

Serum Hormone Levels Associated with Spawning Activity in the Mummichog, *Fundulus heteroclitus*

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Daily collections of the mummichog, *Fundulus heteroclitus*, from field populations during the first 45 days of the breeding season revealed a semilunar cycle in the sperm index. Peaks in the sperm index were preceded by 6 days with peaks in the serum testosterone concentration. Bihourly sampling of field populations during a 72-hr period at the new moon showed both diel cycling and an upward trend in serum progesterone levels in female mummichogs. Male mummichogs had 12-hr cycles in serum 17 β -hydroxy-4-androstene-3,11-dione levels, the peaks of which preceded high tide by 4 hr. The physiological significance of these cycles are discussed. © 1988 Academic Press, Inc.

Seasonal changes in serum androgen and estrogen levels have been described for many species of fish (Fostier *et al.*, 1983). However, hormone oscillations which occur during the breeding season have been examined less frequently. Fish which spawn only once a year, like catfish and trout, exhibit increased estradiol-17 β production associated with vitellogenesis (Lamba *et al.*, 1983; Zohar and Billard, 1984). Other species of fish, like the mummichog, *Fundulus heteroclitus*, have multiple spawning events during the breeding season and undergo cyclical changes in vitellogenesis between spawning events (Selman and Wallace, 1983). Serum concentrations of estradiol-17 β in female mummichogs cycle in phase with the gonosomatic index at the new and full moons (Taylor, 1984; Bradford and Taylor, 1987). This correlation is consistent with the finding that

estradiol-17 β stimulates production of vitellogenin, which is incorporated into the oocytes of mummichogs (Selman and Wallace, 1983).

Diel variations in serum levels of estrogens or androgens have been shown in trout (Schreck *et al.*, 1972), catfish (Lamba *et al.*, 1983), gulf killifish (MacGregor *et al.*, 1983), and carp (Kime and Dolben, 1985; Bienarz *et al.*, 1986) at the time of spawning. Estrogen levels seem to be related to vitellogenesis, but the relationship of androgen levels to gametogenic events is not as obvious.

Male mummichogs exhibit fluctuations in serum levels of sex steroids and the gametogenic condition of the gonads during the breeding season (Cochran, 1987). However, the relationship between serum androgen levels and spermatogenesis appears to be complicated. The annual patterns of change in testosterone (T), 17 β -hydroxy-4-androstene-3,11-dione (11-KT), and 11 β ,17 β -dihydroxy-4-androsten-3-one (11 β -HT) are different. Serum levels of all three androgens peak during the breeding season, but they peak at different times. Whether the fluctuations in sperm index

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and/or hormone levels which occur during the breeding season correlate with environmental parameters, such as lunar cycle or temperature, has not been pursued previously.

This study was initiated to determine whether sperm production and serum androgen levels of mummichogs, collected on a daily basis during the breeding season, follow a pattern similar to that of estrogens and oogenesis in females (Bradford and Taylor, 1987). As other species possess diel cycles of serum estrogen and androgen levels, we also examined both female and male mummichogs for 72 hr during a predicted lunar spawning event to ascertain whether serum sex steroid concentrations changed cyclically, and, if so, whether those changes could be correlated with gametogenic events.

MATERIALS AND METHODS

Materials. Isotopes (^3H]testosterone (sp act $\mu\text{Ci/mM}$), ^3H]estradiol (sp act $\mu\text{Ci/mM}$), ^3H]progesterone (sp act $\mu\text{Ci/mM}$), ^3H]cortisol (28.9 Ci/mM), ^3H]cortisone (28.9 Ci/mM) were purchased from New England Nuclear and were repurified each month. Cortisol and cortisone were converted to $11\beta\text{-HT}$ and 11-KT , respectively, for use as recovery standards as described previously (Cochran, 1987). Unlabeled steroids were purchased from Steraloids (Wilton, NH).

