SOME EFFECTS OF METHALLIBURE (I.C.I. 33,828) ON
THE STICKLEBACK, GASTEROSTEUS ACULEATUS L.

by

BARBARA AGNES MARY CAREW
B.Sc., Memorial University of Newfoundland,
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in the Department
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Zoology

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
August, 1968
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Department of Geology

The University of British Columbia
Vancouver 8, Canada

Date 16 August 1968
ABSTRACT

Treatment of male sticklebacks with metallibure brings about a marked reduction in the level of prespawn-ing aggressiveness. Gametogenesis in the testes of the testes of the treated fish is slowed down so that after 30-38 days of treatment most testes contain spermatozoa and spermatogonia while the testes of the controls are more mature and contain spermatocytes and spermatozoa or spermatozoa only. Forty-nine days of treatment result in testes with spermatogonia, spermatocytes and spermatozoa, control testes are completely mature and contain only spermatozoa. Metallibure causes no significant increase in thyroid epithelial cell height evidence that the I.C.I. compound is not having a direct goitrogenic effect such as is found when fish are immersed in a solution of thiourea. The adenohypophysis of metallibure treated fish contains less basophilic material than that of untreated controls.
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*Veterinary Medical Department, Ayerst Laboratories, Montreal, P.Q.

**Endocrinology Study Section, Department of Biochemistry, Emory University, Atlanta, Georgia
I. INTRODUCTION

A number of studies have helped elucidate the characteristic reproductive behavior of the three-spined stickleback *Gasterosteus aculeatus* L. (Tinbergen and ter Pelkwick, 1937; van Iersel, 1953, 1959). Briefly the sequence is as follows: In the late winter or early spring the fish show a decreased tendency to school. The males defend discrete territories against intruders of either sex although they are particularly aggressive towards other males. During this time the silvery winter fish develop characteristic breeding coloration. The lateral and dorsal surfaces of both males and females darken to a mottled green-black. The throat region of the males becomes red and their eyes change from silver to bright blue. Later the males collect algae and glue it together to form a nest with a sticky mucous secretion produced by the kidneys at this stage. After tunneling through the nest the male is ready to court a gravid female. The male leads the female to the nest with a distinctive zig-zag swimming motion. When the male points to the nest entrance the female enters and in response to his prods, spawns. The male follows through the nest and fertilizes the eggs. He then chases the female away. The male continues to defend the territory and cares for the clutch of eggs by fanning water over them with his
rapidly moving pectorals. When the eggs have hatched the male guards the young for a few days. This completes the reproductive cycle.

The preceding description of reproductive behavior applies to the salt water form of *Gasterosteus* which enters fresh water to breed. I have used only the freshwater form which lives all year in fresh water. Hagen (1967) who studied both forms in the Vancouver area, finds the aggressive territorial and reproductive behavior of both very similar and he can detect no behavioral barriers to interbreeding between the two forms.

The role of the endocrine system in this behavior sequence is evidently complex and at present only partially understood. Techniques used in endocrinological studies rely heavily on observations of the effects of removal of an endocrine gland and subsequent replacement therapy involving injections of gland extracts or synthetic hormones. Removal of the gonads of sticklebacks is a comparatively simple operation and has been successfully carried out by a number of workers (Baggerman, 1957; Hoar, 1962 a, b; Wai and Hoar, 1963); removal of the pituitary, however, is difficult and the operation has not yet been successful with *Gasterosteus*. Investigators have therefore used photoperiod control to regulate pituitary activity.

Baggerman (1957) showed that the seasonal reproductive cycle of stickleback is largely controlled by the changing
light conditions while the effects of temperature are additive. It is assumed that short photoperiod (8 hr of light alternating with 16 hr of darkness) suppresses the high gonadotrophin production characteristic of breeding fish, while long photoperiod (16 hr of light alternating with 8 hr of darkness) readily brings fish into reproductive condition. Investigators of the endocrinology of stickleback reproduction have used the differences in response, to various treatments, of short photoperiod physiologically hypophysectomized fish compared with long photoperiod fish.

Wai and Hoar (1963) used gonadectomy and subsequent treatment with methyl testosterone (a synthetic androgen) on long and short photoperiod sticklebacks. They found the high level of pre-spawning aggressiveness, characteristic of territorial fish prior to nest building, appeared in gonadectomized fish under long photoperiod but not in gonadectomized fish under short photoperiod. The addition of methyl testosterone did not greatly affect the aggressiveness of these castrated fish. The androgen treatment did, however, restore the secondary sex characters (mucous secreting kidney tubules and nuptial coloration) which had been lost after castration. Castrated fish showed neither nest building nor sexual behavior. Methyl testosterone treatment, however, resulted in the appearance of both these behavioral sequences in males under both photoperiod regimes. These behavior patterns reappeared more quickly and in more of the long photoperiod fish.
The evidence from the above studies is that pituitary gonadotrophins control pre-spawning aggressiveness, while gonadal steroids control secondary sex characters, and both gonadotrophins and androgen act synergistically to produce nest building and sexual behavior (Hoar, 1965).

To obtain additional evidence of the endocrine control or pre-spawning behavior and gametogenesis, a pharmacological technique of suppressing gonadotrophin was sought. Hoar, Wiebe and Wai (1967) discussed preliminary results obtained using methallibure (I.C.I. 33,828), a non-steroid, non-hormonal compound, on three species of fish. These experiments, using sticklebacks, goldfish and surfperch strongly indicate that methallibure selectively inhibits pituitary function. The gonads of the treated fish showed division of the primary germ cells but no maturation of these cells. Controls exhibited normal germ cell maturation.

The data reported by Hoar et al. (1967) on sticklebacks were considered preliminary. Mortality was high in the treated fish and the sample size was small. However, as well as the effect on gametogenesis, there was an indication of an effect on gonadal steroid production. The cells of the brush border segment of the kidney tubules remained at a size characteristic of immature fish in treated sticklebacks. The cells of this segment of the kidney become large with granular cytoplasm under the influence of androgen.

Wiebe (1968) continued the investigation on surfperch Cymatogaster aggregata. He found treatment with methallibure
resulted in testis atrophy, regression of the interstitial cells and lack of increase in size of the fleshy modifications of the anal fin. These modifications of the anal fin are a secondary sex structure which develop under the influence of male gonadal steroid. No marked anti-thyroid effect was detected when the methallibure treated fish were compared with a group treated with the goitrogen, thiourea. Wiebe (1967) reported that there was a significant drop in the reproductive behavior of methallibure treated fish compared with untreated controls.

The present study continues the investigation of the effects of methallibure on *Gasterosteus* to further the understanding of the control of the pituitary gonadotrophins on prespawning aggressive behavior and testis physiology. These two aspects of the study are closely interrelated. The work to date suggests that methallibure acts directly on the pituitary gonadotrophs rather than the tissues of the testis (Leatherland and Pandey, 1968). This has been investigated histophysiologically by examining sections of the testis (gametogenic tissue), the height of the kidney tubules (as a measure of steroid effects), and the pituitary (for basophils) in treated fish and untreated controls.

