rank sum tests, $p<0.05$). Bars with different letters indicate temporal statistical differences for females only (KW one-way ANOVA on ranks, $p<0.050$; Dunn's tests, $p<0.050$). There was no difference among sample times for males.

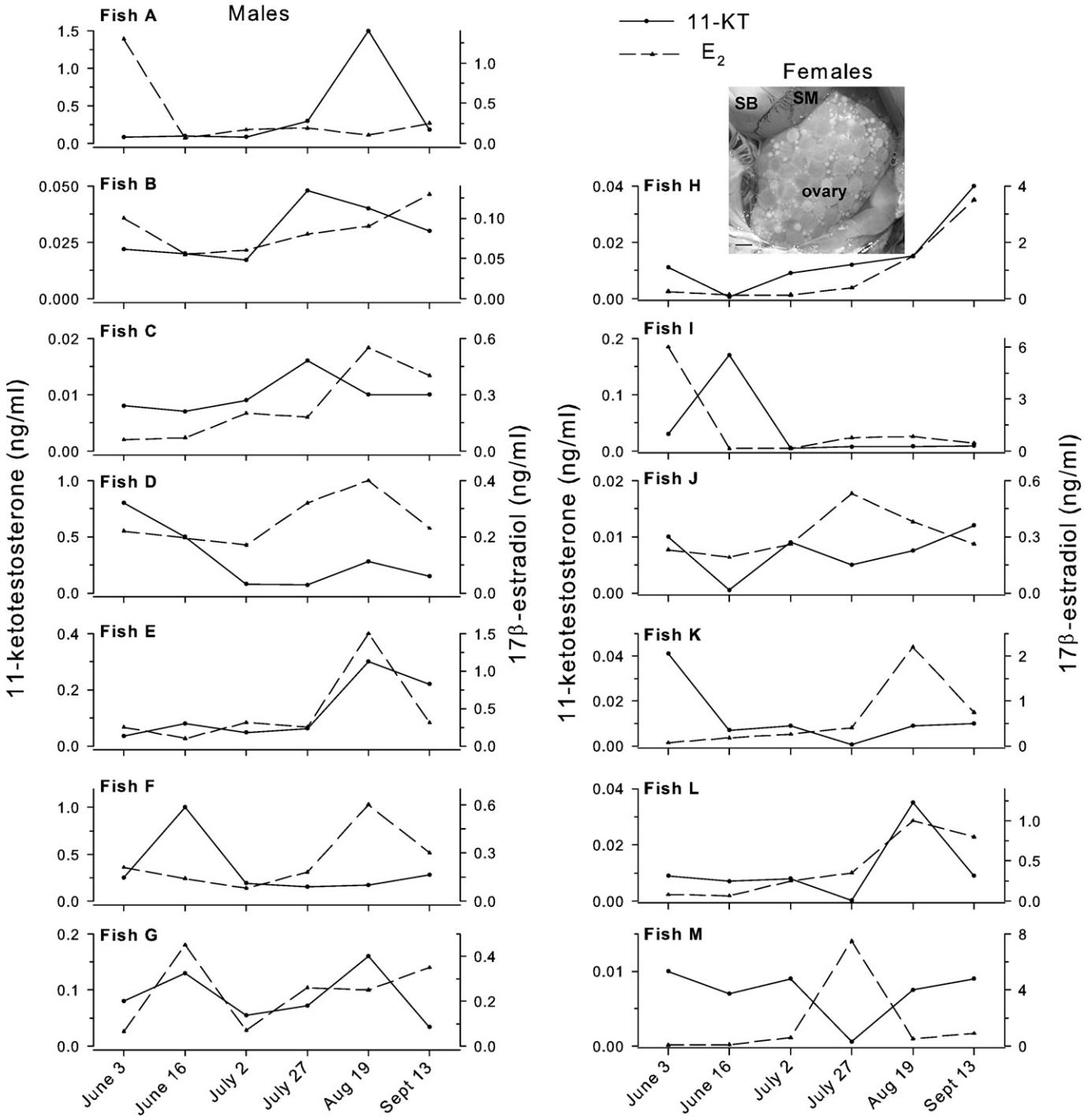

Fig. 2. Inter-individual and temporal variation in circulating 11-KT and E_2 concentrations in serially sampled 2007 *outdoor* toadfish. Each graph represents a different individual measured across the six sampling times and shows both 11-KT (solid line) and E_2 (dashed line). Note that the y-axis scales differ to illustrate the temporal variations within each individual. Inset shows the ovary of Fish H one week after the Sept 13 sampling and illustrates eggs of various sizes, including large ones 3–4 mm in diameter. SB, swim bladder; SM, sonic muscle; Scale bar=3 mm.