Animals. Mature male and female mummichogs (5–25 g) were collected in minnow traps in Parish Creek, (Shady Side, MD). Male mummichogs were collected at 9:00 AM each day from April 15 through May 31 for a daily sampling regime. Male and female mummichogs were also collected every 2 hr, beginning at 9:00 AM, May 6th through 9:00 AM, May 9th for the 72-hr study of hormonal and gonadal changes associated with lunar spawning events. All traps were sampled repeatedly during the 72-hr period; however, no trap was sampled a second time until all of the traps had been sampled once. All fish were eliminated from a trap at the time of sampling, but only those of the appropriate size were kept as samples. Traps were then rebaited with chicken livers to attract fresh fish. No fish entered the traps during the night. All blood samples were taken within 15 min of collection. Blood was collected by severing the tail and allowing the blood to flow by capillary action from the caudal peduncle into a 200- μl pipet. The blood from at least five fish was pooled in 1.5-ml plastic microfuge tubes and

centrifuged at 10,000g for 5 min in an Eppendorf microfuge. Serum was removed and stored frozen at -40° until assayed. Five serum pools were obtained for each time point.

Plasma extraction and chromatography. Approximately 2000 dpm of recently purified tritiated $11\beta\text{-HT}$, 11-KT , T, estradiol, or progesterone was added to $16 \times 125\text{-mm}$ screw-cap glass tubes containing serum samples. This tracer served as an internal standard for recovery estimation. Samples consisted of 0.1 ml serum mixed with 0.4 ml phosphate-buffered saline with gelatin (PBSG) (Cochran, 1987). The samples were extracted twice by vortex mixing for 1 min with a 10- vol excess of anhydrous ethyl ether, followed by centrifugation at 3000g. The aqueous layer was frozen, and the organic phase was decanted and dried. Androgens were first separated on HPLC, then measured by radioimmunoassay (RIA) (Cochran, 1987).

Extracted samples of serum estradiol and progesterone were resuspended in 0.4 ml PBSG, vortexed, and incubated in a water bath at 45° for 30 min before 0.1-ml aliquots were removed from each tube to calculate recoveries. The antibody made to estradiol was kindly supplied by Dr. Donald Thompson (Thompson and Honey, 1984), and the antibody to progesterone was supplied by Dr. G. W. Niswender (Elbaum *et al.* 1975). The estradiol antibody was used at a 1:4000 dilution, binding approximately 39% of the radioactivity when ^3H]estradiol was added. The progesterone antibody was used at a 1:10,000 dilution, binding approximately 45% of the radioactivity when ^3H]progesterone was added. Standard curves for estradiol and progesterone ranging from 0 to 1000 pg were determined in duplicate, together with the unknown plasma samples. Unlabeled estradiol and progesterone were recrystallized to a constant melting point prior to their use.

Overnight incubation and separation of free from bound steroid have been described previously (Cochran, 1987). The supernatants collected into scintillation vials were mixed with RIA Solve II (Research Products International Corp., Mt. Prospect, IL), and counted to 2% accuracy in a Packard Tri-Carb liquid scintillation spectrometer (Model 4530). The concentrations of estradiol and progesterone were calculated by interpolation of the standard curves using the Rodbard NIH program. Final adjustments were made for the size of the sample extracted, the size of assay aliquot, and the recovery estimation. Two 0.5-ml water samples, treated as unknowns, served as a check on the system blank through each assay group. Generally, blank values were below the assay sensitivity and therefore were not subtracted from the values of unknowns. Because the mass of tracer added for recovery estimation was small, no corrections were made in the assay.

The sensitivity, accuracy, and precision of the estradiol and progesterone assays have been described previously (Thompson and Honey, 1984; Elbaum *et al.*

1975). The specificity of the respective antisera, with regard to cross-reactivity to other steroids, has also been described in those same papers. Estimates of estradiol and progesterone in a pooled female serum sample were made using direct extraction, elution from a μ Bondapak C18 column with methanol-water (85:15, v/v), and elution from an Altex ultrasphere column (Beckman) with acetonitrile-water (1:1, v/v). The estimates were respectively estradiol, 17.7 ± 1.3 , 16.5 ± 2.3 , 17.1 ± 4.2 ng/ml; progesterone, 110 ± 23 , 113 ± 14 , 112 ± 17 ng/ml.

Gonadal analyses. The sperm index (gonosomatic index * sperm/testes; Cochran, 1987) of five males and the gonosomatic index, GSI, [(gonad weight/total body weight) * 100] of five females were determined at each time point.

Physical measurements. During the 72-hr experiment, the relative tide height, temperature, and conductivity were measured every 2 hr. Relative tide height was determined by reading a fixed scale attached to a piling. Temperature and conductivity of the water were monitored continuously by a submerged conductivity-temperature instrument (Inter-Ocean Systems, San Diego, CA).