At present, pituitary gonadotrophin is thought to control directly the increased aggressiveness of male sticklebacks when they set up and maintain discrete territories prior to spawning. If methallibure is blocking the action of gonadotrophin then presumably treated stickle-
backs should be less aggressive than untreated controls. In brief, the primary objective of the study was to investigate the effects of methallibure on pre-spawning aggressive behavior and to investigate the results in terms of the gonadotrophic control over this behavior and testicular physiology.
II. METHODS AND MATERIALS

Freshwater sticklebacks type B' (Heuts, 1947) were caught at various times throughout the year in the Little Campbell River south of Vancouver, British Columbia. They were kept until needed for the experiments in large tanks of running dechlorinated water. Although the tanks were housed in the laboratory, the photoperiod was regulated at the natural length by a photocell placed in a south window. The water temperature varied between 5° and 14° C depending on the time of year. At the outset of an experiment, fish were placed in groups of two or four in 16 litre glass aquaria (45 litre glass aquaria were used in one experiment, No. 8). Dechlorinated water fortified with 20 ppm calcium chloride was continually aerated and filtered through glass wool; the filters were changed once a week. During the experiments the aquaria were kept in an air-conditioned room which maintained water temperature at 20 ± 1°C. The photoperiod provided 16 hr of light alternating with 8 hr of darkness. Illumination (50-55 ft-c) was provided by fluorescent lamps. All aquaria contained sand and water sprite (Ceratopteris sp.). The plants covered about 75% of the water surface.

The fish were fed frozen brine shrimp (Artemia salina) once a day. As a precaution against fungal infection, 2-3 drops of 1% malachite green solution were usually added to each aquarium at the beginning of the experiments.
A. **Sex Determination**

Male fish were used in the experiments. Usually, males could be distinguished from females by the greater amount of red pigmentation in the throat region. However, in one experiment (No. 5) no trace of color could be seen, so a tiny incision was made in one side of the fish. Males were distinguished from females on the basis of the difference in shape and pigmentation of the gonads. The males were kept in 50% sea water for 48 hr after this operation before they were placed in groups of two in the glass aquaria. A preparation called Orabase* was used over the incision. This preparation adhered to and closed the wound for an hour or two, after which it dropped off.

B. **Behavior Observations**

In the behavior studies, all aquaria were observed for periods of five minutes two or three occasions each week. The behavior observed was of two main types: (1) **Aggressive behavior** and (2) **Nest-building behavior**.

Three components of aggressive behavior were distinguished: (a) **Approach** where one fish darts at another but stops before reaching it, this type of behavior is very common when two or more fish have established territories in a single tank; (b) **Bite** where one fish makes a dart at another and bites it once or several times; (c) **Chase** where the aggressive fish pursues the subordinate one who must

* Squibb Oral Protective Paste
seek shelter. The nest building (van Iersel, 1953) consisted of: (a) Sand-digging where a male, in a head down posture, picks up a mouthful of sand, turns, swims away and spits it out; (b) Searching where a male picks up small pieces of roots or algae and spits them out; (c) Bringing material where a male brings pieces of algae or plant to the nest-site; (d) Gluing where a male swims over the nest area while secreting a mucous from the kidney and (e) Fanning where a male adopts a head down position at 30°-40° to the horizontal at the nest entrance and maintains this position by the combined forward swimming movements of the tail and the backward swimming movements of the pectorals.

Quantative data were obtained by the use of an Esterline-Angus pen recorder with the keys numbered so that each number corresponded to a particular behavior movement. Thus, when a behavior pattern was observed the appropriate key was depressed. The frequency of the various patterns was recorded.

The fish in each aquarium were assigned a level of aggression for each observation period, based on the following criteria (Hoar, 1962b).

**Level 0:** No attacks, fish frequently swim around in a loose group.

**Level I:** One or two attacks per minute (ten or fewer in five minutes); loose schooling, no social order; no evidence of dominance or subordination.
Level II: Three or more attacks per minute, fish of equal or near equal status, no dominance or subordination.

Level III: Territorial relationship but no particular dominance, one fish has a preferred area and bites fish entering this area but does not fight vigorously or subordinate other fish.

Level IV: A dominance-subordinate relationship is well marked; the dominant fish attacks other fish vigorously, drives them into corners and sometimes kills them. When more than one dominant is in the tank the territorial boundaries are sharp.

The number of aggressive acts is usually greatly reduced at Level IV, since a tank often contains a single dominant male with all subordinates in hiding, so that encounters between fish are few. Consequently, in most experiments once a week, a "standard" female or juvenile fish was used. The "standard" fish was placed inside a glass tube (6.5 cm in diameter) and tubes with fish placed in the middle region of all tanks which were rated Level IV during the previous observation period. Usually the "standard" fish would actively swim up and down inside the glass tube. The number of attacks on the tube in five minutes was recorded.

C. Methallibuine Injection and Immersion

Several dosages and frequencies of dose of Methallibuine were tested (Table I). In most experiments a
fine suspension of methallibure in distilled water or freshwater teleost saline* was prepared with a small amount (one drop per 5 ml) of the wetting agent "Tween 80". Either 0.05 or 0.025 ml of suspension was injected intra-peritoneally with a 26 G. needle. Successive injections were made on alternate sides of the fish. In the first experiments there was some leakage of injected suspension so "Orabase" was applied to the hole made by the needle. In experiments No. 6 and 7 the use of "Orabase" was considered unnecessary because the injected volume was so small.

In three of the eight experiments methallibure was added to the ambient water of a group of experimental fish. Details of the amount added are in Table I.

D. Luteinizing Hormone Injection**

In experiments No. 6 and 7, one group of fish was injected with 0.20 mg of LH in 0.025 ml distilled water twice a week. A second group in No. 6 and 7 was injected with a mixture of methallibure and LH (0.20 mg LH + 0.20 mg methallibure).

E. Thiourea Immersion

In experiment No. 8, a group of fish was treated with thiourea by immersion with 1.5 gm of thiourea added to the 45 litre tanks every week for seven weeks.

*1 litre of freshwater teleost saline contains 5.50 gm NaCl, 0.14 gm KCl, 0.12 gm CaCl₂ in distilled water.

**NIH-LH-B5 Bovine
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* 16 litre
** 45 litre

Summary of Experiments Performed

TABLE 1
F. General Condition of Fish

Only fish which appeared healthy were used for experiments. However, there were mortalities among both experimental and control fish in most experiments. In some cases the fish developed fungal infection but in others the cause of death was not apparent. The initial injection doses of methallibure may have been too high but in later experiments mortalities were about equal in experimental and control groups. The exceptions were groups injected with LH alone or in combinations with methallibure. The higher mortality in these groups was probably due to the joint effects of LH and long photoperiod (natural gonadotrophin at high levels) since the dose of LH was the same as that used by Hoar (1962b) on sticklebacks kept under short photoperiod, when it had no ill effects.

