

A STUDY OF THE EFFECTS OF PROLACTIN AND TESTOSTERONE
ON THE PARENTAL BEHAVIOUR OF THE MALE STICKLEBACK

Gasterosteus aculeatus L.

by

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B.A., University of British Columbia, 1962

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Zoology

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
September 1964

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ABSTRACT

Male stickleback did not show a greater increase in displacement or parental fanning when injected with prolactin than they did when injected with saline solvent alone.

Displacement fanning was higher in gonadectomized fish with pituitary activity suppressed by short photoperiod than in gonadectomized fish with an active pituitary under long photoperiods when both groups were treated with the same testosterone concentration. Injection of pituitary fractions into the short photoperiod fish reduced fanning to the levels found under long photoperiods.

Normal males under long photoperiod showed significantly higher fanning than any of the gonadectomized groups indicating that methyl-testosterone in concentrations used did not fully replace the effects of the normal gonad. Exposure of males to a wide range of testosterone concentrations indicated that a relatively high concentration of testosterone was required for normal fanning. Low levels of fanning occurred after gonadectomy of fish in breeding condition in both "displacement" fanning and parental fanning stages, indicating the gonad was not required for performance of the fanning pattern as such but was required for fanning to reach levels approaching these found in normal fish.

No evidence was found that prolactin initiates or maintains the parental fanning cycle. Testosterone however was necessary for normal fanning levels.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. W. S. Hoar for suggesting the problem and for his time and valuable suggestions.

Special thanks are due to Frank van Neetten who prepared the histological material on stickleback kidney, and to Bill Woodall for his help in obtaining fish.

I wish to acknowledge the financial support provided by a National Research Council Studentship.

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INTRODUCTION

Care of eggs or young by the parent animal is a specialization which occurs time and again throughout the animal kingdom. Many of the advances made by the vertebrates in their evolution have been related to improved care of the young, for example, the cleidoic egg allowing colonization of land, or the evolution of mammary glands and placental development in mammals. Increasing survival of the young is the essence of the evolutionary process. The examples above are of physiological mechanisms of parental care but just as important are behavioural mechanisms which permit the internal fertilization required for cleidoic eggs or viviparity, or lead to the selection or building of a safe place for the young, incubation of the eggs or suckling of the young. Even within one group, such as the teleosts, many mechanisms, physiological and behavioural, may be evolved to increase survival of the young. Some Cichlids and catfish protect the eggs in their mouths, Cichlids (Baerends and Baerends van Roon, 1950) and Sticklebacks (Tinbergen, 1951) may herd and protect schools of newly hatched young, Cottids dig burrow for their eggs (Morris, 1954) and Discus fish Symphsodon discus nourish the young with a secretion of the skin (Egami and Ishii, 1962).

Two of the most common dangers to young fish and eggs are predation by the many scavengers of the seas and lakes, and the

ever present difficulty of the low oxygen concentration of many natural waters. The first problem is often overcome by construction of a "nest" to protect the eggs from predators, and perhaps to keep them from being carried away by water current, beyond the protection of the parent or into unsuitable environments. The construction of a nest, confining eggs to an area of limited water circulation enhances the second problem, oxygen. This problem is then often overcome by the parent fanning fresh water with its fins to circulate it through the nest in order to ventilate the eggs, or young. This is found in a wide variety of fish, for example wrasse (Feidler, 1962), Cottids (Morris, 1954), Badis badis (Barlow, 1964) and the Sticklebacks (van Iersel 1953, Morris 1958).

All these behavioural mechanisms must have some physiological background related to sense receptors, the central nervous system, the endocrine system or the effector organs. In a few cases some of this background has been explored experimentally, as in the relationship between the pituitary hormone Prolactin and "broodiness" in the jewel fish (Noble Kumpf and Billings, 1938), testosterone and nest building in the male threespined stickleback Gasterosteus aculeatus (Wai and Hoar, 1963) and fanning in the wrasse Crenilabrus ocellatus (Fiedler, 1962) which seems to be related again to prolactin.

The male threespine stickleback shows several patterns of behaviour associated with the care of eggs and young, (van Iersel, 1953). It migrates to a suitable location, builds a nest, induces the female to lay eggs in the nest, then fans water over the eggs until they hatch; after hatching it keeps the young in the region of the nest for a time until they are free swimming.

The object of this study is to investigate the endocrine background of the fanning behaviour of the male G. aculeatus with special reference to prolactin and testosterone. Prolactin has been implicated in parental behaviour in fish: wrasse (Feidler, 1962), jewel fish (Noble et al, 1938) and discus fish (Egami and Ishii, 1962), birds; pigeons and hens and mammals (Lehman, 1961). Testosterone which controls many activities characteristic of male animals and which has been shown to control an earlier pattern in this sequence, nest building by the stickleback (Wai and Hoar, 1963) was also investigated with reference to fanning in the stickleback.

MATERIALS AND METHODS

I. COLLECTION AND MAINTENANCE OF FISH

The stickleback used in this study were of both the plated and unplated forms (Heuts, 1947) of Gasterosteus aculeatus L. They were collected by dip net or by lift net from drainage ditches on the Musquem Indian Reserve (fresh water) and from floats in Coal Harbour (brackish water); both locations are in Vancouver, B.C. Heavy infestation with parasites was sometimes found, especially in Coal Harbour fish. Only fish which appeared healthy were used in experiments.

Before being used in experiments the stickleback were sometimes held in cooled (10° - 15°C) recirculating sea-water tanks under natural photoperiod. However, they were usually placed in observation tanks immediately after capture. All fish whether in holding or observation tanks were fed exclusively on frozen brine shrimp (Artemia salina).

For observation during an experiment, fish were placed in 16 litre glass aquaria with sand, and plants suitable for nest building and for sheltering females, subordinate males. The water in the aquaria was continuously filtered through glass wool and aerated. The aquaria were illuminated (50 - 55 ft.-C.) by overhead fluorescent lights and the photoperiod was

controlled by an electric time switch to suit the particular experiment. During experiments, all fish were kept in fresh dechlorinated water with Ca^{++} ion added as CaCl_2 to bring the Ca^{++} concentration up to 20ppm. This permitted 100 per cent survival of anadromous fish while the Ca^{++} concentration does not exceed that found naturally in many British Columbia streams and lakes. This was required because normally anadromous Gasterosteus aculeatus show a very high mortality when placed in dechlorinated Vancouver tap water with its low ion content (Smith, 1962). Problems of supply necessitated the use of anadromous stickleback during the winter months.