Statistical analysis. Data were initially screened using a Z test of proportions to determine whether significant differences existed between different collection points. If such differences were shown to exist, the data were examined for cyclicity using periodogram analysis, one of the preferred techniques for spectrum analysis of time series data (Enright, 1965) or autocorrelation regression (SAS Institute, Inc., Cary, NC). The following *a priori* assumption was made: the majority of the time variability of the data was contributed by oscillations of the periods in the experimental range. Significant lags shown by autocorrelation regression indicate a temporal relationship between data points. These analyses were used to determine which data sets warranted further analyses. Data sets exhibiting peaks above background were then modeled using the equation $y = u + A \cos wt + B \sin wt + Et$ (Bloomfield, 1976). Parameters were generated by least-squares estimation (Statgraphics, STSC, Inc., Rockville, MD) and coefficients examined for significance (Student *t* test) in order to test the null hypothesis.

Following statistical analysis, parameters with significant demonstrated cyclicity were graphed in a figure for a single cycle. To illustrate the cyclicity of the parameters, the mean values of each time point from three cycles were averaged and plotted as the smooth curve of the sliding average of three points (SIGMA-PLOT, Jandel Corp., Sausalito, CA).

RESULTS

Semilunar study. A Z test of proportions of the mean daily sperm indices from April

15 through May 31 indicated that significant ($P < 0.001$) changes in the sperm index had occurred (Fig. 1). Periodogram analysis of the data showed the sperm index increased and declined with a cycle of approximately 15 days (Fig. 2). Peak sperm index values corresponded approximately to the times of the full and new moons of the semilunar spring tide cycle.

Serum concentrations of T, 11 β -HT and 11-KT fluctuated significantly ($P < 0.001$) during the 45-day period (Fig. 1). Serum concentrations of 11 β -HT ranged from a high value of 43.5 ± 14.3 ng/ml (mean \pm SD) to a low value of 4.7 ± 1.6 ng/ml. Testosterone levels ranged from 8.09 ± 2.75 to 0.62 ± 0.33 ng/ml, and 11-KT levels ranged from 11.39 ± 0.33 to 0.30 ± 0.10 . Periodogram analyses of the fluctuations in 11-KT serum concentrations did not reveal any cyclical pattern of change. The serum 11 β -HT and testosterone data were indicated to have a 15-day cycle. However, when the data were subjected to regression analyses, only testosterone proved to have a highly significant ($P < 0.01$) 15-day cycle. Comparison of the mean serum testosterone concentrations along with the mean sperm indices as 15-day cycles (Fig. 2) shows the peak serum testosterone concentrations precede the rise in the sperm index by approximately 6 days. This was confirmed by analysis of covariance.

72-Hr study. Both salinity and temperature increased over the 72-hr collection period (Fig. 3). However, changes in temperature and salinity did not correlate with tidal changes. The relative tide height was greater at night than during the day (Figs. 3 and 7).

Female GSI's (range 11.3–28) and the percentage of fish with ovulated eggs (range 80–100%) did not change over the 72-hr period. Figure 4 shows the serum concentrations of estradiol and progesterone for this same period. Autocorrelation regression of serum estradiol concentrations indicated no significant lags.