G. Histology

All fish were killed by an overdose of tricaine methane sulfonate (Sandoz MS 222), the gonad and body weight recorded and their tissues fixed in Bouin's picric acid-formal-acetic acid solution. The tissues were embedded in paraffin using routine procedure and stained usually with haematoxylin and eosin, less frequently with picro-aniline blue. Sections of the various tissues were cut at 5-10μ. In experiments No. 1 to 4 only the gonads were sectioned, in No. 5 both gonads and kidneys and in No. 6-8 gonads, kidneys and thyroids were sectioned. In experiment No. 8
pituitaries were also sectioned and stained with alcian blue-PAS-orange G.
III. RESULTS

A series of eight experiments was carried out between October 1966 and April 1968. The details are summarized in Table I. In each of the experiments one or two groups of fish were treated with methallibure. In all, five groups of fish were injected with methallibure and three groups were immersed in a dilute suspension of the compound. Two groups of fish were injected with Luteinizing Hormone (LH) and two groups with a mixture of equal amounts of LH and methallibure. A single group of fish was immersed in a dilute solution of thiourea.

The first two experiments form a preliminary study. They helped familiarize me with the various behavior patterns of the sticklebacks, the general care of the fish and the technique of injection. The experiments No. 3 to 8 formed the basis of the detailed study (Table I).

A. Behavior Studies

1. Prespawning Aggressiveness
   a) Methallibure Treatment

   Since two methods of treatment with methallibure were used (immersion and injection) it was important to determine if equivalent results were obtained with both methods. Therefore, data from experiments No. 6, 7 and 8 on the levels of aggressive behavior were evaluated for both methods and it was found that both resulted in a marked drop
in the level of aggressiveness after four weeks of methallibure treatment (Fig. 1). The aggressive level of the controls increased over the same four week period.

To determine the effects of injection, data were combined from experiments No. 6 and 7 (Fig. 1). At the beginning (Week 0), before injections were begun, four tanks were at Level I (two experimental, two control) and twelve tanks were at Levels III and IV (seven experimental, five control). After two weeks of treatment the change in aggressiveness was already apparent with an increase in the controls and a decrease in the experimentals. Eight of the experimental tanks were between Levels 0 and II with no territoriality present, while only one control tank was between these levels. The remaining six control tanks and the one experimental were between Levels III and IV.

After four weeks of treatment the groups injected with methallibure were all at Levels 0 and I, with a maximum of ten aggressive acts in five minutes. All the controls were at Levels III and IV. From the second week to the fourth week there was a slight drop in level of two of the control tanks although all control tanks exhibited territorial behavior while none of the experimentals did.

When data from experiments No. 7 and 8 were combined almost the same results were obtained after four weeks of immersion in methallibure. In this case (Fig. 2) before treatment four tanks of fish were at Level I (three control, one experimental) while eight were at Levels III and IV.
Figure 1

Aggressive levels of sticklebacks injected with methallibure compared with controls.

A. before the treatment was begun
B. two weeks after the first injection
C. four weeks after the first injection

Open bars, methallibure injected; bars with slanted lines, control.
Figure 2

Aggressive levels of sticklebacks immersed in methallibure compared with controls.

A. before the treatment was begun
B. two weeks after the first addition
C. four weeks after the first addition

Bars with dots, methallibure treated; bars with slanted lines controls.
(three control, five experimental). After two weeks of treatment there had been only a slight change in level of aggressiveness. After four weeks of treatment there had been a more marked change, and three tanks were at Levels O and I (all experimental), six control tanks were at Levels III and IV and two experimental were at Level III. The immersion technique was not quite as effective as injection and it was slower to show its effects but after four weeks of treatment no tanks treated with methallibure showed dominance and subordination. Thus, treatment of sticklebacks with methallibure for four weeks can be seen to bring about a decrease in aggressive behavior, while at the same time, the untreated fish show an increase in aggressive behavior.

The data from experiment No. 7 were used to compare the number of aggressive acts, performed in the test period, of the two methallibure treated groups with the controls (Table II). The amount of aggression for each tank was the total number of bites, approaches and chases observed in five minutes. Each tank was observed twice a week. From the four tanks at each treatment a weekly average (+ the Standard Error of the mean) was computed and is given in Table II. From the second through to the fifth week, one of the weekly observation periods included observations with the "standard" fish in the glass tube in all aquaria that were at Level IV the previous observation period.
TABLE II

Amount of Aggressive Behavior in Methallibure-treated and Controls

<table>
<thead>
<tr>
<th>WEEK</th>
<th>TREATMENT</th>
<th>Control</th>
<th>Meth. Inj.</th>
<th>Meth. Imm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>3.8 ± 0.9</td>
<td>5.3 ± 1.1</td>
<td>12.3 ± 5.3</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>9.3 ± 3.8</td>
<td>4.5 ± 0.3</td>
<td>4.5 ± 2.6</td>
</tr>
<tr>
<td>2*</td>
<td></td>
<td>22.3 ± 10.2</td>
<td>6.8 ± 2.8</td>
<td>26.5 ± 4.8</td>
</tr>
<tr>
<td>3*</td>
<td></td>
<td>22.0 ± 3.2</td>
<td>9.8 ± 9.1</td>
<td>17.5 ± 8.4</td>
</tr>
<tr>
<td>4*</td>
<td></td>
<td>5.3 ± 1.7</td>
<td>1.3 ± 0.9</td>
<td>8.0 ± 6.4</td>
</tr>
<tr>
<td>5*</td>
<td></td>
<td>19.8 ± 6.8</td>
<td>1.5 ± 0.7</td>
<td>4.0 ± 2.8</td>
</tr>
</tbody>
</table>

All values based on average amount of aggressive behavior in 5 min ± S.E. of 4 tanks each observed twice per week.

* Tubes with standard fish were used in one of two weekly observations - weeks 2 - 5

No significant difference at 0.05 among means for any week.
These data show that the number of aggressive acts is relatively low in the methallibure injection group throughout the five weeks of treatment especially in the last two weeks (1.3 ± 0.9 and 1.5 ± 0.7). The group immersed in methallibure show an increasing number of aggressive acts during the first three weeks of treatment. After this the number of attacks gradually decreases to 4.0 ± 2.8 and five weeks of treatment. The controls also exhibit an increasing number of aggressive acts during the first three weeks. The high number is maintained during weeks three and five but drops during week four. This drop is found in all groups during the fourth week but only the control group rise to high number 19.8 ± 6.8 during the fifth week. However, due to the small sample size and the variability between tanks the mean of either methallibure treated group is not significantly different from the mean of the control group for any week (at 0.05 level using the 'least significant difference').

b) Other Treatments
   i) Luteinizing Hormone

Fish were injected with LH alone and in combination with equal concentrations of methallibure in experiments No. 6 and 7. The effects of these treatments on levels of aggressive behavior are compared with the controls in Fig. 3. Before injections were begun four tanks were at Levels 0 and I (three LH and one control); the remaining twenty
Figure 3

Aggressive levels of sticklebacks injected with LH and LH + METH compared with controls.