The male stickleback were observed for periods ranging from 10 to 30 minutes depending on the experiment. A "hide" was found to be necessary to prevent disturbing fish over long periods of observation; it was arranged so as to permit the observer to enter and leave the room without alarming the fish. Behaviour was recorded by means of a typewriter, at two-second intervals during an observation. The intervals were timed by a metronome fitted with an electric contact and a buzzer. The metronome was set to tick every second but the electric contact was made only at one extreme of the pendulum's arc therefore activating the buzzer every two seconds. The total observation period (e.g. 15 minutes) was timed on a stop watch thus allowing correction of behaviour frequencies or durations if the

metronome varied from the proper interval. Behavioural information was recorded in the form of abbreviations, e.g.

. . p pu fn fn fn p. indicates that at the first two intervals the fish did not show nesting or parental activity; at the third it was in a position over the nest and pointing its head at the nest; at the fourth it was pushing the nest with its snout, at the fifth, sixth and seventh observations it was fanning the nest, etc. These abbreviations were then totalled either manually or by means of electric contacts on the typewriter keys connected to automatic counters. Several items of behaviour were recorded:

1) Building and Maintenance of Nest

tmt - testing material for building

bmt - bringing material to the nest

rmt - removing material from nest

d - sand digging

p - pointing at nest with snout from a position above the nest

pu - pushing at nest with snout, not distinguished from "boring" (van Iersel, 1953)

g - glueing nest material with glue-like kidney secretion

2) Parental

fn - fanning water over the eggs

c - catching young

sp - spitting young out

Apart from "p" these behaviour patterns are described in the literature as distinct items (van Iersel, 1953). The two major variations between the criteria in this study and those used by van Iersel are the failure to distinguish between pushing and "boring" in the present study, and the use of the term "fanning bout" to refer only to the duration of a period of uninterrupted fanning rather than as van Iersel (1953) uses it to refer to a period of fanning interrupted only by short periods of nesting activity.

Calculations performed on the data were:

1. Per cent of time spent fanning

$$\frac{\text{number of 2 second intervals spent fanning} \times 100}{\sum \text{all 2 second intervals}}$$

- when calculating mean "percent of time fanning"
for a group of observations:

$$\frac{\sum \text{Percent of time spent fanning}}{\sum \text{Observations}}$$

2. Average length of fanning bouts

$$\frac{\text{number of 2 second intervals spent fanning}}{\text{number of fanning bouts}}$$

- when calculating average bout length
for a group of observations:

$$\frac{\sum \text{Average bout length per observation}}{\sum \text{Observations showing fanning}^*}$$

* If the observations which showed no fanning were included it would distort the average bout length.

Statistics (Steel and Torrie, 1960) applied were:

1. Standard deviation of the mean

$$s_{\bar{x}} = \sqrt{\frac{s^2}{n}} \quad \text{where } s^2 = \text{variance, } n = \text{number of observations.}$$

2. 95 per cent Confidence interval on the mean

$(s_{\bar{x}}) (t \text{ for } n-1 \text{ d.f.})$ where the confidence intervals of two samples overlap the means, the means are not significantly different.

3. "t" test on difference of means

$$t = \frac{\bar{d}}{s_{\bar{d}}} \quad \bar{d} = \bar{x}_1 - \bar{x}_2 \quad s_{\bar{d}} = \sqrt{s^2 \frac{n_1 + n_2}{n_1 n_2}}$$

When pituitary hormones were used they were injected (after anaesthetizing fish in 0.01 per cent Tricaine methane sulphonate, Sandoz) by means of a 0.25 ml. tuberculin syringe, through a 30 guage needle into the body cavity. When several injections were to be given to the same fish the injection site was alternated between right and left sides.

II. EXPERIMENTS TO TEST THE EFFECTS OF PROLACTIN ON FANNING

Because of widespread effects of prolactin on both the behaviour and physiology of parental activity in vertebrates, it was decided to test this hormone as a possible controlling agent for the fanning of the male stickleback.

1. Prolactin injections on parental fanning.

The first experiment was designed to show whether regular injection of a suspension of prolactin in water would increase the normal parental fanning of the male fish. Male stickleback were kept, one fish per tank in observation aquaria under 16-hour photoperiod (16 hours light, 8 hours dark). When a fish built a nest it was given an injection of either prolactin or distilled water every third day until the experiment ended, unless it was an uninjected control fish. Fish under all three treatments were allowed to fertilize eggs, then were observed twice a week for 10 minutes each. Numbers of fish used are summarized in Table I. Prolactin dosage was: 15 mg./ injection given suspended in .025 ml. distilled water (Panlitar lot #R10109, Armour Drug Co.). Fish in both injected classes received up to 8 injections (depending on date of nest building).

2. Prolactin injections during "displacement" fanning.

The second group experiments served as a control of the saline solvent in which prolactin was dissolved, and utilized a

a somewhat higher dosage of prolactin. If prolactin initiated parental fanning its injection might lead to an increase in "displacement" fanning to a level resembling early parental fanning. (Displacement fanning has been defined by van Iersel, 1953, as fanning which occurs after the nest is completed but before eggs are present in the nest.) The fish which had all built nests (but had not spawned) were injected as above, with either prolactin (of) 0.67 Kreb's saline solution. Prolactin (Panlitar lot #R10109) was dissolved in the Kreb's solution and frozen in small amounts to be thawed as needed. Dosage was 25 mg. in .025 ml. of solution. Six fish were used initially, one per tank; three were given prolactin and three were given injections of Kreb's solution. Behaviour was recorded for two 10-minute periods (for a total of 20 minutes per day) on days that the

TABLE I

Numbers of Fish Used in Each Category of Experiment to
Test Effect of Prolactin on Parental Fanning

<u>Treatment</u>	<u>No. of Fish Used</u>	<u>No. of Fert. Eggs</u>
Prolactin	6	4
Distilled water	5	5
No injection	10	6

fish was not injected. Surviving fish received a total of six injections. One prolactin fish and one Kreb's solution fish were permitted to fertilize and raise eggs before the experiment but eggs had completed development or died before experiment began.

3. Prolactin injection during the parental fanning cycle.

A third series of prolactin experiments tested the effect of a single large injection during the regular cycle of parental fanning; fish were injected once on the third or fourth day after egg laying. They were observed daily one and one-half hours per day throughout the cycle with several observations on the day of injection, both before and after the fish was injected. Prolactin dosage was 0.20 mg. given in 0.01 ml of Kreb's solution (National Institute of Health, ovine, lot #23116). Seven fish were used, four with prolactin and three with solvent. Later two fish were tested with a needle prick substituted for an injection; two normal fish were also observed during undisturbed fanning cycles.