In contrast, changes in serum progester-

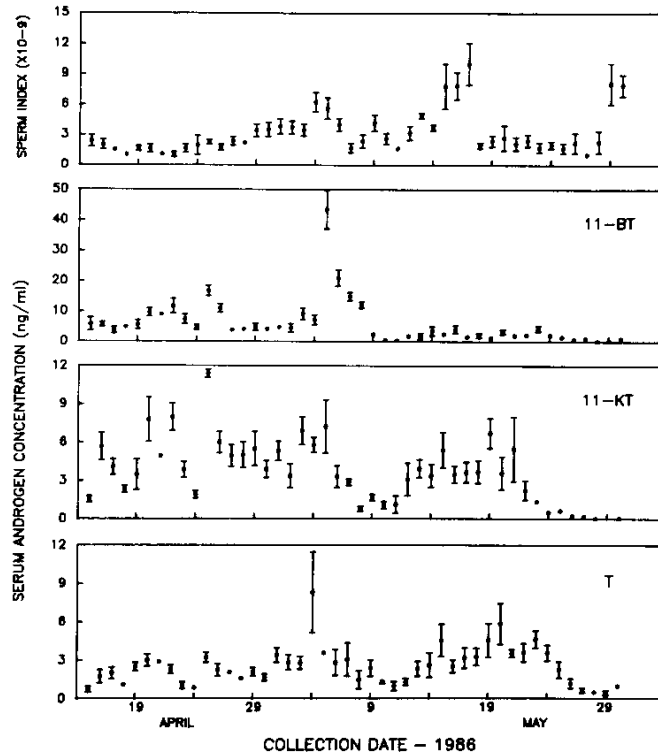


FIG. 1. Sperm index and serum androgen concentrations (mean \pm SEM of five samples) from mummichogs collected in Parish Creek during the first half of the breeding season. The new moon was May 8, and full moons appeared on April 24 and May 23.

one levels were more regular on both an hourly and daily basis. Figure 4 shows that progesterone levels increased during the 3 days of spawning activity. Further inspection of the progesterone data by autocorrelation regression indicated significant ($P < 0.05$) lags at the 1-, 2-, and 12-sample periods. Using the slope from the linear regression to detrend the progesterone data reveals a diel cycle (Fig. 5). The data shows that serum progesterone levels rose from midday through the afternoon and evening, peaking at 0100 hr. The concentration then fell through the early morning hours.

Least-squares regression analysis of the sperm index (Fig. 6) indicated a continuous decline during the first 48 hr of this study, from a high value of $6.63 \pm 0.92 \times 10^{-9}$ to

a low value of $0.83 \pm 0.16 \times 10^{-9}$. During the remaining 24 hr the sperm index remained fairly constant. The decline in the sperm index probably represented spawning activity, although no actual spawning was seen.

Analysis of covariance of serum concentrations of 11β -HT, 11-KT, and T during the 72-hr study (Fig. 6) did not indicate any correlation between the serum androgen levels. Periodogram analysis of serum levels of 11-KT over the 72-hr period indicated both 12- and 24-hr cycles. Plotting the mean serum 11-KT concentrations concomitant with the relative tide height over a 24-hr period (Fig. 7) shows the peaks in 11-KT concentrations precede the maximum tide height by approximately 4 hr. Analysis of

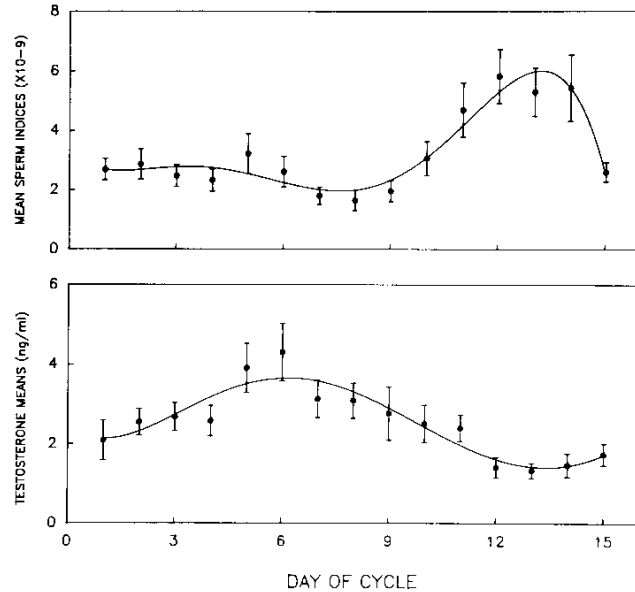


FIG. 2. Daily mean values for sperm indices and serum testosterone concentrations graphed as a 15-day cycle. Each point represents the average of 3 daily means \pm SEM for the first 45 days of the breeding season. The computer-drawn line shows the smooth curve of the sliding average of three points.

covariance confirmed the phase relationship of the peaks in serum 11-KT levels with the tide peaks.

DISCUSSION

Measured values for the sperm index and serum sex steroid levels were highly variable. The principal source of this variability was most likely the genetic differences in (a) the ability of the natural population to respond to photoperiodic stimulation and (b) the capacity for gonadal steroid output (Desjardins and Lopez, 1983). Additional variability might arise from the tendency of testicular androgen output to be episodic in most vertebrate species (Roosen-Runge, 1977). Because the testicular androgen secretion of one individual is unlikely to be synchronized with any other individual, the average level of androgen in the blood will be highly variable.