A. before the treatment was begun
B. two weeks after the first injection
C. four weeks after the first injection

bars with open circles, LH injected;
bars with horizontal lines, LH + METH injected;
bars with slanted lines, controls.
tanks (five LH, six control and nine methallibure and LH) were at Levels III and IV. After two weeks of injection both the controls and the LH group showed a rise in level of aggression but the LH + METH group showed a drop in level. Six of the seven control tanks were at Level IV, as were five of the seven LH tanks. Only three of the nine LH + METH were at Level IV. After four weeks of treatment a number of fish had died but of those which remained all the controls and LH tanks were at Levels III and IV as were three of the five LH + METH.

These data indicate that treatment with LH + METH results in a level of aggression intermediate between the high levels with LH alone and the low levels with methallibure alone.

ii) Thiourea Immersion

A group of fish was immersed in thiourea in experiment No. 8 and the level of aggressive behavior compared with the controls and the methallibure treated group (Fig. 4). Over the four week period the thiourea group maintained a high level of aggression. During this time one group of controls increased in level and both groups of methallibure treated fish dropped in level. Thus it is concluded that treatment with thiourea has no effect on level of aggression.

2. Nest Building

Although the level of aggression was high in many
Figure 4

Aggressive levels of sticklebacks immersed in thiourea and methallibure compared with controls.

A. before treatment was begun
B. two weeks after the first addition
C. four weeks after the first addition

bars with dots, methallibure treated;
bars with cross hatching, thiourea treated;
bars with slanted lines, controls.
tanks and discrete areas were defended, nest building was infrequently observed. In all experiments only three control fish, four LH, three LH + METH, two thiourea, one methallibure immersion and no methallibure injected built nests.

B. Histology

1. Testis and Kidney

The cyclical changes which annually occur in the stickleback testis were classified by Ahsan and Hoar (1963) into four distinct stages of maturation and are as follows:

I **Inactive** (Fig. 6) is characteristic of the postspawning period during August and September. Spermatogonia are most prominent in the seminiferous tubules but there are also a few nests of primary spermatocytes. The interstitial tissue is indistinct or absent. A few sperm from previous cycles may be present.

II **Slight or Mild Activity** (Fig. 7 & 14) found in nature from October through December. Primary and secondary spermatocytes predominate; spermatogonia are less common; there are a few interstitial cells.

III **Moderate Activity** (Fig. 8, 12 & 13) seen under natural conditions from January through March. The early stages of spermatogenesis are still evident but spermatids and spermatozoa are conspicuous and the interstitial cells are more numerous.
Figure 5 - Diagram of Stage 0
This stage is characterized by seminiferous tubules ringed with spermatogonia two or three cells thick. The centers of the tubules are filled with spermatozoa or spermatids. Few spermatocytes or interstitial cells are present.

Figure 6 - Diagram of Stage I
Spermatogonia are most prominent in the seminiferous tubules but there are also a few primary spermatocytes. Interstitial cells are indistinct or absent.
Figure 7 - Diagram of Stage II

Primary and secondary spermatocytes predominate; spermatogonia are less common; there are a few interstitial cells.

Figure 8 - Diagram of Stage III

The early stages of spermatogenesis are still present but spermatids and spermatozoa are more numerous. More interstitial cells are present.
Figure 9 - Diagram of Stage IV

The seminiferous tubules are packed with spermatids and spermatozoa. Earlier stages are difficult to find and interstitial cells are numerous.
IV Fully Active (Fig. 9, 15 & 17) characteristic of the breeding season from mid-April to early August. The seminiferous tubules are packed with spermatids and spermatozoa. Earlier stages are difficult to find and interstitial cells are numerous.

In the present study, a fifth stage, defined as Stage 0 (Fig. 5, 10, 11 & 15), was found in many methallibure treated fish. This stage was characterized by seminiferous tubules ringed with spermatogonia two or three cells thick. The centers of the tubules were filled with spermatozoa or spermatids. Very few, if any, spermatocytes were present and few interstitial cells were evident.

In the present study testes were categorized according to one of the above levels of maturation by examination of seven or eight longitudinal sections through the middle region of the organs.

It can be seen from Table I, the experiments of 31 to 38 days in duration, i.e. No. 3, 5, 6 & 7, were carried out at different seasons of the year. The fish were therefore at different stages of testis maturation depending on the time of year. However, Baggerman (1957, 1966) finds that at any time of year sticklebacks exposed to 16 hr of light and 20°C will attain full breeding condition in 2 - 4 weeks. Also, I find most of the control fish are mature or maturing in all experiments after 30 - 38 days exposure to long photoperiod and 20°C. Perhaps the fish may vary in their responsiveness to methallibure treatment at different
Figure 10 - **Stage 0** Photomicrograph of the testis of a fish treated with methallibure for 31 days x 200

Figure 11 - **Stage 0** Same testis as Fig. 10 x 900

Figure 12 - **Stage III** Photomicrograph of the testis of a fish treated with methallibure for 49 days x 200

Figure 13 - **Stage III** Same testis as Fig. 12 x 900

- `spgon.` - spermatogonia
- `spzoa.` - spermatozoa
- `sptid.` - spermatid
- `1 spcyt.` - primary spermatocyte
- `2 spcyt.` - secondary spermatocyte
- `sp. duct.` - main sperm duct
Figure 14 - **Stage II** Photomicrograph of the testis of a control fish under long photoperiod 32 days x 200

Figure 15 - **Stage IV** Photomicrograph of the testis of a control fish under long photoperiod 49 days showing main sperm duct and several branches to seminiferous tubules x 200

Figure 16 - **Stage 0** Photomicrograph of the testis of a methallibure treated fish showing regression of the interstitial cells x 900

Figure 17 - **Stage IV** Photomicrograph of the testis of a control fish showing well developed interstitial cells x 900
times of year but since the histology of the treated group was also quite consistent it was considered justifiable to combine the results from these experiments. The number of fish examined from each experiment is given in Table III and the results of the histological examination are given in Table IV.

a) Methallibure Treatment on the Testis

Samples of three or four fish were examined after 12 to 22 days of immersion in, or injection with methallibure. All 12 and 22 day control testes were at Stage IV showing fully active testes characteristic of breeding fish. Fish treated with methallibure for 12 days were also at Stage IV as were four of the six fish examined after 22 days of treatment. The remaining two fish were at Stage 0. It was concluded that methallibure treatment brought about no detectable change in testis histology during the first 12 days of treatment but two of the six fish examined show a treatment effect after 22 days of treatment.