2.4. Hormone assays

Plasma E_2 and 11-KT levels were measured with enzyme-immunoassay (EIA) kits (11-KT EIA kit, Estradiol EIA kit; Cayman Chemical, Inc., Ann Arbor, MI, USA). 11-KT was measured because it is thought to be the major active androgen in male teleost fishes and often circulates in higher concentrations than T (Kime, 1993; Borg, 1994). Plasma cortisol levels were also measured for some fish, but will be reported as part of a separate study. A 10 µL aliquot of plasma from each sample was extracted three-times with 200 µL diethyl ether. Diethyl ether was then evaporated under a fume hood, samples reconstituted with 200 µL of assay buffer, and the kit protocol followed. Recovery efficiencies of the extraction were 87% (11-KT) and 88% (E_2). Ultrapure water (Cayman Chemical, #400000) was used to make all reagents and buffers, and samples were run in duplicate. Following development, the plates were read at 405 nm (Sunrise plate reader). Steroid concentrations were determined based on the standard curve obtained for each assay. Intraassay coefficients of variation were 3.1–6.8% (11-KT) and 4.5–8.1% (E_2), and interassay coefficients of variation were 6.7% (11-KT) and 11.1% (E_2).

2.5. Statistical analyses

Linear regressions were used to test for relationships between fish body size and hormone concentrations. Spearman rank order correlation examined the relationships between steroid levels, average daily water temperature, daylength, and previously entrained tidal amplitudes. Statistical comparisons among sexes, sampling times, and fish groups were made with non-parametric Mann–Whitney rank sum tests or Kruskal–Wallis (KW) 1-way analysis of variance (ANOVA) on ranks with subsequent Dunn's test for multiple comparisons because data did not meet the assumptions of normality (Kolmogorov–Smirnov test) and equal variance required for parametric tests. Comparisons among serially sampled 2007 *outdoor* and *indoor* fish were made with Friedman repeated measures ANOVA on ranks with subsequent Tukey's test for multiple comparisons. Coefficient of variation (CV), which is a dimensionless ratio of standard deviation to mean, was also calculated to examine inter-individual variations among serially sampled 2007 fish. All statistical comparisons were performed with Sigma Stat 3.1 (Systat Software, Inc., San Jose, CA, USA), and all steroid concentrations in the text are reported as medians unless otherwise noted.

3. Results

3.1. Experiment 1 — Geographic variation

There was no difference in 11-KT levels between 2007 male *wild-MA* (median: 0.08 ng/mL) and *wild-NJ* (median: 0.12 ng/mL) toadfish (Mann–Whitney rank sum test, T=103.0, $p=0.472$), or in E_2 levels between 2007 female *wild-MA* (median: 0.18 ng/mL) and *wild-NJ* (median: 0.18 ng/mL) toadfish ($n=9$) (Mann–Whitney rank sum test, T=55.0, $p=0.670$) (data not shown).

3.2. Experiment 2 — Inter-individual variation, temporal variation, and environmental correlations in serially sampled toadfish

3.2.1. Inter-individual variation

Serial blood sampling of 2007 *outdoor* fish showed variations in 11-KT and E_2 levels among individuals (Figs. 1 and 2). In male 2007 *outdoor* fish, 11-KT levels ranged from 0.01–1.50 ng/mL (medians: 0.07–0.29 ng/mL) and E_2 concentrations ranged from 0.05–1.50 ng/mL (medians: 0.10–0.48 ng/mL). Male inter-individual variations within a sample period ranged from 2 to 9-fold for E_2 (CV range=0.17–0.96) and 9 to 188-fold for 11-KT (CV range=0.53–1.94). The highest 11-KT level measured at each sample date was from a different male, with over 100-fold inter-individual variations between lowest and highest titres in four (June 3, 16; July 2; Aug 19) out of the six sampling times (CV>1.00) (Fig. 2).

Within males, overall inter-individual variation was higher for 11-KT compared to E_2 (comparison of CV, Student's *t*-test, T=2.84, $p=0.017$), but there was no difference in inter-individual variation between *indoor* and *outdoor* fish ($p>0.050$). Overall inter-individual variation in 11-KT was also greater in males compared to females (comparison of CV, Student's *t*-test, T=2.38, $p=0.038$), but there was no difference in E_2 between the sexes (comparison of CV, Student's *t*-test, $p=0.062$).

In female 2007 *outdoor* fish, E_2 concentrations ranged from 0.07–7.50 ng/mL (medians: 0.15–0.91 ng/mL) and 11-KT levels ranged from <0.01–0.04 ng/mL (medians: <0.01–0.03 ng/mL) (Figs. 1 and 2). Female inter-individual variations within a sample period ranged from 3 to 86-fold for E_2 (CV range=0.42–1.75) and 2 to 14-fold for 11-KT (CV range=0.25–1.00). The two greatest inter-individual variations in female E_2 levels were at the earliest sample time on June 3 (CV=1.60) and later on July 27 (CV=1.75). The ovaries of all *outdoor* females were also examined one week after the last blood sampling period in September 2007 and contained oocytes of various sizes, including larger 3–4 mm

