STEROID ASSAYS AND THEIR USEFULNESS IN FISHERIES RESEARCH[1]

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ABSTRACT

Techniques used to quantify circulating levels of steroid hormones in teleostean fishes may help solve problems in fisheries research. Competitive protein binding assay used to determine levels of androgens, corticoids, and radioimmunoassay for estrogens allow analysis of relatively large numbers of samples within a short period of time.

Results obtained by these methods indicate that rainbow trout (*Salmo gairdneri*) have endocrine responses to various stressors. Repeated bleeding causes a marked lowering of plasma androgens in the male while corticoids are increased in both sexes. Gonadectomy reduces androgens in the male more rapidly than in the female; estrogen levels are not appreciably altered. Carbon dioxide anesthesia greatly elevates plasma corticoids while tricaine methanesulfonate has no apparent effect on corticoids or androgens.

Such information may be of use to researchers wishing to establish background levels of these hormones in extirpation experiments or in adminstration studies influencing the endocrine system. Steroid assays may also be of value in determining effects of sampling techniques or other experimental conditions.

INTRODUCTION

"Hidden factors" in experimental design can have a significant impact on results obtained from investigation of several aspects of fish biology. The intent of this paper is to present a concept for experimental design which should be considered during the initial phases of an investigation. Current techniques for hormone quantification are described, and results obtained from these methods are used to illustrate possible sources of error in experimental biology. Primarily, organismic responses to unknown impacts of methodology are explored using steroid hormones of fish as examples. Recent studies involving determination of androgens, estrogens, and corticoids in rainbow trout (*Salmo gairdneri*) are used to illustrate such problems confronting investigation of fish.

The researcher should identify possible sources of error influencing his results other than his specific treatments under investigation. Examples of questions which must be answered for experimental design in physiological studies are: (1) Blood samples are often required - what effect does the bleeding have on the fish? (2) Organs are commonly removed to eliminate the influence of their products - is the level of the product actually reduced by extirpation? (3) Anesthetics are frequently employed in physiological and field studies - what influence does the anesthetic have on experimental results? Endocrine responses in fish are currently receiving attention and potentially may be used to supply an index of the physiological state of the organism.

Techniques now available for quantification of steroid hormones requiring minute sample volumes may be used to answer some of the above questions. Previous assay systems were limited because they required large volumes of plas-

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ma, thus restricting work to individuals of large species. Individuals often had to be sacrificed to obtain the sample, or samples had to be pooled, thereby eliminating detection of between individual variability. The amount of sample required also denied the possibility of serially sampling the same individual. Two relatively new methods, radioimmunoassay for estrogens and competitive protein binding for androgens, overcome these restrictions and have recently been adapted for use on fish.

TECHNIQUES

Blood is generally obtained from fish by cardiac puncture or puncture of the caudal artery with a heparinized syringe. Plasma, obtained by centrifugation, is stored at -15 C; freezing for prolonged periods does not noticeably alter steroid levels.

Competitive Protein Binding Assay

This method was first proposed by Murphy (1964). It is based on competition for binding sites on plasma proteins which specifically bind certain steroids between the endogenous hormone and a known quantity of the same hormone radioactively labeled. Corticoids (Murphy, 1967) and androgens (Murphy, 1968) are assayed by this method. The assay techniques and their applications will be discussed in the light of some of my current studies on rainbow trout. I have found that 0.05 ml or less plasma is required to estimate corticoids and 0.05-0.2 ml plasma to determine androgens in this species.

For trout hormones these assays consist of double extraction with chloroform for androgens or ethanol for corticoids (Schreck *et al.* 1972, in press a). Fagerlund (1970) reported a competitive protein binding assay for salmonids which is specific for cortisol and cortisone. Dried extracts are shaken with steroid-specific binding proteins. Sex hormone-binding globulin (third trimester human pregnancy plasma) saturated with ^{3}H-testosterone is used in the androgen assay. For corticoids, corticoid-binding globulin (intact male dog serum) saturated with ^{3}H-cortisol is employed. Following incubation of the globulin-labeled hormone complex with the extract, Florisil^{R} (magnesium silicate) is added to remove the free fraction of steroid (radioactive and from the extract) not bound to the protein. The radioactivity in the protein fraction is counted in a scintillation spectrophotometer and the level of steroid in the extract determined from a standard series ranging from 0-4 ng authentic testosterone or cortisol. Steroid concentrations of less than 5×10^{-11} grams/ml plasma can be detected using this method. Results of the assay are also reproducable (t-test, $\alpha = .05$).

Radioimmunoassay

This estrogen assay was first suggested by Abraham (1969). It is very generally similar to the above system except that competition between the known radioactive steroid and the plasma hormone is for binding sites on an antiestrogen antibody. Schreck *et al.* (in press b) discusses a radioimmunoassay of estrogens in rainbow trout. In this species 0.2 ml plasma is adequate. A small amount (300 cpm) ^{3}H-estradiol-17β is injected into the plasma. Recovery of the label is used to estimate extraction efficiency. The plasma is extracted twice with ether. The dried test fraction is placed in an antiestrogen antibody coated tube with a known amount of ^{3}H-estradiol-17β . The particular antibody used was prepared by Ekre and Foote (1971). After incubation, the unbound fluid is counted in a scintillation spectrophotometer and the level of plasma steroid determined from a standard curve ranging from 12.5 to 400 pg. This method is sensitive to the 1.25×10^{-13} gram/ml plasma level and results are reproducable.