After 30 - 38 days of treatment there was a very marked difference between treated and control fish. In the testes of most control fish gametogenesis was well underway or complete and 20 of the 22 fish examined were at Stages III and IV. However, gametogenesis is severely retarded in fish injected with methallibure and only 3 of the 23 examined are at Stages III and IV. The largest group of 15 are at Stage 0, showing maturation of about half the germ cells to
### TABLE III

**Number of Fish Used for Examination of Testis Histology**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exp. No.</th>
<th>Duration in Days</th>
<th>No. of Fish</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4a</td>
<td>12</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>22</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>31</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Inj. &amp; Uninj.</td>
<td>5 a &amp; b</td>
<td>36 &amp; 38</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>32</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>35</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>49</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Meth. Inj.</td>
<td>4a</td>
<td>12</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>22</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Meth. Imm.</td>
<td>5 a &amp; b</td>
<td>36 &amp; 38</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>32</td>
<td>5</td>
<td>5</td>
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<tr>
<td></td>
<td>7</td>
<td>35</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>L.H. Inj.</td>
<td>6</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>35</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>L.H. + Meth. Inj.</td>
<td>6</td>
<td>32</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>35</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Treatment</td>
<td>Duration of Treat. in Days</td>
<td>Stages of Testis Stimulation</td>
<td>Brush Border Seg. Kidney</td>
<td>Histology of the Testis and the Kidney</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------------</td>
<td>------------------------------</td>
<td>-------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td></td>
<td>No. Fish</td>
<td>0</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>*30</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Meth. Inj.</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>*30</td>
<td>23</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Meth. Imm.</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>*30</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>LH</td>
<td>*30</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Meth. + LH</td>
<td>*30</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thiourea</td>
<td>49</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 30 to 38 days

** mean ± S.E.
spermatozoa and the remainder at the spermatogonia level.

Results were very similar with the immersion technique. No fish were at Stages III and IV and four of the seven examined were at Stage 0. After 49 days of immersion in methallibure there were still differences between treated and control fish. All control fish were completely mature and at Stage IV. Methallibure treated fish, however, were less mature and six of the seven examined were at Stage III. In these testes about half the germ cells were mature sperm and the rest in various intermediate stages (primary and secondary spermatocytes and spermatogonia). The block in gametogenesis appears to have been less complete after 49 days of treatment than after 30 - 38 days.

b) Methallibure Treatment on the Kidney

The height of the cells of the brush border segment of the kidney increases two or three times when male sticklebacks become mature and start to build nests. The height of these cells increases with high levels of androgen in the testis. (Wai and Hoar, 1963). To determine the effects of methallibure on steroid production, samples of kidney from fish treated for 30 or 49 days with methallibure were examined and compared with controls (Table III).

A mean cell height for each kidney was computed from the minimum cell height of the brush border segment of 25 kidney tubules. The kidneys were classified into two groups, those with and those without mucous cells in the collecting tubules. The mean for each group and each treatment (+ the
Standard Error) was computed. The presence of clear mucous cells in the collecting tubule as well as the granular appearance of the convoluted tubules indicate steroid stimulation.

After 30 days of methallibure treatment mucous cells were not present in any of the kidneys of treated fish but they were only present in two of the control fish. The cell height of controls without mucous cells and that of the methallibure group were not significantly different. These date indicate low steroid levels in both treated and control groups after 30 days.

However, after 49 days four of the five control fish sampled exhibit a marked stimulation of the kidney tubules (28.26 ± 2.82). There are no mucous cells present in any of the methallibure treated group. These data indicate higher steroid levels in control fish.

In summary, treatment with methallibure either by immersion or injection retards gametogenesis in the testis after 30 - 38 days of treatment. There seems to be a block in the meiotic transformation of spermatogonia into spermatocytes.

Most of the treated fish exhibit testes with germ cells which have completely undergone meiosis (spermatozoa) or cells which have not started the meiotic process (spermatogonia). However, after 49 days of treatment the block appears to have been less effective and germ cell maturation has only slowed down so that while the controls
are mature the methallibure treated group are maturing.

Steroidogenesis has also been affected by methallibure treatment and the cells of the kidneys of the treated groups never attain a height characteristic of actively breeding fish. Controls after 49 days under experimental conditions have kidneys which show signs of stimulation and mucous production.

c) **LH Treatment**

Fish were treated for 30 days with LH. Of the sample of five, three had fully active testes Stage IV while the other two were less active. There was also evidence of increased steroid production since four of the five kidneys examined had mucous cells and granular cells with a height of 17.25 ± 1.50. This indicates a higher steroid production in the LH group than the controls where only two of the 20 examined show mucous cells.

d) **Thiourea Treatment**

Fish treated for 49 days with thiourea were all at Stage IV of testis stimulation as were the control group. Three of the five fish had mucous cells present in the kidneys and a mean cell height of 22.75 ± 3.12 which is characteristic of fish secreting mucus. The states of gametogenesis and steroidogenesis are the same in the thiourea treated group as they are in the control group. It is, therefore, concluded that thiourea has no effect on either steroidogenesis or gametogenesis.
e) GSI

The gonosomatic index or GSI (Gonad Wt X 100/Total Body Wt) was calculated for methallibure, thiourea and control fish for experiments No. 5, 6, 7 and 8 (Table V). The GSI of the methallibure treated fish was just significantly lower than the GSI of the control fish in two experiments (No. 5 and 6) at the 0.05 level. In two later experiments (No. 7 and 8) the GSI of methallibure treated and control fish are not significantly different. So although there are differences in histology between treated and control fish, the weight of the testis of the treated fish does not consistently reflect this difference. The group of fish treated with thiourea have a GSI which is not significantly different from either the controls or the methallibure treated group.

4. Thyroid

a) Methallibure and Thiourea Treatment

The thyroid of Gasterosteus is composed of a number of follicles found scattered under the pharynx at the roots of the afferent branchial arteries. Ten unbroken follicles were chosen at random for each fish. The lowest and the tallest epithelial cells in each follicle were measure and the mean cell height computed (lowest + tallest/2 = mean cell height). This same convention of computing mean follicular cell height has been used by Eales (1965) on Salmo gairdner, and Wiebe (1968) on Cymatogaster aggregata.
### TABLE V

**G.S.I.**

**Comparison of Effect of Methallibure and Thiourea Treatment on Gonasomatic Index**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Control</th>
<th>Methallibure Injection</th>
<th>Methallibure Immersion</th>
<th>Thiourea Immersion</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.53 ± 0.05</td>
<td>0.33 ± 0.09*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.45 ± 0.08</td>
<td>0.21 ± 0.02*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.31 ± 0.03</td>
<td>0.21 ± 0.10</td>
<td>0.21 ± 0.06</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>8</td>
<td>0.38 ± 0.06</td>
<td></td>
<td>0.41 ± 0.06</td>
<td>0.39 ± 0.04</td>
</tr>
</tbody>
</table>

* Significant difference at .05 level

Mean Values ± Standard Error of Mean
Seven fish from each treatment - thiourea, methallibure and untreated controls - were examined and a grand mean for each treatment computed from the mean follicle cell height of each fish. These values are given in Table VI.