III. EXPERIMENTS TO TEST THE EFFECTS OF GONADAL AND PITUITARY

HORMONES ON FANNING

1. Photoperiod, pituitary and gonadectomy with replacement of testosterone

The object of this experiment was to test the effect of the pituitary gland as a whole, through the influence of photo-

period and crude anterior pituitary extract. Luteinizing hormone and prolactin alone were also tested. Gonadectomy with replacement of testosterone was used in an attempt to standardize the level of gonadal hormone in the two photoperiods. Two males were kept in each tank. Pituitary hormones were injected three times per week by the same methods as prolactin in the above experiments. When fish built nests they were observed for periods of twenty minutes each on days that they were not injected. Gonadectomy was performed, after anaesthetizing in M.S. 222, by cutting 1-2 mm slits in the body wall, just over the gonads, which were then pulled out with forceps and cut free well down the seminal duct. In sham operated fish gonads were held with forceps but not cut free, then replaced in fish. All operations were done under a dissecting microscope (10X). Males were easily distinguished from females by their darker pigmentation and smaller size of the testis. After gonadectomy the operated fish were kept in sea water for a week. Only two instances of regeneration were found in close to 200 fish gonadectomized in this manner and re-examined later. Mortality was very low in immature fish.

Testosterone was 17-a-methyl testosterone U.S.P. (Sigma Chemical Co. lot M102B-083). It was dissolved in absolute ethyl alcohol and measured into tanks with a .025 ml tuberculin.

syringe. The dosage was equivalent to 1 part/4 X10⁶ parts of water (Wai and Hoar, 1963). This was given once a week in 0.1ml. of alcohol.

Crude anterior pituitary extract (Bovine, Armour lot #305) luteinizing hormone (N.I.H.-LH-S-7 ovine) and prolactin (N.I.H. ovine lot #23116) were each injected in 0.2mg. doses dissolved in .05 ml. 0.67 Kreb's solution. Treatments and numbers of fish are summarized in Table II.

2. Range of testosterone dosages.

In this series of experiments a wide range of testosterone levels were tested on "displacement" fanning. The fish used were initially immature. All were kept under 16 hour days, two fish per tank. Gonadectomies and sham operations were performed as above. Testosterone, as above, was diluted in absolute alcohol and administered once a week in concentrations to give the following initial concentrations in the water:

Testosterone	No. Fish	Code
1: 4 X 10 ⁶	14	A
1: 4 x 10 ⁷	14	B
1: 4 x 10 ⁸	14	C
1: 4 x 10 ⁹	14	D
Normal	18	N
Sham op.	10	S

The larger number of normal fish was to compensate for the fact that immature stickleback are difficult to sex by eye, hence, in all probability some females would inadvertently find

TABLE II

Summary of Treatments and Numbers of Fish Used
in Experiment to Test Effect of Pituitary
Activity on "Displacement" Fanning

<u>Photoperiod</u>	<u>Operation</u>	<u>Hormones</u>	<u>No. of Fish Used</u>	<u>Code</u>
16 hr.	Normal	0	14	16 N
	Gonadect.	Testosterone	14	16 G
8 hr.	Normal	0	14	8 N
	Sham op.	0	14	8 S
	Gonadect.	Testosterone	14	8 Opit
	Gonadect.	Test. + LH	14	8 LH
	Gonadect.	Test. + LtH	14	8 LtH
	Gonadect.	Test. + Ant.Pit.	14	8Ant.Pit.

their way into the experiment. The lower number of sham operated fish was due to last-minute mortality; it was found that operated and sham operated fish would not stand full sea water for recovery, this late in the season (April) so 12 parts/thousand sea water was used for the last three days of recovery. Fish which built nests were then observed for 20 minutes each three days per week.

Kidney histology: at the termination of this experiment the kidneys of the experimental fish were fixed in Bouin's, dehydrated and imbedded in paraffin. Sections of 10 μ were mounted and stained in Ehrlich's haematoxylin and 2 per cent aqueous eosin solution. The height of granular epithelial cells of the conoaluted tubules was measured with an optical micrometer under high power (10x45).

Periodic observations on all fish were carried out to ascertain presence of nuptial colouring and sand digging.

3. Gonadectomy in the mature phase.

This experiment was designed to test the dependence of fanning, both with and without eggs, on gonadal hormones. For this purpose male stickleback were gonadectomized in the parental phase. The gonads are larger at this time and the fish bleeds more freely. So, modifications in the gonadectomy procedure were required: each opening was closed with one suture,

(surgical silk was used so removal of sutures was unnecessary) and the fish were placed in 12 ‰ sea water after the operation, then returned to their tanks several hours before the first post-operational observation on the day after the operation. Mortality was 50 per cent in spite of these precautions. The stickleback were observed for 20 minutes per day, from one day before gonadectomy in fish without eggs or the day after egg laying in those with eggs, until six days after gonadectomy. In fish with eggs the operation or sham operation was performed on the second or third day after the eggs were laid. After the experiment was terminated, surviving fish were examined for gonad regeneration. In those fish which died before examination the heat 23 - 24°C of the water led to rapid decomposition precluding accurate determination of regeneration.

RESULTS

I. THE EFFECT OF PROLACTIN ON FANNING

1. Effect of prolactin injection on parental fanning.

Due to the failure of most of the fish to raise eggs successfully no real conclusions can be drawn with regard to the effects of prolactin on parental fanning (Table III). The data suggests that a decrease may occur in the treated fish, but the difference is well within normal variation or could be due to the effects of injection as such, or the solvent.

2. Effect of prolactin on "Displacement" Fanning.

To obtain results that were more comparable average values were calculated for both percent of time spent fanning and average length of fanning bouts; this was done for all observations from the time of the second injection (injections every third day). By this time the two fish with eggs had either raised the young or the fish had died. It also gave some time for the hormone to act.

i) Percent fanning. Fish which received prolactin fanned less than those receiving a control injection of saline solvent (Figure 1). While both the numbers of fish and the observed differences were small, these results indicate that prolactin does not increase fanning, and may decrease the percentage of time spent fanning by males without eggs.

TABLE III

Effects of Prolactin in Aqueous Suspension
on Parental Fanning

Treatment	No. Fish Observed	No. Fert. Eggs	No. Raising Eggs Successfully	Av. % Fanning Days 1-8
LtH injection	6	4	1	25.74%
Water injection	5	5	0	0
Uninjected	10	6	1	34.47%

ii) Length of fanning bouts. Differences in the average length of fanning bouts paralleled those found in the average percent of time spent fanning (Figure 1). One cannot say from this experiment whether the change in bout length was a cause or a result of the difference in total fanning.

3. Effects of prolactin injection during the parental fanning cycle

Here each fish was injected once, on day two or three of its parental fanning cycle. It appears from the data in Table IV that prolactin increases percent fanning over that found in solvent injected normal fish, from day two through to day five. This is, however, due to individual variation within the small number of fish used. When the differences between individual fish before and after injection are compared (Table V) it appears that while both prolactin and solvent injections lead to increased fanning the increases are not significantly different, with the solvent leading to a slightly greater increase than the prolactin. Needle pricks alone did not lead to significant increases in fanning so the increases are presumably in response to the injection of saline.