CRITIQUE

Physiological studies on fish frequently require the sampling of blood. The sampling may in itself be a stressing factor, influencing results of a treatment. To evaluate the effects of bleeding, 20 male rainbow trout were bled four times during a 4 hour period (Schreck *et al.*, in press a). Due to the sampling, plasma androgen levels declined from a mean of 45.8 ng/ml initially to 15.0 ng/ml at the fourth sample. Plasma estrogen levels in these fish, however, did not change significantly, having means of 2.4 ng/ml at the first bleeding and 1.8 ng/ml at the last (Schreck *et al.*, in press b).

Plasma corticoid levels respond inversely to the androgens. Sampling blood from 5 rainbow trout of both sexes three times by puncture of the caudal artery causes a significant elevation of corticoids from an initial level of 24.0 ng/ml to 59.2 ng/ml for the third sample taken 24 minutes later. The above experiments indicate that sampling blood alters the physiological state of a fish. Steroid assays may be used to give an index of stress in experiments where fish were serially bled.

Extirpation of an organ to determine responses or functions of fish without the product or action of the organ is frequently employed in experimental studies. The gonads are often the target of surgery to eliminate the influence of endogenous androgen and estrogen. It is generally not known, however, how long it takes for the substances under consideration to be cleared from the fish nor has it been established if the levels are reduced at all. The effects of gonadectomy and laparotomy were contrasted in rainbow trout of both sexes to determine if sex steroid levels were significantly reduced and to observe the rate of decline (Schreck *et al.*, 1972). Androgens required up to 21 days in the 8 orchiectomized trout and up to 42 days in the 7 ovariectomized fish to differ significantly from the controls. Over these time periods plasma androgens of either sex were not reduced below 25% of pre-castration levels. This may indicate a base level of androgen maintained by a non-gonadal, probably interrenal, source.

Plasma estrogen levels in the fish were not altered by castration. Mean presurgery level in the males was 3.7 ng/ml, and 21 days post castration the concentration was 3.9 ng/ml. In the female the mean pre-ovariectomy value was 4.6 ng/ml, and there was no significant change at 21 days post-gonadectomy when the mean was 3.5 ng/ml. Thus the non-gonadal source of estrogen is very important in maintaining plasma concentrations or the clearance rate is very slow. Such information would be of value to researchers considering extirpation studies. Using rapid techniques for analysis one can now ascertain whether or not the "expected" consequences of organ removal have been achieved prior to basing an experiment on such a premise.

The last example of uses of steroid assays concerns the selection of an appropriate anesthetic for a specific experiment. Yearling rainbow trout of both sexes were subjected to carbon dioxide (225 mg/l) anesthesia and tricaine methanesulfonate (40,00 mg/l) anesthesia with 9 fish in each treatment. Controls (5 fish) were treated similarly to the test fish but were not anesthetized. Blood was taken from the caudal artery after loss of righting reflex and after 12 and 24 minutes in the anesthetic. Neither anesthetic influenced plasma androgen levels; no differences due to treatment or time were found. A direct relationship, however, was present between plasma androgen concentration and state of maturity of the fish as demonstrated by Schreck *et al.* (in press a).

A great difference was found, however, between corticoid levels of the fish subjected to the anesthetics. The corticoids in the tricaine methanesulfonate anesthetized group were similar to that of the controls. The mean initial value was 35.1 ng/ml, and the 12 and 24 minute levels were 50.3 ng/ml and 72.4 ng/ml, respectively. Carbon dioxide imposed a great initial stress, elevating the

corticoid level in the first sample to a value equivalent to three bleedings in non-anesthetized trout. The first mean concentration was 61.4 ng/ml. This level increased slightly to 79.7 ng/ml after 12 minutes and then declined sharply to 53.8 ng/ml after 24 minutes. Obviously, fish responded differently to the two anesthetics. It is interesting that carbon dioxide, a substance normally encountered by fish in nature, was more stressful than the synthetic anesthetic in causing corticoids to increase. The use of assays as employed here can provide insight into the proper choice of chemical tools for a specific experiment. They may also be of value in establishing the presence of unwanted environmental conditions such as low oxygen or high carbon dioxide, among others, for a specific physiological response by a fish.

CONCLUSIONS

It is hoped that this paper has provided an insight into areas of experimental design which deserve attention to avoid possible sources of error. In addition, relatively new methods of hormone analyses have been introduced as possible means to approach such problems. Sources of possible "hidden" bias in research found in trout include: (1) effects due to blood sampling technique, (2) false assumptions about an organ-product relationship as found in gonadectomy, and (3) error introduced by a chemical agent such as anesthetics used as a tool or via stressful factors in the ambient environment.

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