The mean follicular cell height (10.53μ) of the thiourea treated group is significantly different from the means of the methallibure treated (5.28μ) and the control (4.92μ) groups. There was marked thyroid hypertrophy in three of the thiourea treated fish examined. The follicles were smaller than those of the controls and had greatly increased epithelial cell heights and decreased colloid content. The other four thiourea treated fish showed little sign of increased thyroid activity compared with the controls. However, even with this variability of response the difference between means of the thiourea treated group and the control group is 5.62μ compared with a least significant difference of 1.11μ at P<0.05. The thiourea treated group also has a significantly different mean from the methallibure treated group at P<0.05.

The methallibure treated group showed no obvious signs of thyroid hypertrophy and the mean follicular cell height was not significantly different from that of the controls indicating that methallibure has no obvious goitrogenic effects.

5. Pituitary

a) Morphology

The pituitary is made up of four main parts, one
TABLE VI

Mean Thyroid Cell Height Following Thiourea and Methallibure Treatment

<table>
<thead>
<tr>
<th></th>
<th>Mean Follicle Cell Height (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.92</td>
</tr>
<tr>
<td>Methallibure</td>
<td>5.28</td>
</tr>
<tr>
<td>Thiourea</td>
<td>10.53*</td>
</tr>
</tbody>
</table>

* Statistically significant difference from controls and methallibure treated at P 0.05 using least significant difference
nervous the neurohypophysis and three glandular, the pro-
adenohypophysis, the meso-adenohypophysis and the meta-
adenohypophysis. In the pituitary of *Gasterosteus*, the
neurohypophysis interdigitates with the adenohypophysis
(Fig. 17). The trophic hormones secreted by the pituitary
are elaborated by various cell types located in different
parts of the adenohypophysis. Both the gonadotrophin(s)
and thyroid stimulating hormone (TSH) are produced by
basophils located in the mesoadenohypophysis (Pickford and
Atz, 1957).

b) **Methallibure and Thiourea Treatment**

Gonadotrophs and thyrotrophs are scattered throughout
the mesoadenohypophysis. With the stain combination
used (alcian blue, PAS, Orange G) they take on alcain blue
and more or less PAS. This gives them a deep purple hue
with pinkish overtones (Fig. 19, 21 & 23 Black). Since both
cell types are found in the same area and have very similar
staining reactions, it was not possible to differentiate
between these two basophils. The effects of methallibure
and thiourea treatments on the granulation of both cell types
will be considered together.

The amount of basophilic material was rated on a
scale of + to +++. Seven or eight fish were examined in
each group. The score for each group was based on the
examination of at least six sagittal sections of the
pituitary. The results are tabulated in Table VII.
TABLE VII

Amount of Granulation in the Basophils
(Thyrotrophs and Gonadotrophs in Mesoadenohypophysis) after Methallilure and Thiourea Immersion.

<table>
<thead>
<tr>
<th></th>
<th>Amount of Granulation in the Basophils of the Adenohypophysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Methallilure</td>
<td>3</td>
</tr>
<tr>
<td>Thiourea</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
</tr>
</tbody>
</table>
The methallibure treated group contained very little basophilic material in the mesoadenohypophysis. All fish were rated either + or ++ (Fig. 18 & 19). The control group, however, had much more basophilic material and six of the fish examined were rated +++ while two were rated +++ (Fig. 20 & 21; Table VI).

Since methallibure treatment was found to retard gametogenesis and steroidogenesis in the testis but not increase follicular cell height in the thyroid, most of the degranulated cells are probably gonadotrophs.

Thiourea treatment results in degranulation in some fish. This group is rated ++, +++ and ++++. It is significant that the fish rated ++ (Fig. 22) exhibited the greatest increase in thyroid follicular cell height and the fish rated ++++ (Fig. 24) the least. Therefore, it is concluded that thiourea results in degranulation of the thyrotrophs while methallibure results in degranulation of mainly the gonadotrophs.
Figure 18 - Photomicrograph of the pituitary of a fish treated with methallibure for 49 days
x 200

Figure 19 - High power of part of the fish of Fig. 18 showing + of basophilic material
x 900

Figure 20 - Photomicrograph of the pituitary of an untreated control fish
x 200

Figure 21 - High power of part of the meso-adenohypophysis of the fish of Fig. 20 showing ++++ of basophilic material
x 900
Figure 22 - Photomicrograph of the pituitary of a fish treated with thiourea for 49 days
   x 200

Figure 23 - High power of part of the meso-adenohypophysis of the fish of Fig. 22 showing ++ of basophilic material
   x 900

Figure 24 - Photomicrograph of the pituitary of a fish treated with thiourea for 49 days showing ++++ basophilic material
   x 900
IV. DISCUSSION

Brown (1963), Harper (1964), Hemsworth, Jackson and Walpole (1968), Paget et al. (1961) and Walpole (1965) provide evidence that methallibure, dithiocarbamoylhydrazine (I.C.I. 33,828) blocks the formation of gonadotrophin of mammals. This non-steroid, non-hormonal compound has the following chemical formula:

\[
\text{CH}_2=\text{CH},\text{CH},\text{NH},\text{CS},\text{NH},\text{NH},\text{CS},\text{NH},\text{CH}_3 \]

Hoar et al. (1967) were the first to use it on teleosts. They report that treatment of sticklebacks, goldfish and surfperch with methallibure interferes with pituitary gonadotrophin function.

Before discussing the main effects of methallibure on the stickleback, it may be of interest to consider the possible side effects of the treatment. The most important of these is the somewhat toxic effect of the compound and the second the possible direct effect of methallibure on the thyroids of the treated fish.

The preliminary study by Hoar et al. (1967) and the present one both show that methallibure treatment is to some degree toxic to sticklebacks. In my early experiments up to 50% of the experimental group died during the course of treatment. In later experiments survival was better and in experiment No. 8 only one treated fish died during seven
weeks of treatment. Not all mortality can be attributed to methallibure since a third of controls died in the early experiments. Although a toxic effect cannot be ruled out, the very low mortality in later experiments suggests that this factor per se was not a prime cause of death in the earlier tests.

Secondly, since methallibure contains the thiourylene group, it might be expected to block the synthesis of thyroxine with resultant increased thyrotrrophic activity of the pituitary and enlargement and hyperplasia of the thyroid. Walpole (1965) found administration of large doses of methallibure to rats caused a slight increase in thyroid weight but normal thyroid histology. Tulloch et al. (1963) found methallibure decreased the uptake of I\textsuperscript{131} by the thyroids of treated rats and mice. Wiebe (1968) reported no change in the thyroid histology of the surfperch Cymatogaster after 40 days of methallibure treatment. Leatherland and Pandey (1968), however, describe an increase in follicular cell height after 56 days of methallibure treatment on Poecilia. The results obtained in the present series of experiments are similar to those found with Cymatogaster; 49 days of treatment caused no significant increase in the mean thyroid follicular cell height of Gasterosteus (Table VI).