Figure 1.

Effects of Prolactin on
Displacement Fanning

- 0 = mean % fanning
- Δ = mean length of fanning bouts
- (N) = number of observations
- σ = standard deviation of the mean
- I = 95% confidence intervals

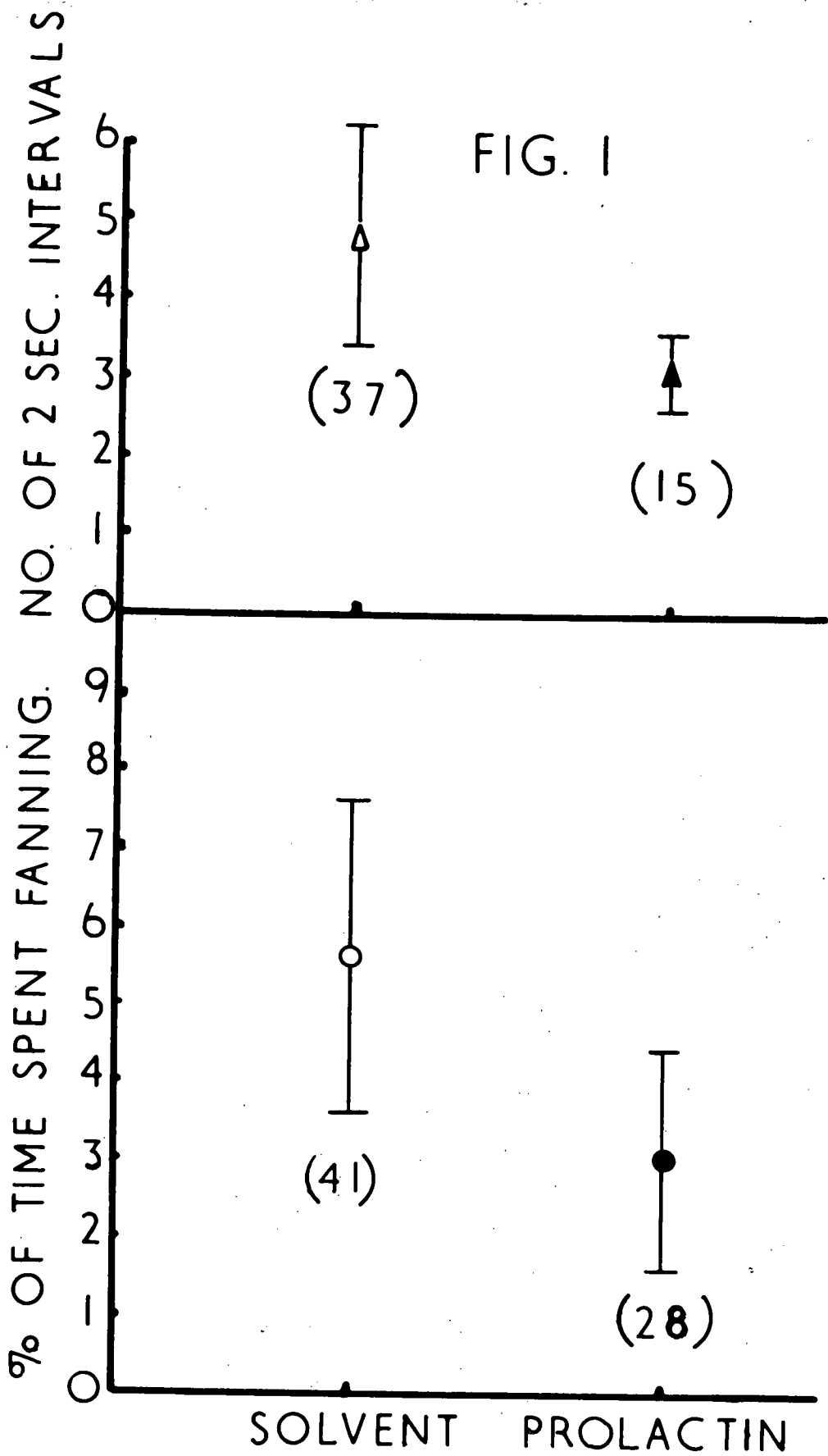


TABLE IV

Effect of Prolactin Injection on Parental Fanning
 Showing Means for Groups of Fish under Different
 Treatments

Day	Treatment	No. Obs.	Mean % Fanning
1	Normal	7	11.6
2	Normal	9	21.2
2	Prolactin	3	30.7
2	Solvent	2	18.0
3	Normal	6	23.8
3	Prolactin	2	30.9
3	Solvent	1	23.7
4	Normal	4	32.3
4	Prolactin	4	39.3
4	Solvent	2	29.8
5	Normal	3	45.1
5	Prolactin	3	34.5
5	Solvent	2	41.4
6	-hatching date		

TABLE V

Differences in Percent of Time Spent Fanning by Individual
Fish Before and After Injection with Prolactin and with
Kreb's Solution

Tank	P R O L A C T I N % of time spent fanning			Tank	S O L V E N T % of time spent fanning		
	Av. Before	Av. After	Diff.		Av. Before	Av. After	Diff.
40	18.1	25.9	+ 7.8	25	12.2	12.1	- .1
59	14.3	8.4	- 5.9	49	22.8	42.5	+14.7
49	28.2	33.9	+ 5.7	34	22.8	28.9	<u>+ 6.1</u>
39	23.2	41.2	<u>+18.0</u>				
		Av.	6.4			Av.	6.9

II. EFFECTS OF GONADAL AND PITUITARY HORMONES ON PARENTAL ACTIVITY IN THE MALE STICKLEBACK

1. Effect of photoperiod, pituitary and gonadectomy with replacement of testosterone

In this experiment fish were kept under 8-hour and 16-hour days while being subjected to gonadectomy and replacement with testosterone, and under 8-hours while being treated with crude anterior pituitary extract, luteinizing hormone and prolactin.

i) Nesting dates.

The dates on which fish completed nests were recorded (Figure 2). There were no obvious differences in the time from the beginning of the experiment to mean date of nest building between the different groups that built nests. Normal and sham operated fish under 8 hours did not build nests, indicating suppression of pituitary gonadotrophins in accordance with the findings of Baggerman (1957) and Hoar (1962).

ii) Percent fanning.

The differences found in the percent of time spent fanning are summarized in Table VI and Figure 3. The normal 16-hour fish fanned significantly (0.001) more than any of the other groups. Fish injected with pituitary hormones showed less fanning than 8-hour fish without pituitary

Figure 2.

