

# Evaluation of Diel Variation in Androgen Levels of Rainbow Trout, *Salmo gairdneri*

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stress of blood sampling by cardiac puncture was evaluated.

A diurnal rhythm in plasma glucocorticoid levels exists in the channel catfish (*Ictalurus punctatus*) (Boehlke et al., 1966). Although definitive proof is lacking, diel periodicity in plasma testosterone possibly occurs in the skate *Raja radiata* (Fletcher et al., 1969). Androgen assay procedures used in prior studies, however, required large, often pooled, volumes of plasma, and thus correspondingly large fish. These methods also had laborious techniques not permitting elaborate experimental design. The competitive protein-binding assay, suggested by Murphy (1968) made it feasible to determine androgen levels in individual small fish.

*Methods.*—Two-year old fall spawning rainbow trout (300-400 mm total length) were kept in a raceway at the Bellvue Experimental Fish Hatchery, Bellvue, Colorado.

The trout were bled via cardiac puncture with a heparinized syringe during their normal spawning season (30 and 31 October 1970). Blood samples were taken from male fish at 1800, 2400, 0600, and 1200 hours during a 24-hour period. Females were sampled only at 1800 hours. In the male trout, called terminal fish, 10 different fish were bled each time at 2400, 0600, and 1200 hours; these 30 individuals were different than the original 28 fish sampled at 1800 hours. In the repeat group, blood (1.0-1.5 ml) was obtained 4 times from the same 20 males, once at each of the sampling periods. Mortality in the repeat group resulted in a decline in number of samples obtained at each successive sampling. Fin clips were used to identify individual repeats, and this group was returned to a section of the same raceway between sampling times.

Sex and maturity of all fish were confirmed at autopsy. Intact fish were weighed and gonado-somatic indices (GSI) determined.

The competitive protein-binding assay as employed in mammals primarily measures testosterone; to a lesser extent a few other androgens and estrogens may be assayed (Horton et al., 1967). Estrogen levels, however, are of negligible magnitude in comparison with androgen concentrations reported here and are too low to influence the androgen determinations. Plasma (0.1 ml), to which a drop of 0.4 N NaOH was added, was double extracted with 2 ml spectrophotometric grade  $\text{ChCl}_3$ . A portion (40%)

EVALUATION OF DIEL VARIATION IN ANDROGEN LEVELS OF RAINBOW TROUT, *SALMO GAIRDNERI*.—Little is known about reproductive endocrinology in teleostean fishes. We, therefore, designed an experiment to determine the possibility of diel variation in androgen levels of male rainbow trout (*Salmo gairdneri*). The circulating levels of male sex hormone were also compared with gonadal development in both sexes, and the androgen response to the

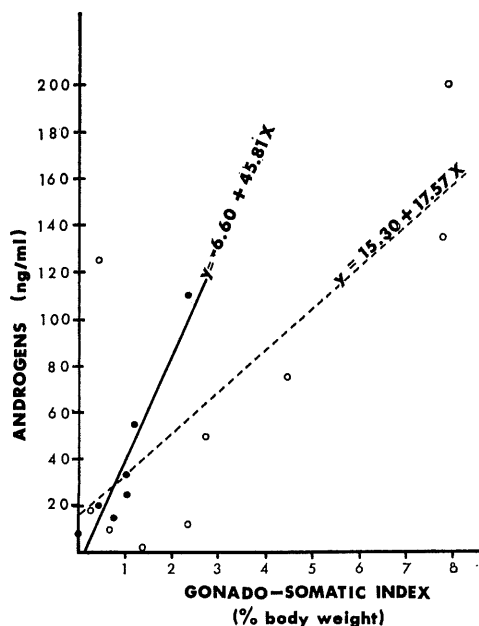


Fig. 1. Relationship of androgen level (ng/ml plasma) and gonado-somatic index in male and female rainbow trout (males—dots and solid line; females—circles and dashed line).