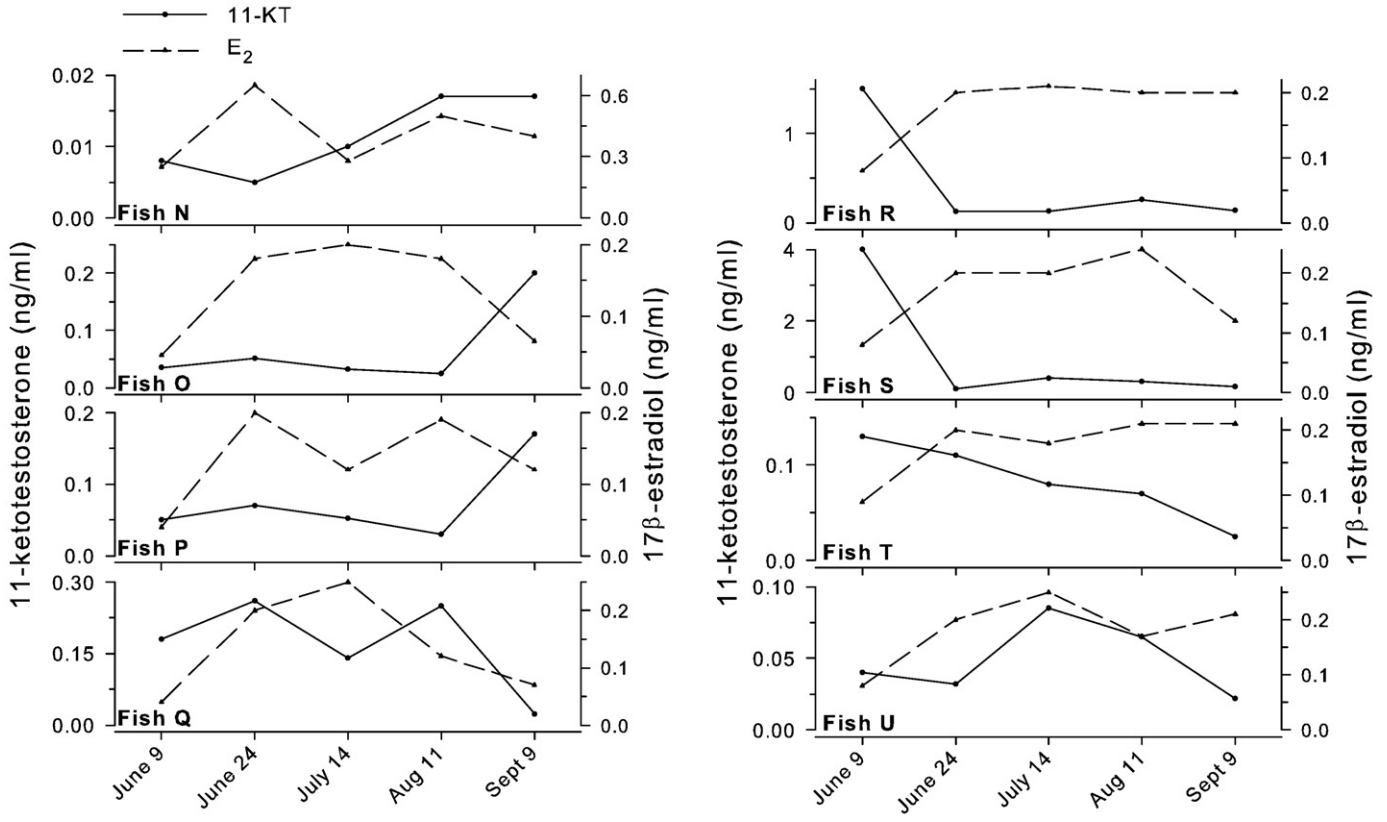

Fig. 3. Inter-individual and temporal variation in plasma 11-KT and E_2 levels in serially sampled 2007 *indoor* oyster toadfish *Opsanus tau*. Each graph represents a different individual measured across the six sampling times for both 11-KT (solid line) and E_2 (dashed line). Note that the y-axis scales differ to illustrate the temporal variations within each individual.

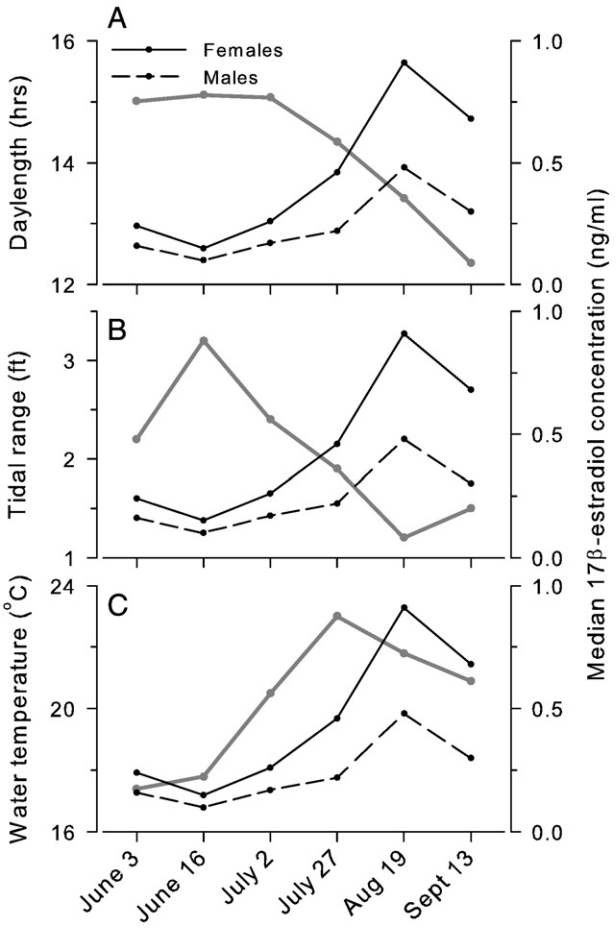

Fig. 4. Relationship between median plasma E_2 concentrations and environmental parameters in 2007 *outdoor* oyster toadfish *Opsanus tau*. A) E_2 concentrations were negatively correlated with daylength (solid gray line) in both males (dashed black line) and females (solid black line). B) E_2 concentrations were also negatively correlated with lunar-associated tidal range (solid gray line) (maximum high tide minus minimum low tide) in males and females. C) E_2 concentrations were positively correlated with average daily water temperatures (solid gray line) in males and females. (Spearman Rank Order correlations, $p<0.050$).