Dates of Nest Building by Fish Under
16-hr. and 8-hr. Photoperiods with
Pituitary Hormones and Gonadectomy
with Replacement of Testosterone

0 = mean nesting date for group
| = range of nesting dates
(N) = number of fish

FIG. 2

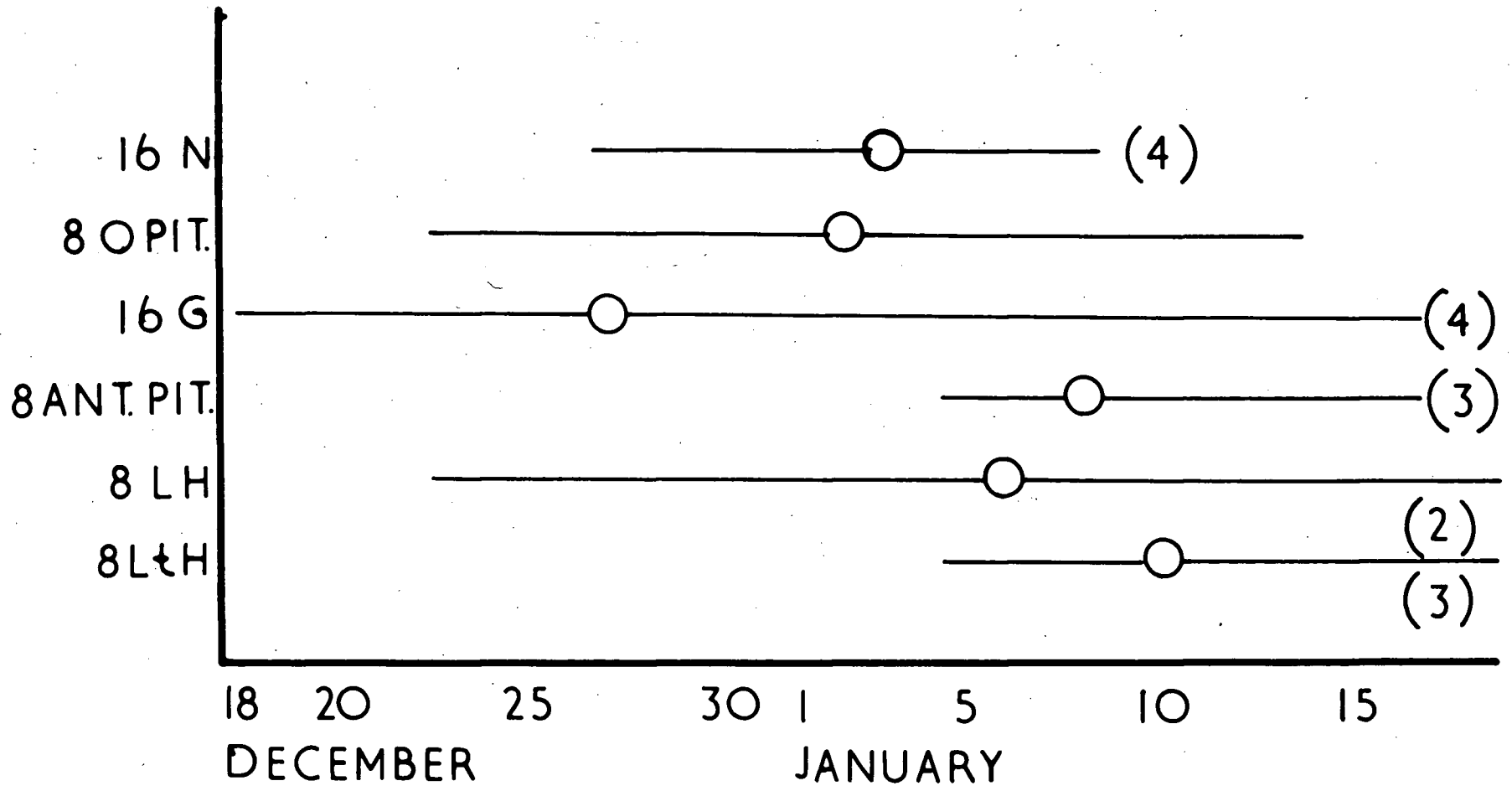


TABLE VI

Comparison of % Fanning 16-hr. and 8-hr.

Photoperiods and Hormone Treatments

(Opit = gonadectomy and testosterone replacement but no pituitary injection)

Photoperiod Hours	Treatment	No. Obs.	Mean % Fanning	t	p
16	Normal	29	4.10	5.19	0.001***
16	Gonadect.	30	0.47		
16	Normal	29	4.10		
8	Opit.	31	1.22	7.27	0.001***
16	Gonadect.	31	0.47		
8	Opit.	30	1.22	1.67	0.1
8	Opit.	30	1.22		
8	Ant. pit.	12	0.02	1.90	0.1
8	Opit.	30	1.22		
8	LH	23	0.12	2.34	0.05*
8	Opit.	30	1.22		
8	LtH	13	0.28	0.23	0.55
8	LtH	13	0.28		
8	LH	23	0.12	0.89	0.4
8	LtH	13	0.28		
8	Ant. pit.	12	0.02	1.24	0.2
8	LtH	13	0.28		
16	Gonadect.	31	0.47	0.54	0.5
8	LH	23	0.12		
16	Gonadect.	31	0.47	1.40	0.2
8	Ant. pit.	12	0.02		
16	Gonadect.	31	0.47	1.32	0.2

injections (but treated with testosterone) but not significantly less than 16-hr. gonadectomized fish which would presumably have high pituitary activity but otherwise be the same as the 8-hour fish. There were no significant differences between the three pituitary injections. These results suggest an inhibitory effect of the pituitary on fanning as well as an excitatory effect by the intact gonad which was not provided by the replacement testosterone.

iii) Length of fanning bouts.

In this experiment the length of the fanning bouts was essentially the same in both 16-hour groups and in the 8-hour 0 pituitary group but was significantly lower in the fish receiving prolactin and luteinizing hormone (Figure 4). Only one observation on fish receiving anterior pituitary extract showed fanning, too few for statistical analysis. The difference between the three groups of injected fish and the 16-hour gonadectomized group may indicate that the difference is due to the injection treatment rather than to the pituitary fractions.

2. Effect of a range of testosterone dosages

In this experiment the fish were all kept under 16-hour photoperiod. Normal and sham operated fish were compared with gonadectomized fish exposed to four different dosages of testost-

Figure 3.