of the combined extracts in  $15 \times 85$  mm culture tubes was evaporated to dryness at 45 C under nitrogen. The residue was shaken with 1 ml  $^3\text{H}$ -testosterone-saturated sex hormone binding globulin (10 ng containing  $5 \times 10^6$  dpm  $1,2$   $^3\text{H}$ -testosterone and 0.3 ml human late pregnancy [third trimester] plasma made up to 100 ml with deionized water). Tubes were shaken gently, placed in a 45 C water bath for 5 minutes, transferred to an ice-water bath for 10 minutes, and shaken gently while in the ice bath. Thereafter, 80 mg washed Florisil (magnesium silicate, 60–100 mesh, Sigma Chemical Co.) was added to a tube which was immediately shaken for 30 seconds and returned to the ice bath for 3 minutes. Immediately following, 0.5 ml supernatant was placed in a liquid scintillation vial to be counted. Intervals between tubes were constant. A standard curve of percentage counts displaced by 0, 1.0, 2.0, 3.0, and 4.0 ng authentic testosterone as determined by the competitive protein binding procedure served for estimation of androgen levels in the samples. Randomization of our samples insured against bias or experimental error. Results of this method are replicable.

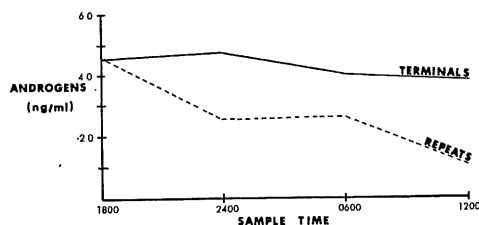


Fig. 2. Androgen levels (ng/ml plasma) of terminal and repeat rainbow trout.

*Results.*—There is a strong correlation between GSI and plasma androgen level in both male and female trout. Data and simple linear regression lines are shown in Figure 1. Regression analysis for males shows that GSI and androgen level are related ( $F = 39.8$ ,  $P < 0.005$ ;  $R^2 = 0.89$ ). The simple linear regression for females is also significant ( $F = 8.95$ ,  $P < 0.025$ ;  $R^2 = 0.56$ ). The slopes of these two lines are significantly different ( $F = 5.8$ ,  $P < 0.025$ ,  $R^2 = 0.56$ ) from each other.

There was no significant difference in androgen level between terminal fish sacri-

TABLE 1. ANDROGEN LEVELS (NG/ML) IN PLASMA OF RAINBOW TROUT AND STATISTICAL ANALYSIS.

Treatment and Time	n	Mean	Range	SD
<b>Terminal males</b>				
1800	28	45.9	8–110	26.6
2400	10	46.8	20–100	24.8
0600	10	39.7	18–65	13.3
1200	10	37.1	20–85	19.1
<b>Repeat males</b>				
1800	20	45.8	15–100	21.1
2400	17	26.2	12–62	13.6
0600	11	33.7	13–100	26.2
1200	9	15.0	5–48	13.5
<b>Repeat males (survivors)<sup>1</sup></b>				
1800	9	41.4	15–78	19.4
2400	9	21.2	12–45	10.7
0600	9	37.7	14–100	27.6
1200	9	15.0	5–48	13.5
<b>Females</b>				
1800	11	78.3	2–200	66.4

<sup>1</sup> Includes only the 9 individuals which survived to be bled four times.

ficed at the four sample times (one-way analysis of variance,  $F = 0.64$ ,  $P > 0.59$ ; Bartlett's test of homogeneity of variance,  $F = 1.74$ ,  $P > 0.16$ ) (Table 1). Consequently, no diel variation could be demonstrated (Fig. 2).

Levels of circulating androgens determined by repeatedly sampling the same fish were significantly different over the time periods (one-way analysis of variance,  $F = 6.24$ ,  $P < 0.001$ ). Bartlett's test of homogeneity of variance ( $F = 2.38$ ) was not significant ( $P > 0.07$ ) but indicated variances might be different between hours. A randomized complete block design analysis of variance was thus carried out on the 9 individuals surviving to be included in all four sampling periods (Table 1). Differences between trout ( $P < 0.01$ ) and between hours ( $P < 0.01$ ) were significant ( $F = 6.54$ ). Consequently, repeated sampling and cardiac puncture of the same trout result in a deviation from the steady state condition.