diameter eggs (Fig. 2 inset), with the exception of a single fish that had only small (1–2 mm dia.) oocytes. This individual also had the lowest E_2 levels at the final sampling date in September.

In 2007 *indoor* fish, male 11-KT levels ranged from 0.01–4.0 ng/mL (medians: 0.05–0.08 ng/mL) and E_2 levels from 0.03–0.65 ng/mL (medians: 0.08–0.20 ng/mL). There was high inter-individual variation in 11-KT levels among *indoor* males (CV range=0.84–2.12) and two of these males had the highest 11-KT concentrations of all fish analyzed in this study (1.5 and 4.0 ng/mL on the June 9 sample time) (Fig. 3).

Inter-individual variations in E_2 levels were also present in male *indoor* fish (CV range=0.40–0.76) (data not shown), but were lower than that seen for 11-KT levels in these same fish (comparison of CV, Student's *t*-test, T=2.54, $p=0.031$). The same two males with the highest 11-KT concentrations also had the lowest E_2 concentrations on June 9 (0.04 ng/mL and 0.09 ng/mL), which was also the sampling date with the greatest inter-individual variation for both hormones (CV=0.76 for E_2; 2.12 for 11-KT).

3.2.2. Temporal variation

Median 11-KT concentrations did not differ across the study period for male 2007 *outdoor* fish (Friedman repeated measures ANOVA on ranks, $\chi^2=7.79$, df=5, $p=0.168$). Median E_2 concentrations in *outdoor* males did vary, but post-hoc comparisons were unable to identify the differences (Friedman repeated measures ANOVA on ranks, $\chi^2=12.98$, df=5, $p=0.024$; Tukey's tests, $p>0.050$) (Fig. 1).

Median E_2 concentrations in female 2007 *outdoor* fish were higher on July 27, August 19, and September 13 compared to June 16 (Friedman repeated measures ANOVA on ranks, $\chi^2=18.00$, df=5, $p=0.003$; Tukey's tests, $p<0.050$) (Fig. 1). Median 11-KT concentrations also varied in these female 2007 *outdoor* fish, but post-hoc tests were unable to identify the differences (Friedman repeated measures ANOVA on ranks, $\chi^2=12.23$, df=5, $p=0.032$; Tukey's tests, $p>0.050$).

In male 2007 *indoor* fish, there was no difference in median 11-KT concentrations across the five sampling dates between June and September 2007 (Friedman repeated measures ANOVA on ranks, $\chi^2=1.13$, df=4, $p=0.889$) (Fig. 3). In contrast, male 2007 *indoor* fish had lower median E_2 concentrations on the first sampling date (June 9) compared to the following three times (June 24, July 14, Aug 11) (Friedman repeated measures ANOVA on ranks, $\chi^2=18.63$, df=4, $p<0.001$; Tukey's tests, $p<0.050$) (data not shown).

Steroid levels were also compared between sexes in the 2007 *outdoor* fish only. Median 11-KT concentrations were approximately ten times higher in males (0.09 ng/mL) compared to females (0.01 ng/mL) (Mann–Whitney rank sum test, T=684.5, $p<0.001$) (Fig. 1). Median E_2 concentrations were twice as high in females (0.42 ng/mL) compared to males (0.21 ng/mL) (Mann–Whitney rank sum test, T=1539.0, $p<0.001$) (Fig. 1). Males had higher 11-KT levels compared to females on five of the six sampling dates (June 16–Sept 13). Females had higher E_2 levels compared to males during the later three sampling times (July 27–Sept 13) (Fig. 1). There was also a weak positive correlation between 11-KT