The 15-day cycle of the sperm index ob-

served in male mummichogs (Fig. 1) corresponds with the previously reported semilunar cycle of the gonosomatic index of females (Taylor *et al.*, 1979; Taylor, 1984; Correll and Cory, 1985; Bradford and

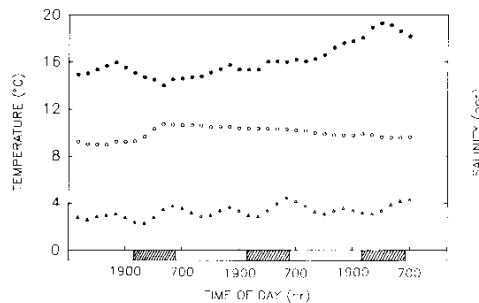


FIG. 3. Mean hourly serum progesterone concentrations graphed as a 24-hr cycle. Each point represents the average of 3 hourly means \pm SEM of detrended serum progesterone levels during the 72-hr collection. The computer-drawn line shows the smooth curve of the sliding average of three points. Night-time is indicated by the hatched portion below the graph.

Taylor, 1987). Interestingly, the sperm index rose abruptly just prior to a precipitous drop indicating sperm was lost from the testes. It is important to note that the homogenization resistant heads, which were counted to obtain the sperm index, represent the endpoint of the maturation process. Thus, the rise in the sperm index seems to indicate that the completion of sperm maturation occurred just prior to the semilunar spawning activity.

Previously observed fluctuations in the sperm index during the breeding season (Cochran, 1987) were associated with major fluctuations in the serum concentrations of T, 11 β -HT, and 11-KT. Although daily determinations of serum levels of 11 β -HT and 11-KT (Fig. 1) revealed significant variations, these changes were not cyclical and did not correlate with the sperm index. Serum T concentration, however, did vary with a semilunar periodicity (Fig. 2). The peak in T concentration preceded an increase in the sperm index by 6 days. This may be an indication of a direct effect of

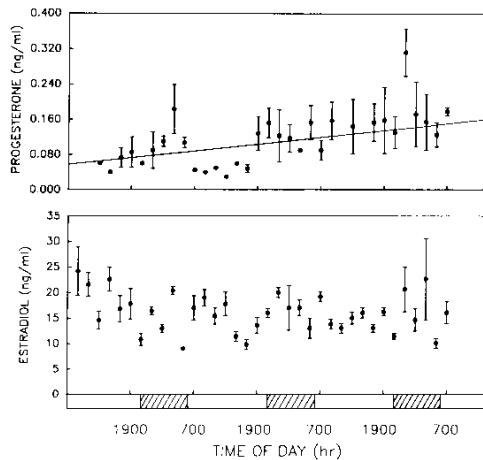


FIG. 4. Water temperature (closed circles), salinity (open circles), and relative tidal height (triangles) during the 72-hr collection (May 6-9). The numbers for all three scales are the same. Thus, temperature is in $^{\circ}\text{C}$, salinity in parts per thousand, and tide height is in absolute numbers. Night-time periods are indicated by the hatched portions below the graph.

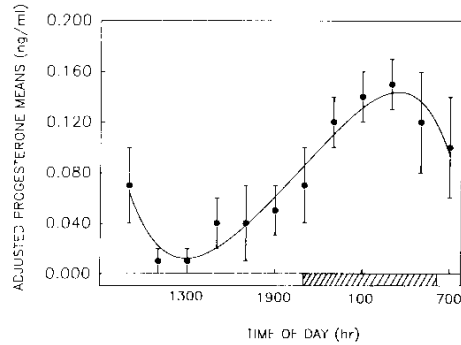


FIG. 5. Serum concentrations of progesterone and estradiol of female mummichogs collected in Parish Creek every 2 hr during a 72-hr collection. Mean \pm SEM from five serum pools at each time point. The regression line, $y = 0.002x + 0.08$, indicates the upward trend in serum progesterone concentrations. Night-time periods are indicated by the hatched portions below the graph.

testosterone on spermiogenesis as has been suggested in other species of fish (Fostier *et al.*, 1983). Indeed, Lofts *et al.* (1966) demonstrated that injections of methyl testosterone could induce regressed testes of hypophysectomized *Fundulus heteroclitus* to produce sperm.