One individual repeat at 0600 had 100 ng/ml plasma androgen, twice that of the next highest level. This value raises the mean of the 0600 group to what appears to be a peak (Table 1). Repeats have individual patterns in androgen concentrations. There is a general decline in androgen levels due to repeated sampling from a mean of 46 ng/ml at 1800 to 11 ng/ml at the fourth bleeding (Fig. 2), ignoring the one divergent individual.

Male ( $\bar{x} = 45.9$ , 8–110 ng/ml) and female ( $\bar{x} = 78.3$ , 2–200 ng/ml) androgen levels did not differ significantly within the population sampled. It is interesting to note that the variances of the males ( $S^2 = 707.6$ ) and females ( $S^2 = 4409.0$ ) were not equal (Bartlett's test,  $F = 14.17$ ,  $P < 0.0002$ ). The nonsignificant difference between males and females was thus based on the method of Cochran and Cox (1957) to compare means with unequal variances ( $t' = 1.52$ , ns).

*Discussion.*—There is a direct relationship of plasma androgen concentration to gonad development in both sexes of trout. Idler and Tsuyuki (1959) using a bioassay reported an increase in androgen activity of the testes with increased gonad weight in sockeye salmon (*Oncorhynchus nerka*) over a period of spawning migration. Plasma androgen levels in this salmon indirectly support this contention (Schmidt and Idler, 1962). Idler et al. (1971) found an increase in both

testicular and peripheral plasma androgens of Atlantic salmon (*Salmo salar*) with maturation time. We establish that the higher the GSI of either sex the higher the plasma androgen concentration in trout.

The positive regression of androgen level with GSI secondarily supports the demonstration by Idler et al. (1971) that primary tissue producing male sex hormones in teleosts is gonadal. Because fish with a high GSI were fully mature, it is doubtful that the regression lines could be extrapolated further. This relationship may find application where one wishes to assess development of a fish's gonad without killing the animal. Through the determination of circulating androgen levels it might be possible to distinguish between different sympatric races of a species with different spawning times.

Although duplicate samples showed variability of the assay to be negligible, the large variability in androgen levels among individual rainbow trout is important in the evaluation of cycles or periodicities. Large numbers of samples should be used and pooled samples avoided. Using Stein's (1945) two-stage method we have calculated that approximately 120 male or 880 female samples would be needed to obtain a 95% confidence interval of  $\pm 10$  ng/ml.

We could demonstrate neither a diel periodicity in androgen levels of male trout nor a difference between the sexes with respect to male steroid levels.

The androgen levels of fish bled more than once do not follow the steady-state pattern (Fig. 2). It is reasonable to suspect that other forms of stress can bias comparisons of androgen levels as well. For example, is the drop in plasma testosterone of skate from 0930 to 1730 hours (Fletcher et al., 1969) a normal occurrence or was it induced by the concurrent infusion experiments or the initial withdrawal of blood? This decline in androgen concentration was similar to that of our repeat fish. Idler and Truscott (1966) suggest that porcine ACTH can depress testosterone in male skate while increasing testosterone glucuronoside in females. Consequently other endocrine factors induced by treatment may influence androgen patterns.

Due to repeated sampling, the decline in androgen levels is physiologically reasonable. The synthetic machinery and release of androgen apparently cannot replace that lost to the sample quickly enough to rebuild the

titer by the next sampling period. Blood volume of rainbow trout is about 2–3.5% of their body weight (Schiffman and Fromm, 1959; Conte et al., 1963). Thus approximately 10% of the trout's blood was removed at each sampling time and the blood volume was reduced to about one-half after the fourth sample. Some of the lost volume is probably replaced by other body fluids, water, or new blood. The ability of the testes to maintain a certain androgen titer may actually be less than would appear from the data. Androgens are determined on a ng/ml basis; the testis, however, is secreting its product into a progressively smaller volume of blood. The curve in Fig. 2 would then actually decline more rapidly and to a lower level. Differences observed in the data can be accounted for by variations in size and physiological makeup of the individual fish.

Caution in interpretation of data concerning androgen levels obtained by repeated sampling of the same fish or from fish subjected to different experimental conditions or stress should be exercised. Small sample sizes should be avoided.

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