The effects of methallibure on the thyroid of Gasterosteus were compared with those obtained when a group of fish was treated with the goitrogen thiourea. Thiourea acts by blocking the iodination of tyrosine which is one
step in the synthesis of thyroxin: (Hoar 1966). Thiourea treatment has been used on a number of species of fish and usually results in an increase in follicular epithelial cell height and in some cases marked hyperplasia (Atz (1953) Astyanax, Chambers (1953) Fundulus, Wiebe (1968) Cymatogaster). Treatment of sticklebacks with thiourea for 49 days resulted in a significant increase in mean follicular cell height. The variability in amount of stimulation between individuals would suggest that the concentration of thiourea used (0.003%) was only just effective. Kinnear (1960) reports that a solution of 0.0025% thiourea is the most dilute that prevents any synthesis of thyroxine by the flounder Platichthys.

Thiourea has been found by Chambers (1953) to result in loss of weight, retardation of growth and depletion of liver glycogen in Fundulus. Barrington and Matty (1952) found that minnows immersed in thiourea do not feed well and that spermatogenesis is arrested in the testis at the spermatocyte stage. No side effects are observed using a solution of thiourea on Gasterosteus. The fish feed well and only spermatozoa are found in the testes of both thiourea treated and control fish. It may be significant that the dosages used by Chambers and other early workers were about ten times greater than those used in the present study.

In summary, the effects of methallibure and thiourea on Gasterosteus are very different. Methallibure brings about retardation in testis gametogenesis and no significant
increase in thyroid epithelial cell height. Thiourea, however, has no observable effect on the gametogenetic process but brings about a significant increase in thyroid epithelial cell height. Methallibure is concluded to have no observable goitrogenic action. Therefore, in spite of the possible toxic effect, the use of methallibure is considered to have yielded useful information about the hormonal effects of the gonadotrophins on the behavior and various tissues of Gasterosteus.

A. Prespawning Aggressive Behavior

All the present evidence indicates that prespawning aggressive behavior in Gasterosteus is under direct control of the pituitary via an LH-like gonadotrophin. Hoar (1962a, b) demonstrated that both castrated and normal male sticklebacks held under short photoperiod, 'physiologically hypophysectomized' exhibited a low level of aggressiveness but normal and castrated males under long photoperiod exhibited a high level of aggressiveness. Injection of the short photoperiod fish with mammalian LH or chorionic gonadotrophin resulted in an increase in level of aggressiveness. Wai and Hoar (1963) concluded that treatment of castrated long and short photoperiod sticklebacks with methyl testosterone did not affect their level of aggressiveness.

Treatment of Gasterosteus with methallibure resulted in a marked drop in level of aggressiveness of the controls increased (Fig. 1 & 2). The drop in aggressiveness is
associated with a loss of territorial behavior on the part of the treated fish and an increase in the number of control fish which defend a discrete area. The amount of biting and chasing is consistently less (than in the controls) in the fish injected with methallibure and less, in two of the last three weeks, in fish immersed in methallibure (Table II). When a group of fish is treated with a mixture of methallibure and mammalian LH the level of aggressiveness is higher than that in fish treated with methallibure alone (Fig. 1, 2 & 3) but not as high as that of the controls. The treatment combination of LH + METH did not seem to be tolerated at all well by the fish so that the effects on the behavior by this replacement therapy must be considered highly suggestive rather than conclusive.

The study indicates that methallibure treatment on long photoperiod fish results in the same kind of reduction in aggressiveness which is characteristic of fish kept under short photoperiod. Treatment with LH + METH results in a level of aggressiveness intermediate between the methallibure treated and the control groups.

It is very difficult to determine whether methallibure affects only prespawning aggressive behavior since nest building and reproductive behavior occur in only a few fish - whether experimental or control. The height of the cells of the kidney tubules of fish treated with methallibure for 30 - 38 days is low but so is the height of the kidney cells of the controls (Table IV). These results
indicate that steroid levels are probably low in both groups. Then it is not surprising that the sequences of reproductive behavior which are thought to be under steroid or steroid + gonadotrophin control are for the most part absent. However, a lowering in the level of gonadotrophins by methallibure could logically be expected to lower steroid production and indirectly affect the whole cycle of reproductive behavior. Wiebe (1967) finds such a result with *Cymatogaster*. As with *Gasterosteus*, certain elements of reproductive behavior appear to be under direct gonadotrophic control and others under direct gonadal steroid control. Treatment with methallibure eliminates all reproductive behavior since it blocks the gonadotrophin which in turn regulates the gonadal steroids.

B. Testis and Kidney

Spermatogenesis and steroidogenesis in the testis of fish are controlled by the interaction of the gonadotrophin of the pituitary and the androgens of the testis. Much has been learned about the role of the gonadotrophins by the removal of the pituitary and subsequent replacement of the various hormones. Dodd (1965) summarizes the results of hypophysectomy by a number of workers and concludes that the pituitary through the gonadotrophins is necessary for the initiation and maintenance of spermatogenesis in the lower vertebrates (except the cyclostomes) but its control is probably limited
to phophase of the first meiotic division. There is much
evidence, he finds, for the maintenance of spermatogenesis
in the absence of the pituitary. He suggests that
gonadotrophin acts indirectly by stimulating the secretion
of steroid. Further evidence comes from the known ability
of a variety of steroids to stimulate cell division.

Methallibure has been found to have marked effects
on spermatogenesis and steroidogenesis by Hemsworth et al.
(1968) on rats and by Hoar et al. (1967) on three species
of fish.

Treatment of Gasterosteus with methallibure resulted in no
changes in histology after 12 days. After 22 days of
treatment only two of the six experimental fish showed a
treatment effect. The other four treated and all control
fish were completely mature. After 30 - 38 days of
methallibure treatment the histology of control and treated
groups was very different indicating that methallibure
treatment brings about a depression in gonadotrophic
activity. A reduction rather than a complete block is
postulated since some spermatogenesis seems to occur in
treated fish especially in the group treated for 49 days.

Any reduction in gonadotrophin would most affect the
spermatogonia to spermatocyte transformation since this
stage appears from work on hypophysectomized fish to be the
most dependent on gonadotrophin. Therefore, it is postulated
that any cells which were spermatocytes before the treatment
would proceed at the normal rate to become spermatozoa
and cells which were spermatogonia would remain at this stage or undergo the initial phase of meiosis at a much slower rate than normal.

It appears to take about 30 days of methallibure treatment to obtain a widespread difference in histology between treated and control fish. However, during the 49 days of methallibure treatment it is postulated that enough gonadotrophin is present to allow most spermatogonia in the treated fish to become spermatocytes. The germ cells of the controls, however, have completely undergone spermatogenesis and are mature sperm.