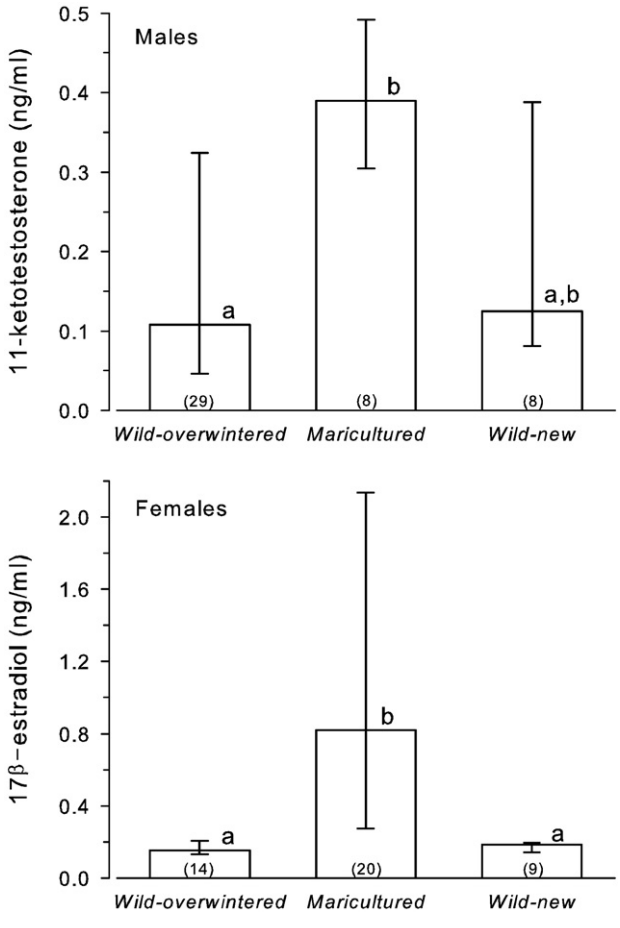

Fig. 5. Subpopulation variation in male 11-KT and female E_2 levels among *wild-new 2006*, *wild-overwintered 2006*, and *maricultured* oyster toadfish *Opsanus tau*. Data are plotted as medians (bars) and 25–75th quartiles (error bars). Sample sizes are indicated in parentheses within each bar. Bars with different letters indicate statistical differences (KW one-way ANOVA on ranks, $p<0.050$; Dunn's tests, $p<0.050$).

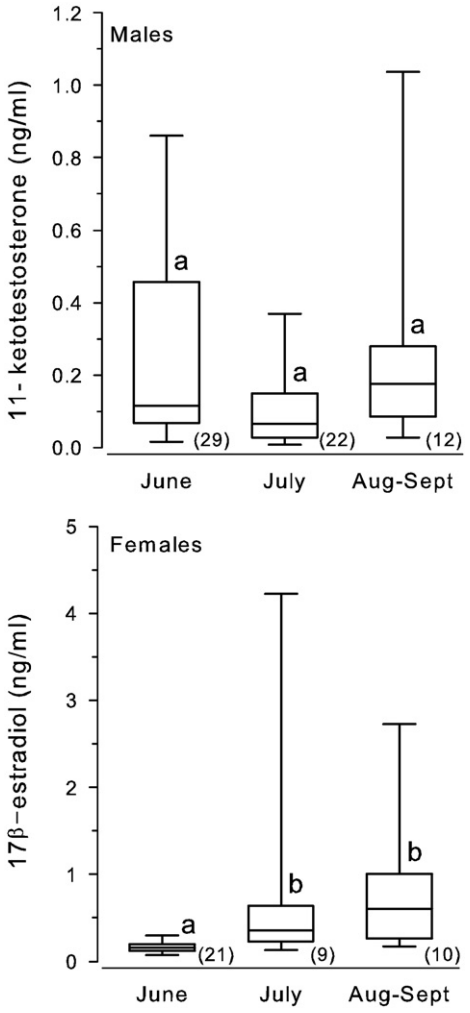

Fig. 6. Population-level variation in plasma 11-KT concentrations in male and E_2 concentrations in female oyster toadfish *Opsanus tau* sampled in June, July, and August–September. Data are combined 2006 and 2007 with maricultured fish removed, and plotted as medians (solid line) with 25th–75th quartiles (box boundaries), and 10th–90th percentiles (error bars). Bars with different letters indicate statistical differences (KW one-way ANOVA on ranks, $p<0.050$; Dunn's tests, $p<0.050$). Sample sizes are shown in parentheses.

and E_2 concentrations in both males (Spearman Rank Order correlation, $r=0.32$, $p=0.046$) and females (Spearman Rank Order correlation, $r=0.36$, $p=0.042$) (data not shown). There was no relationship between body size (SL or body weight) and steroid levels (E_2 or 11-KT) for either males or females used in this study (linear regressions, $p>0.050$) (data not shown).

3.2.3. Environmental correlations

Circulating E_2 levels in 2007 *outdoor* fish were negatively correlated with both daylength (sunrise to sunset) and maximum tidal amplitude differences (maximum high tide minus minimum low tide) associated with the lunar cycle in both males (Spearman Rank Order correlations, daylength: $r=-0.55$, $p<0.001$; lunar tide: $r=-0.56$, $p<0.001$) and females (Spearman Rank Order correlations, daylength: $r=-0.61$, $p<0.001$; lunar tide: $r=-0.64$, $p<0.001$) (Fig. 4A,B). In contrast, there was no correlation between daylength or tidal amplitudes and E_2 levels in 2007 *indoor* males (Spearman Rank Order correlations, $p>0.050$). In addition, there was no correlation between 11-KT levels and daylength or tidal amplitude in either 2007 *indoor* or *outdoor* fish (Spearman Rank Order correlations, $p>0.050$).

Water temperatures ranged from 14 to 25 °C over the May–September 2007 study period. Circulating E_2 levels were positively correlated with water temperature in both females (Spearman Rank Order correlation, $r=0.51$, $p=0.003$) and males (Spearman Rank Order correlation, $r=0.37$, $p=0.019$) maintained in the *outdoor* tank in 2007 (Fig. 4C). E_2 levels were also positively correlated with water temperature in 2007 *indoor* males (Spearman Rank Order correlation, $r=0.35$, $p=0.019$). In contrast, there was no correlation between circulating 11-KT levels and water temperature in any 2007 fish (Spearman Rank Order correlations, $p>0.050$).