Mean Percent of Time Spent Fanning
Under 16-hour and 8-hour Photoperiods
by Gonadectomized Fish with Testosterone
and Pituitary Hormones

○ = mean

□ = one standard deviation of the mean

⊥ = 95% confidence interval on the mean

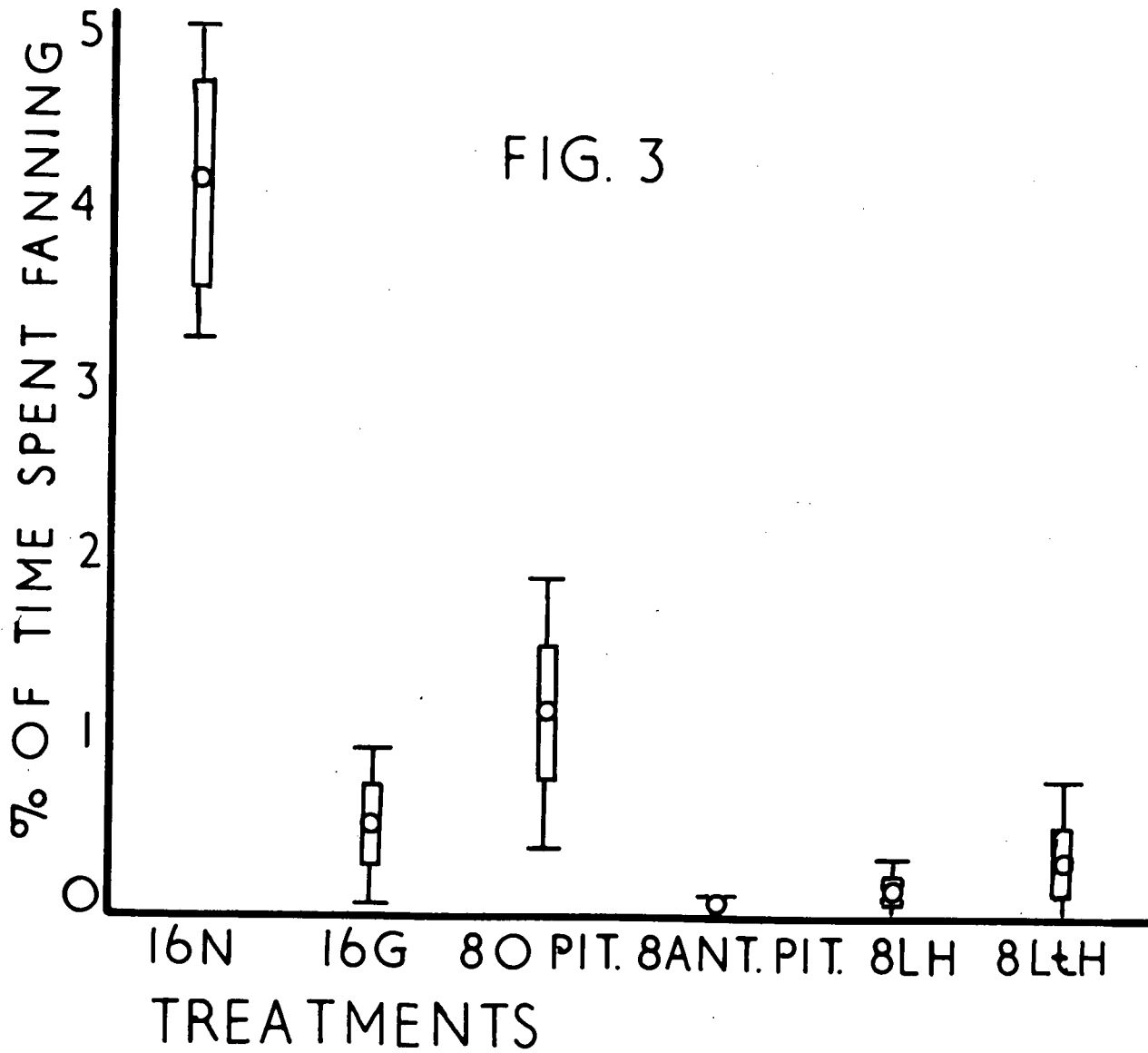


Figure 4.

Comparison of Mean Length of Fanning Bouts Under
16-hour and 8-hour Photoperiod with Injection of
Pituitary Hormones and Gonadectomy with Replacement
of Testosterone




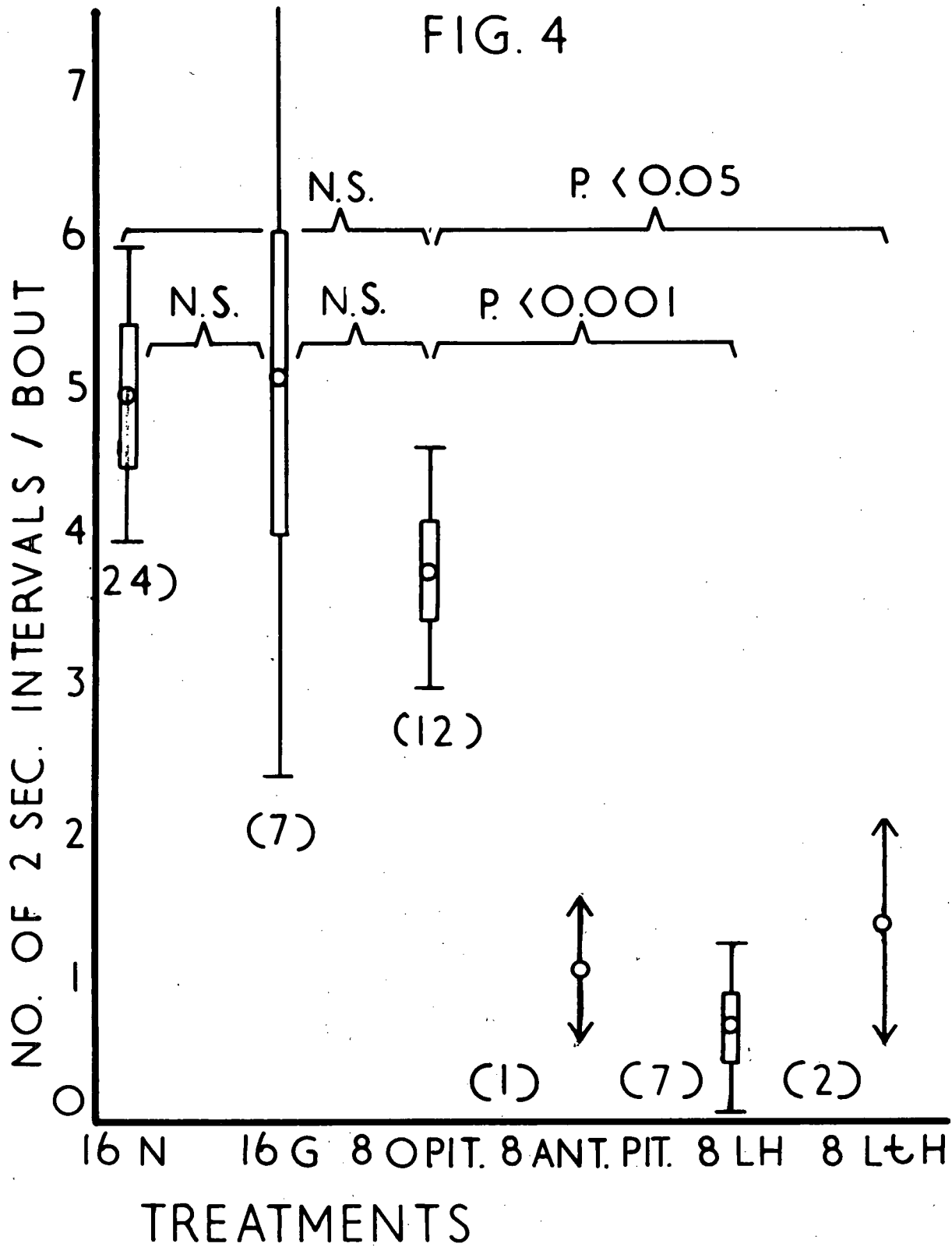
- 0 = mean
-  = standard deviation of the mean
-  = 95% confidence interval on mean
-  = significance of difference
between bracketed means on a
t test
- (N) = number of observations