Gonadotrophins also regulate the development of the interstitial cells in the testis. Since these cells develop in seasonal fish during the breeding season, are sudanophilic and cholesterol positive, they are thought to produce steroid (Marshall, 1960). Ahsan (1966), Lofts, Pickford and Atz (1966), Sundararaj and Nayyar (1967) and others find that these cells (or lobule boundary cells in fish which do not have interstitial cells) regress after hypophysectomy and it might, therefore, be expected that methallibure treatment would regress the interstitial tissue of Gasterosteus.

The evidence with respect to this theory is indirect. In male sticklebacks during the breeding season, the interstitial tissue develops, red coloration appears in the skin and the cells of the brush border segment of the kidney tubules become filled with secretory granules and clear
mucous cells appear in the collecting tubule. These changes are controlled by androgen elaborated in the interstitial cells.

Fish treated with methallibure never showed the increased cell height or granular cytoplasm in the cells of the brush border segment of the kidney tubule. The mean values are in the range of values found by Wai and Hoar (1963) to be typical of mature but sexually quiescent males. The control fish varied somewhat in their response to long photoperiod. When one group was exposed to this light regime for 30 - 38 days only 2 of the 13 examined exhibit kidney histology typical of breeding males but when another group was exposed to long photoperiod for 49 days four of five fish examined showed evidence of mucus production by the kidney (Table IV). The mean cell height of this latter group is in the range characteristic of breeding males (Wai and Hoar, 1963).

Methallibure treated males retained their red coloration throughout the experiments. This would suggest that androgen is probably being produced at a low level by the treated fish and may imply that a certain amount of gonadotrophin is also present. However, levels of gonadotrophin do not appear to be high enough to promote spermatogenesis at the rate found in control fish or to lead to the levels of androgen necessary to bring about the increase in kidney tubule cell height characteristic of breeding males.
When hypophysectomized fish are treated with reproductive hormones stimulation of gametogenesis and steroidogenesis results. Ahsan (1966) finds both processes stimulated in hypophysectomized lake chub treated with either mammalian LH or a crude extract of whole salmon pituitaries. Lofts et al. (1966) report that methyl testosterone stimulates both processes in hypophysectomized Fundulus. However, Sundararaj and Nayyar (1967) find testosterone propionate treatment restores spermatogenesis in hypophysectomized catfish but the interstitial cells are atrophic. Human chorionic gonadotrophin induces significant dose dependent weight increments in the testes and restores spermatogenesis and the secretory activity of the interstitial cells.

In the present study bovine LH was used in an attempt to counteract the effects of methallibure. Unfortunately, a large number of the treated fish died during the course of the experiment. However, three of the five surviving LH injected fish were at Stage IV of testis maturation and four of the five kidneys examined showed clear mucous cells and granular brush border cells. Only two fish injected with LH + METH were examined, both were at Stage IV of testis stimulation. One fish showed kidney tubules with mucous cells, one did not. The small sample indicates that METH + LH is more like the control group and seems to promote testis maturation and steroidogenesis; any conclusion must remain tentative at this stage.
C. Pituitary

The mesoadenohypophysis of teleosts appears to contain all the cell types that are associated with the mammalian pars anterior. There are at least two types of basophils and these have been identified as gonadotrophs and thyrotrophs (Pickford and Atz, 1957). Van Mullem (1959) gives histological evidence for the presence of LH and FSH in the mesoadenohypophysis of *Gasterosteus* but the differentiation of cells producing the gonadotrophins from each other and those producing FSH is not definite.

After 49 days of methallibure treatment in *Gasterosteus* there was extensive degranulation of the basophils of the mesoadenohypophysis (Table IV and Fig. 17 & 18). The effects on gametogenesis and steroidogenesis in the testis (Table IV) are suggestive of less gonadotrophin as are the lower levels of aggressive behavior characteristic of the methallibure treated fish (Fig. 1 & 2). This explanation implies that degranulation of the basophils means hypo-activity or a block in the elaboration of gonadotrophin. Such an explanation has been put forth before to explain degranulation especially the cyclic degranulation associated with reproduction. For example, Sokol (1961) finds conspicuous cyclical changes in the basophils of the mesoadenohypophysis of *Fundulus*. These cells are small and degranulated during the quiescent winter period and large and fully granulated in the breeding season. Sokol considers the majority of these cells to be
gonadotrophs. Additional evidence for the anti-
gonadotrophic effects of methallibure on the pituitary
comes from work by Brown (1963) on rats. He finds
significantly less FSH in the pituitaries of animals treated
with methallibure than is present in the pituitaries of
control animals.

Some of the degranulated cells in the meso-
adenohypophysis of methallibure treated *Gasterosteus*
may be thyrotrophs since thyrotrophs cannot be distinguished from
gonadotrophs on the basis of the stain combination used.
Possible evidence that both basophils may be affected comes
from experiments of Leatherland and Pandey (1968). They
find that the gonadotrophs of methallibure treated guppies
are less numerous, significantly smaller and less aldehyde
fuchsin positive than those of the controls; the thyrotrophs,
on the other hand, are significantly larger and more
aldehyde fuchsin positive than those of the controls. They
conclude the gonadotrophs are hypo-active while the
thyrotrophs are hyper-active. However, they also find that
methallibure treatment brings about a significant increase
in thyroid epithelial height. Such an increase in thyroid
cell height is not found in *Gasterosteus* treated with
methallibure. These considerations lead me to conclude that
most of the degranulated basophils in methallibure treated
sticklebacks are probably gonadotrophs.

Degranulation of basophils also occurs when a group
of fish is treated with thiourea. The amount of degranulation
is variable but it is correlated with the increase in thyroid epithelial cell height. Thus, fish exhibiting the most degranulation (++ in Table VII) also show the greatest thyroid epithelial cell height. Therefore, the degranulated cells are very probably thyrotrophs. A number of other workers have also found degranulation of the thyrotrophs in fish treated with thiourea or thiouracil (Atz, 1953 on Astyanax; Barrington and Matty, 1955 on Phoxinus and Grosso, 1961 on Lebistes).
V. CONCLUSIONS

Treatment of sticklebacks with methallibure results in:

(1) A marked reduction in the level of prespawning aggressiveness in treated fish.

(2) A decrease in the rate of transformation spermatogonia to spermatocytes so that after 30' - 38 days most of the testes of the treated fish contained spermatogonia and spermatozoa while the testes of the controls contained spermatocytes and spermatozoa or spermatozoa only. After 49 days, the testes of the treated group contained spermatogonia, spermatocytes and spermatozoa while those of the controls contained only spermatozoa.

(3) No evidence of stimulation of the cells of the brush border segment of the kidney tubules of treated fish under long photoperiods.

(4) A marked decrease in the granulation of the pituitary basophils.

(5) Some degree of toxicity but no evidence of a direct effect of the treatment on the thyroid comparable to that of thiourea.

In brief, it is concluded that methallibure partially blocks gonadotrophic activity probably at the level of the pituitary. This treatment affects prespawning aggressive behavior and testis physiology in a manner similar to an exposure to short photoperiods.
VI. BIBLIOGRAPHY