3.3. Experiment 3 — Subpopulation variation

Median E_2 concentrations in females differed among *wild-new 2006* (0.18; range: 0.12–0.23 ng/mL), *wild-overwintered 2006* (0.15; range: 0.07–0.40 ng/mL), and *maricultured* (0.82; range: 0.15–5.0 ng/mL) toadfish (KW 1-way ANOVA on ranks, $H=19.8$, $df=2$, $p<0.001$) (Fig. 5). *Maricultured* females had higher median E_2 concentrations compared to both *wild-overwintered 2006* and *wild-new 2006* animals (Dunn's tests, $p<0.050$). Median 11-KT concentrations were also higher in *maricultured* males (0.39; range: 0.28–0.60 ng/mL) compared to *wild-overwintered 2006* males (0.11; range: <0.01–1.10 ng/mL) (KW 1-way ANOVA on ranks, $H=6.7$, $df=2$, $p=0.035$; Dunn's test, $p<0.050$), but not *wild-new 2006* males (0.13; range: 0.02–0.52 ng/mL) (Dunn's test, $p>0.050$) (Fig. 5).

3.4. Experiment 4 — Population-level temporal variation

Because median 11-KT and E_2 concentrations did not differ among 2007 (both *indoor* and *outdoor*) and 2006 (both *wild-new 2006* and *wild-overwintered 2006*) fish (KW 1-way ANOVA on ranks, $p>0.050$), they were combined by sex and month of sample to test for population-level temporal variations in steroid levels (Fig. 6). While non-parametric ANOVA showed that median 11-KT concentrations in males varied across the pooled monthly sampling times (KW 1-way ANOVA on ranks, $H=6.71$, $df=2$, $p=0.035$), subsequent multiple comparisons did not reveal any differences (Dunn's tests, $p>0.050$). In contrast, median E_2 concentrations in females were higher in July and August–September compared to June (KW 1-way ANOVA on ranks, $H=21.82$, $df=2$, $p<0.001$; Dunn's tests, $p<0.050$) (Fig. 6).

4. Discussion

Serial sampling of oyster toadfish showed marked inter-individual variations in male 11-KT and female E_2 levels in two separate groups of fish maintained under different holding conditions (2007 *indoor* and *outdoor*). Male 11-KT levels did not vary seasonally, but female E_2 levels were elevated towards the end of the reproductive season coincident with maturation of oocytes in preparation for winter quiescence. Plasma E_2 concentration, but not 11-KT, was positively correlated with water temperature and negatively correlated with daylength and the lunar cycle in both males and females. *Maricultured* toadfish raised under constant conditions had higher levels of both 11-KT and E_2 that should be taken into account when using these fish for neuroethological studies. These results highlight the importance of studies that collectively examine circulating sex steroids from an individual, population, and ecological perspective.

4.1. Geographic variation

The absence of a geographic difference in circulating 11-KT and E_2 concentrations in the oyster toadfish at our single sampling time during the reproductive period in June 2007 indicates that these two subpopulations (*wild-MA* and *wild-NJ*) have similar hormone profiles.

This is supported by the similarities in population structure (Avise et al., 1987) and the seasonal reproductive cycles described in both MA and more southern locations such as Virginia (e.g. Gray and Winn, 1961; Hoffman, 1963; Edds-Walton et al., 2002; Fine et al., 2004). However, this should be interpreted with caution because changes in circulating sex steroid levels are 1) often brief or pulsatile and thus cannot be fully interpreted from a single sample time, 2) can be influenced by transport and holding conditions, and 3) are not a direct indicator of overall health or physiological condition. Nevertheless, this finding at least partially justifies using fish from southern NJ as an alternate source of experimental toadfish at MBL to reduce the demand on local MA populations.

4.2. Inter-individual variation

Inter-individual variation in 11-KT levels was high among males from both 2007 *indoor* and *outdoor* holding conditions. It is unknown whether the range of circulating hormone concentrations experienced within a single toadfish over the time period measured is biologically meaningful, but it supports the idea of transient changes in male 11-KT levels, and highlights the importance of examining the same individuals over time. For example, the male with the highest 11-KT value measured on each date was a different toadfish (2007 *outdoor*). There are numerous possible factors that could account for this variation, including physiology (i.e. differences in spermatogenic stage among individuals, Hoffman, 1963; Fine et al., 2004), behavior (i.e. dominance hierarchy, Oliveira et al., 2002; vocalization behavior, Remage-Healey and Bass, 2005; parental care, Knapp et al., 1999), or intrinsic genetic differences (see Williams, 2008; Kempenaers et al., 2008). The male toadfish that were guarding embryos on June 16 (Fish C,E) and July 2 (Fish B) had relatively low 11-KT levels on those dates, which is consistent with other studies that show reduced 11-KT concentrations as parental care progresses (Knapp et al., 1999). The number of vocalizations (boatwhistles and grunts) from 2007 *outdoor* fish was highest around the July 2 sample date (Maruska and Mensinger, in press) when all males (Fish A-G) had relatively low circulating 11-KT levels. Androgen levels also did not correlate with the seasonal cycle in boatwhistle parameters (duration and fundamental frequency) in a previous study on *O. tau* (Fine et al., 2004), but studies in other batrachoidids show that rapid (seconds to minutes) elevations in 11-KT regulates vocalization behavior (Remage-Healey and Bass, 2005), which would have been undetected by our sampling regime. While our methodology did not allow precise dissection of all of the cause and effects of individual steroid variation, the variability revealed by the serial sampling, and the formal analysis of inter-individual variation, argues that individual tracking of steroid levels under controlled environmental or social settings could be useful to identify the source(s) of individual variation and phenotypic quality.