During the 72-hr period at the new moon, when the fish were actively spawning, the sperm index fell continuously. It was not possible to determine whether individuals engaged in multiple spawning episodes, or only some of the males in the population spawned at any given high tide. However, as females are thought to spawn repeatedly (Taylor, 1984), it is reasonable to assume that the males do also.

The values obtained for female GSI's, the percentage of fish with ovulated eggs, and serum estradiol levels were consistent with the work of Bradford and Taylor (1987). Our results have extended their data by providing a record of bihourly data during a spawning peak (Fig. 4). Their experiments indicated that estrogen levels may be involved with the vitellogenic phase of oocyte maturation. Estrogen levels were reported to peak just prior to spawning,

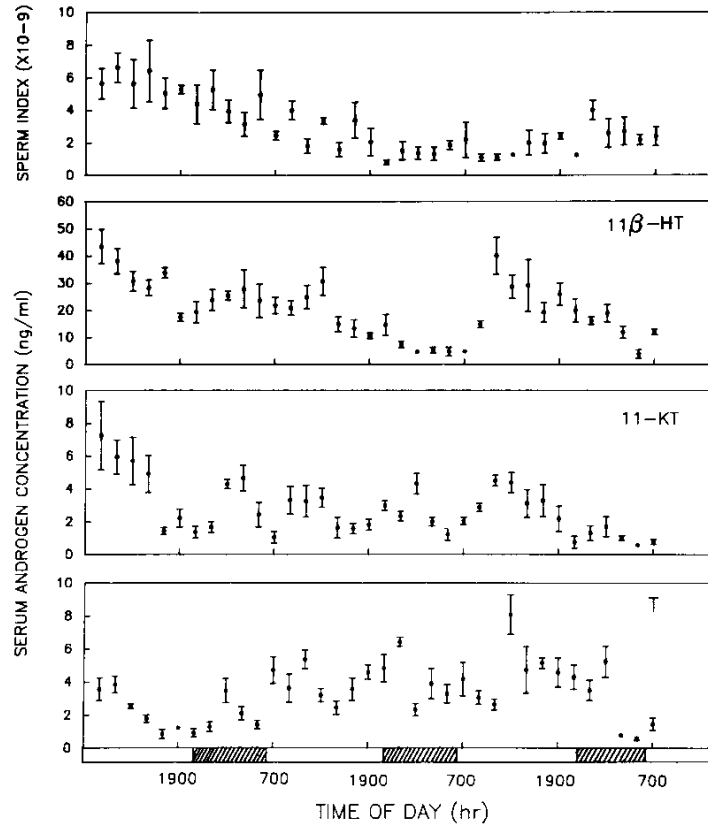


Fig. 6. Sperm index and serum concentrations of T, 11-KT, and 11 β -HT (mean \pm SEM of five samples) of mummichogs collected in Parish Creek every 2 hr during a 72-hr collection (May 6-9). Night-time periods are indicated by the hatched portions below the graph.

which is similar to the male testosterone data presented in this study. It is likely that estradiol is not involved in the final maturation and ovulation of the oocytes, because the data (Fig. 3) indicates no diel cyclicity during spawning periods.

Progestin levels associated with oocyte maturation have been reported to increase during spawning (Schmidt and Idler, 1962, Campbell *et al.*, 1980; Kagawa *et al.*, 1981; Sower and Schreck, 1982, Zohar and Billard, 1984), and, as expected, the serum progesterone concentration tended to increase over the 72-hr collection period (Fig. 3). When this trend was removed from the data, serum progesterone levels were seen

to exhibit a diel change (Fig. 4). This cyclicity is consistent with studies showing diel fluctuations in sex steroid levels in other species of seasonally breeding fish (Schreck *et al.*, 1972; Lamba *et al.*, 1983; MacGregor *et al.*, 1983; Zohar and Billard, 1984; Bieniarz *et al.*, 1986). A number of studies have indicated that progestins are involved in the final maturational processes of oocytes (Schmidt and Idler, 1962; Campbell *et al.*, 1980; Kagawa *et al.*, 1981). Although these data are consistent with those findings, progesterone is not thought to play a direct role in mummichog oocyte maturation (Taylor, 1986). Progesterone, produced by cumulus cells, is probably