FIG. 4



erone ranging from $1:4 \times 10^6$ to $1:4 \times 10^9$. Fish that built nests were observed three times a week for 20 minutes each day.

i) Nesting dates.

The dates on which fish completed nests under the different treatments were recorded and compared. - Only normal, sham operated and fish in the two highest dosages of testosterone (A and B) built nests. A clear separation between the fish treated with testosterone and the fish with intact gonads (normal and sham operated fish) is shown in Figure 5. This may have been due to the treated fish receiving a relatively high amount of testosterone as soon as treatment started whereas it took some time for the long photoperiod to stimulate hormone production in the gonads of the normal and sham-operated fish.

ii) Colouration and sand digging.

Periodic checks were made on the colouration of all the fish in this experiment. At the same time the amount of sand digging as shown by the sand in the aquarium was noted. Table VII shows that there were no clear trends and that a majority of fish in all groups showed nuptial colouration, both blue eyes and red throat throughout most of the experiment. This would seem to indicate that the lowest testosterone dosage given ($1:4 \times 10^9$) was more than sufficient to cause these colour changes. The colouring of the male stickleback has definitely

Figure 5.

Mean Nesting Dates of Stickleback in a
Range of Testosterone Concentrations

N = normal unoperated

S = Sham operated

A = $1:4 \times 10^6$ Testosterone

B = $1:4 \times 10^7$ Testosterone

O = mean, corrected to
nearest day

σ = standard deviation
of mean

\bar{I} = 95% Confidence
intervals on mean

$\underbrace{\quad}$ = significance of
difference between
bracketed means on
t test

(N) = number of observations

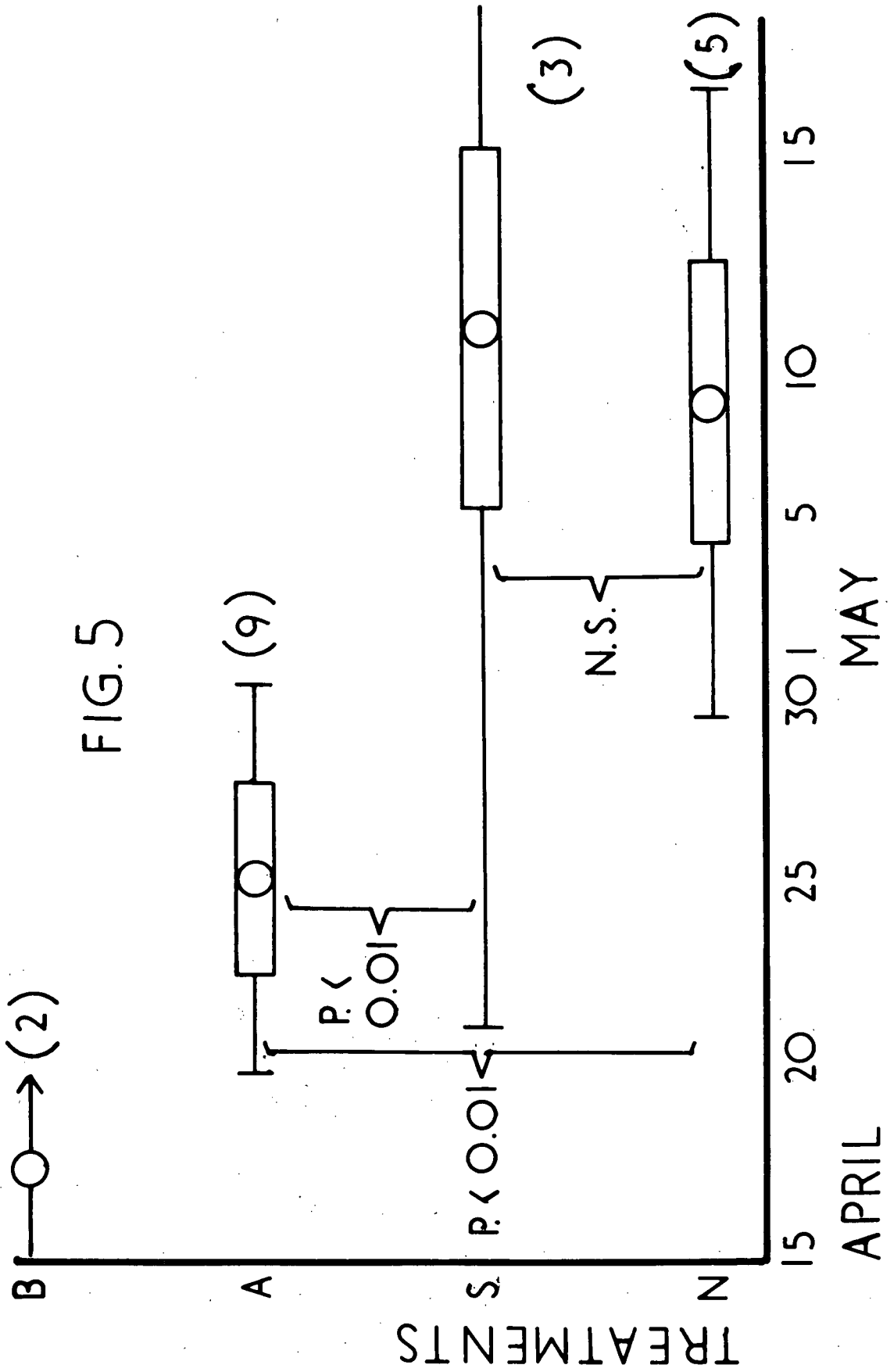


TABLE VII

Percent of Surviving Fish Which Showed Nuptial
Colouration with a Range of Testosterone Dosages

	<u>April 11</u>		<u>April 29</u>		<u>May 20</u>		<u>May 27</u>	
	eyes	throat	eyes	throat	eyes	throat	eyes	throat
N	23%	7%	36%	27%	62%	62%	88%	100%
S	43%	14%	43%	43%	60%	60%	80%	60%
A	52%	48%	69%	81%	80%	80%	67%	67%
B	38%	31%	55%	45%	80%	80%	100%	100%
C	67%	25%	83%	42%	71%	42%	67%	67%
D	36%	43%	50%	43%	100%	56%	88%	88%

been shown to be under control of gonadal hormones (Ikeda, 1933).