There were also variations in female toadfish E_2 concentrations among 2007 *outdoor* individuals, but overall inter-individual variation was lower than that observed for 11-KT in males. Inter-individual variation in female E_2 levels was highest on the July 27 sample date. This is likely due to a single gravid female that had the highest E_2 level in the study, which was sampled along with several post-spawn females on the same date (evidenced by nests with eggs). Female toadfish are thought to start gonadal recrudescence and vitellogenesis at the end of the breeding season prior to the winter quiescent period to ensure availability of large mature eggs for spawning in the spring (see Section 4.3 below), and E_2 levels are often correlated with developing oocytes (Sisneros et al., 2004). Thus, inter-individual variation in E_2 levels among females is likely more related to reproductive condition, but we cannot rule out possible behavioral or genetic differences as mentioned above for males.

Serial sampling of toadfish also revealed that many individuals showed high 11-KT levels when E_2 concentrations were low (and vice versa) on a given sample date, while a smaller proportion showed coincident 11-KT and E_2 fluctuations (see Figs. 2 and 3). Testosterone can be converted to both 11-KT (via 11β hydroxysteroid dehydrogenase) and E_2 (via aromatase), and the relationship between plasma 11-KT, T, and E_2 levels may depend on relative enzymatic activities associated with reproductive physiology and behavior. While we cannot associate specific phenotypic traits with these patterns of plasma steroid levels, they also would not have been observed without repeated measures on the same individuals. There is increasing evidence that relative hormone levels within an individual, rather than absolute hormone levels, determines phenotypic trait values (Williams, 2008) and thus future incorporation of inter-individual variation in studies of hormone titres (as in the present study) is necessary before we can fully understand the complexity of endocrine life-history traits in any species.

4.3. Temporal variation

4.3.1. Males

11-KT is the primary active male androgen in many teleosts, including batrachoidid fishes, where levels often exceed those of T (Kime, 1993; Borg, 1994; Oliveira et al., 2002; Modesto and Canario, 2003; Sisneros et al., 2004a; Remage-Healey and Bass, 2005). 11-KT levels show distinct brief peaks during the early spawning period in oyster toadfish (Fine et al., 2004) and during the peak spawning period in the Lusitanian toadfish (Modesto and Canario, 2003). In the present study, we saw no distinct population peak in 11-KT levels in any subgroup of toadfish but did measure high 11-KT values in individual males that were comparable to those of other studies. It was hypothesized that the 11-KT peak early in the reproductive season of the Virginia *O. tau* population may be important for induction of spermatogenesis and initial development of structures important for reproductive behaviors, with mating calls and nests extending into the fall (Fine et al., 2004). In contrast, spawning and advertisement vocalizations (boatwhistles) are on the wane in late June and generally absent from July through September in the more northern MA toadfish population. Therefore, it was surprising that a peak was not seen early in the season and suggests that baseline population levels may suffice in colder water populations, or that the peak occurred earlier (e.g. May) and was missed by our sampling.

4.3.2. Females

Female oyster toadfish had two-fold higher median levels of E_2 compared to males, as well as elevated levels at the end of the reproductive season in Aug–Sept. We did not detect high E_2 levels early in the study period (with the exception of a single fish with 6.0 ng/mL on June 3), so it is possible that many individuals already spawned prior to our early June sample, which is also consistent with recently captured fish releasing eggs during or just after transport. The increased E_2 levels in Aug–Sept is likely related to gonadal recrudescence and vitellogenesis associated with the growth of new oocytes at the end of the breeding season as daylength and tidal range decline. Previous studies on this species have also found evidence of gradual egg growth in the fall, with many females carrying mature-appearing eggs over the winter for spawning the following spring (e.g. Fine et al., 2004). Consistent with these observations, many of the *2007 outdoor* females had ovaries with developing oocytes of all sizes, including large, maturing eggs (yellow and 3–4 mm in diameter) in September. Fine et al. (2004) hypothesized that developing eggs and steroid synthesis in the fall is needed in these temperate fish because there is insufficient time in the spring to develop a full clutch of 5 mm diameter eggs. This may be especially true at the northern limits of the range, as MA toadfish are thought to undergo a quiescent period during the winter characterized by inactivity and cessation of feeding for several months, and anecdotal reports suggest they burrow into the estuarine sediments during this time (see Mensinger et al., 2001, 2003). Thus the fall oocyte growth may be necessary to ensure availability of developed eggs for the spring spawn.