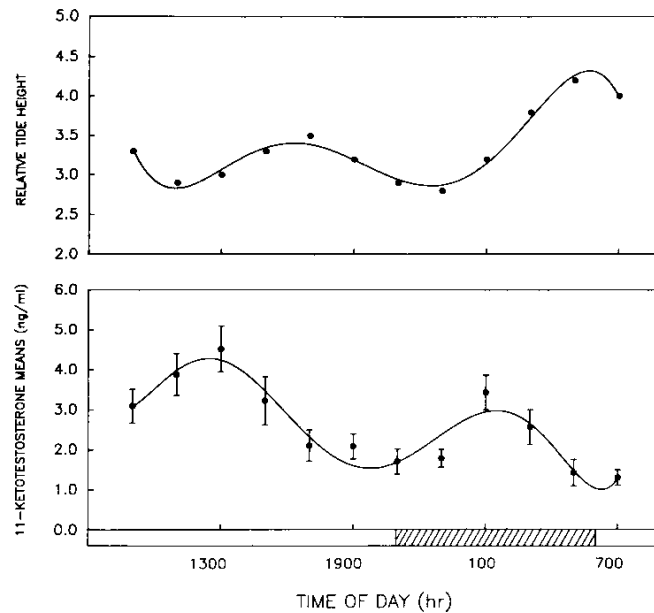


FIG. 7. Mean hourly serum 11-KT concentrations and relative tidal height graphed for a 24-hr period. Each point represents the average of 3 hourly means \pm SEM of serum 11-KT levels and relative tidal height during the 72-hr collection. The computer-drawn line shows the smooth curve of the sliding average of three points. Night-time periods are indicated by the hatched portions below the graph.

converted to $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one by the follicle cells at the time of spawning. However, attempts at measuring the maturational progestin in mummichog serum have not been successful (Taylor, personal communication).

The diel rise in progesterone suggests a daily daytime maturational phase, while the overall upward trend during spawning may reflect incomplete clearance of progesterone following ovulation. The overall pattern raises an interesting question: If progestins inhibit gonadotropin stimulated ovulation in fish, as in mammals, would the incomplete clearance of progesterone on a daily basis limit the number of ovulatory events during any one semilunar spawning peak, and thus give rise to the semilunar cycle?

In male mummichogs, serum androgen concentrations during the 72-hr study (Fig.

6) as well as the semilunar study (Fig. 1) were not covariant. This finding was consistent with previous work which indicated a lack of correlation on a seasonal basis (Cochran, 1987). The fact that serum levels of all three androgens oscillate independently of one another suggests (a) different sources of androgen production, (b) different metabolic clearance rates for each of the androgens, or (c) differential control of testicular androgen production. The first possibility, that circulating androgens are not all produced by the testes, has been suggested previously. Kime (1978) has suggested that at least some of the circulating 11β -hydroxy androgens may represent breakdown products of corticosteroids from the interrenal. Changes in serum levels of 11β -HT in male mummichogs are consistent with changes in serum concentrations of cortisol (Leach and Taylor,

1977; Bradford and Taylor, 1987). Serum concentrations of cortisol, a corticosteroid associated with stress in mummichogs (Leach and Taylor, 1980), might be expected to increase during stressful activity, such as spawning. The changes in 11β -HT concentrations may thus represent an indirect measure of cortisol.

The second possibility, differential metabolic clearance rates for the three androgens, has some support in published reports about androgen clearance rates in mammals. For instance, testosterone and androstenedione have very different metabolic clearance rates in monkeys (Franz and Longcope, 1979).

The third possibility is differential regulation of testicular androgen production. This seems the least likely possibility as there is no evidence of such an occurrence among mammals, reptiles, or birds (Roosen-Runge, 1977; Setchell, 1978). Steroidogenic gonadotropin seems to control androgen production by making more or less pregnenolone available to the biosynthetic pathway (Pedersen and Brownie, 1987); it is difficult to conceive of a mechanism by which fluctuations in gonadotropin levels alone could account for differential production of androgens. Some as yet undescribed mechanism for regulating specific steroidogenic enzymes would have to be postulated.

Serum levels of 11-KT exhibited a tidal cycle of change in which the maximum concentrations of 11-KT preceded the high tides by 4 hr. If 11-KT is associated with spermiation in mummichogs, as has been suggested in other species of fish (Fostier *et al.*, 1983), then perhaps the increased concentrations of 11-KT cause the spermatozoal cysts in mummichogs to release sperm into the efferent ducts. This would have the effect of preparing male mummichogs for spawning at high tide.

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