4.4. Environmental correlation

Reproduction in seasonally breeding temperate marine fishes requires synchronization of spawning-related physiology and behavior among individuals and sexes within the population to increase the chances of successful mating. One common strategy to synchronize reproductive activities is to use environmental cues such as photoperiod, water temperature, lunar, semilunar, or tidal rhythms (e.g. Taylor, 1984; Greeley et al., 1988; Takemura et al., 2004a). Circulating E_2 levels in oyster toadfish were positively correlated with water temperature, and negatively correlated with daylength and previously entrained tidal changes associated with the lunar cycle. Fluctuations in plasma steroid levels according to lunar and semilunar spawning cycles are evident in several teleosts, often correlated with gonadal recrudescence, and may be regulated by *zeitgebers* (external entraining agents) associated with lunar cycles. (Greeley et al., 1988; Emata et al., 1991; Rahman et al., 2000, 2001; Takemura et al., 2004a; Wang et al., 2008). The incidence of grunt vocalizations in the same group of 2007 *outdoor* toadfish used in the present study was also positively correlated with daily water temperature, daylength, and lunar phase (Maruska and Mensinger, in press). The fact that *indoor* fish did not show the same correlations could be attributed to the reduction of entraining cues such as sunlight and moonlight. In other fishes, perception of moonlight intensity and plasma melatonin production is involved in synchronization of gonad development and spawning (e.g. Takemura et al., 2004b). Despite the observed correlations between steroid levels and environmental parameters in the toadfish, it should also be noted that other seasonal behavioral and physiological changes may contribute to variations in steroid levels.

4.5. Subpopulation variation

In wild populations, female toadfish release all or some of their eggs during spawning in the early spring, may resorb unspawned eggs, and then appear to begin egg growth, vitellogenesis, and steroid production in the fall prior to overwintering (Fine et al., 2004). In contrast, many gravid *maricultured* females suffer from a condition described as "egg-bound" where eggs are not released and cannot be physically extruded by abdominal pressure (MRC staff, pers. comm.). This has resulted in the mortality of over 50% of the female *maricultured* fish compared to less than 10% of the male fish. Additionally, very few *maricultured* females release eggs during the spawning months rendering them unsuitable for brood stock. As most researchers desire 20–25 cm fish, the *maricultured* fish were placed under constant temperature (25 °C) and 16:8 h photoperiod to stimulate growth. This prevented them from entering their winter quiescent period but deprived them of natural seasonal triggers. One of the most common problems of captive-grown female fishes is the absence of final oocyte maturation after vitellogenesis, which is likely caused by a lack of the normal pituitary gonadotropin II (or luteinizing hormone, LH) surge seen in wild populations (Zohar, 1989). The higher median E_2 concentrations in female *maricultured* toadfish may be caused by the "egg-bound" condition if ongoing vitellogenesis is present within the ovary without the required stimulus for final oocyte maturation (Nagahama, 1994). In many other fishes, exposure to exogenous estrogenic compounds also results in high mortality rates (e.g. Robinson et al., 2007; Jukosky et al., 2008). Given the positive correlations between water temperature, daylength, and circulating E_2 concentrations, it is also plausible that the high E_2 levels in *maricultured* females are related to the constant long photoperiod and high water temperatures, part of a stress response, or possibly due to increased aromatase (steroidogenic enzyme that converts T to E_2) activity.

Median 11-KT concentrations were also higher in male *maricultured* toadfish. It is likely that *maricultured* fish do not undergo the same seasonal variations in gametogenesis, steroid production, and reproductive behaviors as their wild counterparts because necessary environmental or behavioral cues are absent. The elevated hormone levels and gamete retention observed in *maricultured* toadfish emphasizes that the conditions that promote faster growth (e.g. high temperature and long photoperiod) can also have unintended physiological consequences. Future studies will attempt a brief winter chill and warm-up with ambient photoperiods to mimic natural conditions with the expectation of reducing the high steroid levels found in *maricultured* fish.

The oyster toadfish has served as an important marine biomedical model for studies of vestibular function (Mensinger et al., 1997; Rabbitt et al., 2001), acoustics and vocal communication (Fay and Edds-Walton, 1997; Edds-Walton et al., 2002; Edds-Walton and Fay, 2005; Bass et al., 2008; Fine and Thorson, 2008), lateral line (Palmer et al., 2005), muscle physiology (Rome and Klimov, 2000; Rome, 2006; Mitchell et al., 2008), and nerve regeneration (Mensinger et al., 2000), and the mariculture program was established to provide researchers with a year-round supply of *O. tau* to help alleviate pressure on declining wild populations (Mensinger et al., 2001, 2003). However, the elevated levels of circulating E_2 in females and 11-KT in males should be carefully considered by researchers that may use these *maricultured* fish for experimentation, especially in light of the known effects of steroids on vocalization, auditory processing, and muscle hypertrophy found in this and other batrachoidid fishes (Fine and Pennypacker, 1986; Brantley et al., 1993; Sisneros and Bass, 2003; Sisneros et al., 2004b; Lee and Bass, 2005; Remage-Healey and Bass, 2005, 2006, 2007).

These data on individual and population-level variations in circulating steroid levels in the oyster toadfish provide important new information on the endocrine reproductive cyclicity of this model vocal teleost and set the framework for future studies to examine the relationship between hormones, reproduction, environmental parameters and behavior (including vocal communication) in an evolutionary context.

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