In sand digging, a behaviour pattern which is part of agonistic behaviour as well as nest building, was more frequent although not significantly so in group C fish than those in group D.

iii) Percent of time spent fanning.

The average percent of time spent fanning by fish with intact gonads (normal and sham-operated) and gonadectomized fish treated with $1:4 \times 10^6$ testosterone are compared in Figure 6. No fanning was observed by fish in lower concentrations of testosterone. The stickleback in testosterone fanned significantly less than those with gonads, though the difference was not so great as in the previous experiment (Figure 3.) where the 16-hour normal and 16-hour gonadectomized fish are comparable to normal and group A fish in this experiment. The difference between the results of the two experiments lies in the higher fanning by 16-hour normal fish in the first experiment; the fish in $1:4 \times 10^6$ testosterone fanned at essentially the same levels in both experiments. The difference is, therefore, probably due to a differential stimulus to the gonads of the normal fish, in spite of the fact that both sets of fish were kept in the same room, under the same photoperiods and temperatures although at different seasons (Experiment II(1) December-January, Experiment

II(2) April-May). This conclusion is further supported by the onset of nest building in Experiment II(2) normal fish which was later relative to testosterone treated fish than in Experiment II(1).

iv) Average length of fanning bouts.

There were no significant differences between the lengths of the fanning bouts in the three groups of fish that built nests and fanned (figure 7.).

v) Histology of kidney tubules.

Kidneys from two fish under each treatment were preserved in Bouin's solution then sectioned and stained. The height of the granulated cells in the convoluted tubules of the stickleback kidney has been shown (Wai and Hoar, 1963) to be dependant on testosterone dosage. So, this procedure was used to check on the dosage received by fish under different treatments. Fish in the different concentrations of testosterone did respond differently in the manner to be expected (Figure 8.). The fact that fish with intact gonads showed somewhat lower cell height than those in $1:4 \times 10^6$ of testosterone although they fanned more indicates that fanning is not entirely dependent on testosterone level, or that the synthetic testosterone does not duplicate natural product from the fish.

Figure 6.

Percent of Time Spent Fanning in a Range
of Testosterone Concentrations (16-hr.)

N = normal unoperated fish


S = Sham operated

A = $1:4 \times 10^6$ Testosterone conc.

O = mean

= one standard deviation of
the mean

I = 95% confidence
interval on the
mean

 = probability difference
in means not due to
chance, t test.

(N) = number of
observations

FIG. 6

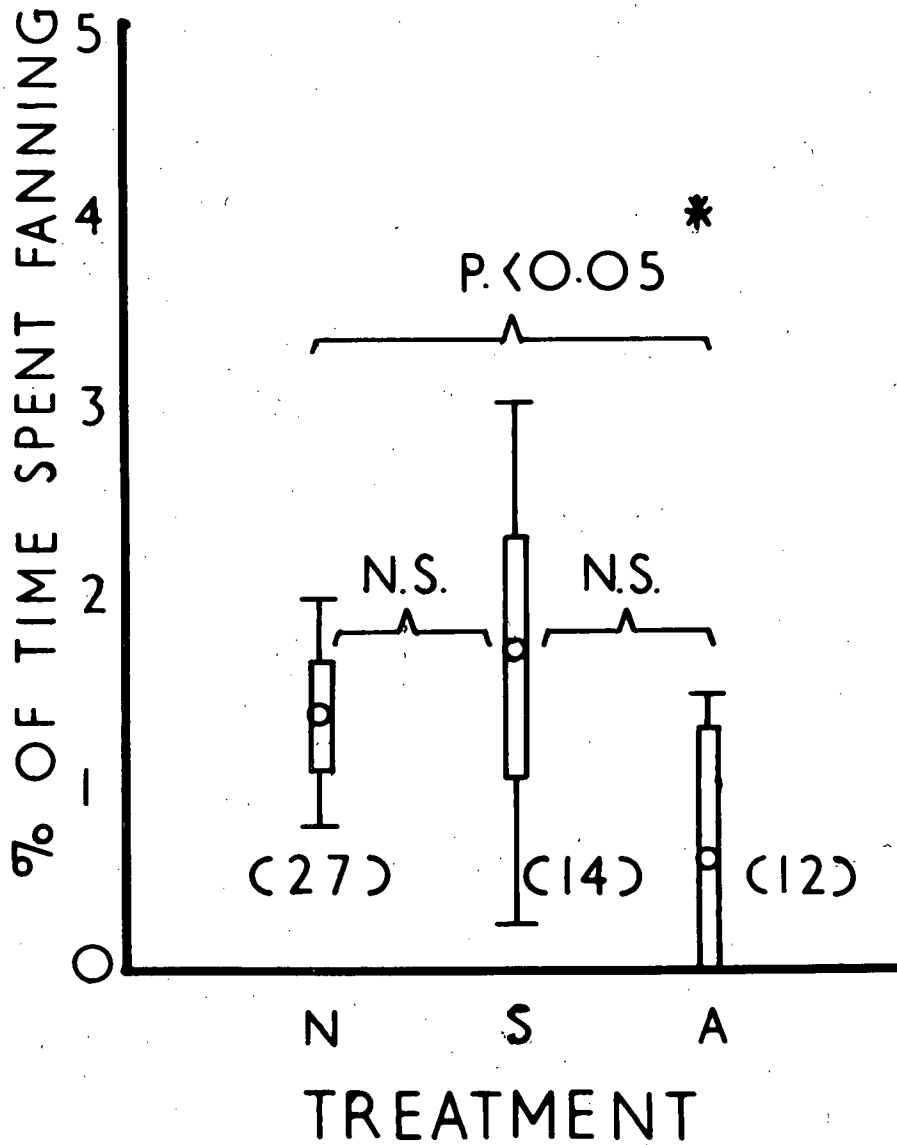


Figure 7.

Average Length of Fanning Bouts in a Range
of Testosterone Concentrations


- N = normal unoperated fish
- S = Sham operated
- A = $1:4 \times 10^6$ Testosterone concentration
- O = mean
- = one standard deviation of the mean
- I = 95% confidence interval on the mean
-  = Probability difference in means not due to chance
- (N) = number of observations (only those which showed fanning were used)

FIG. 7

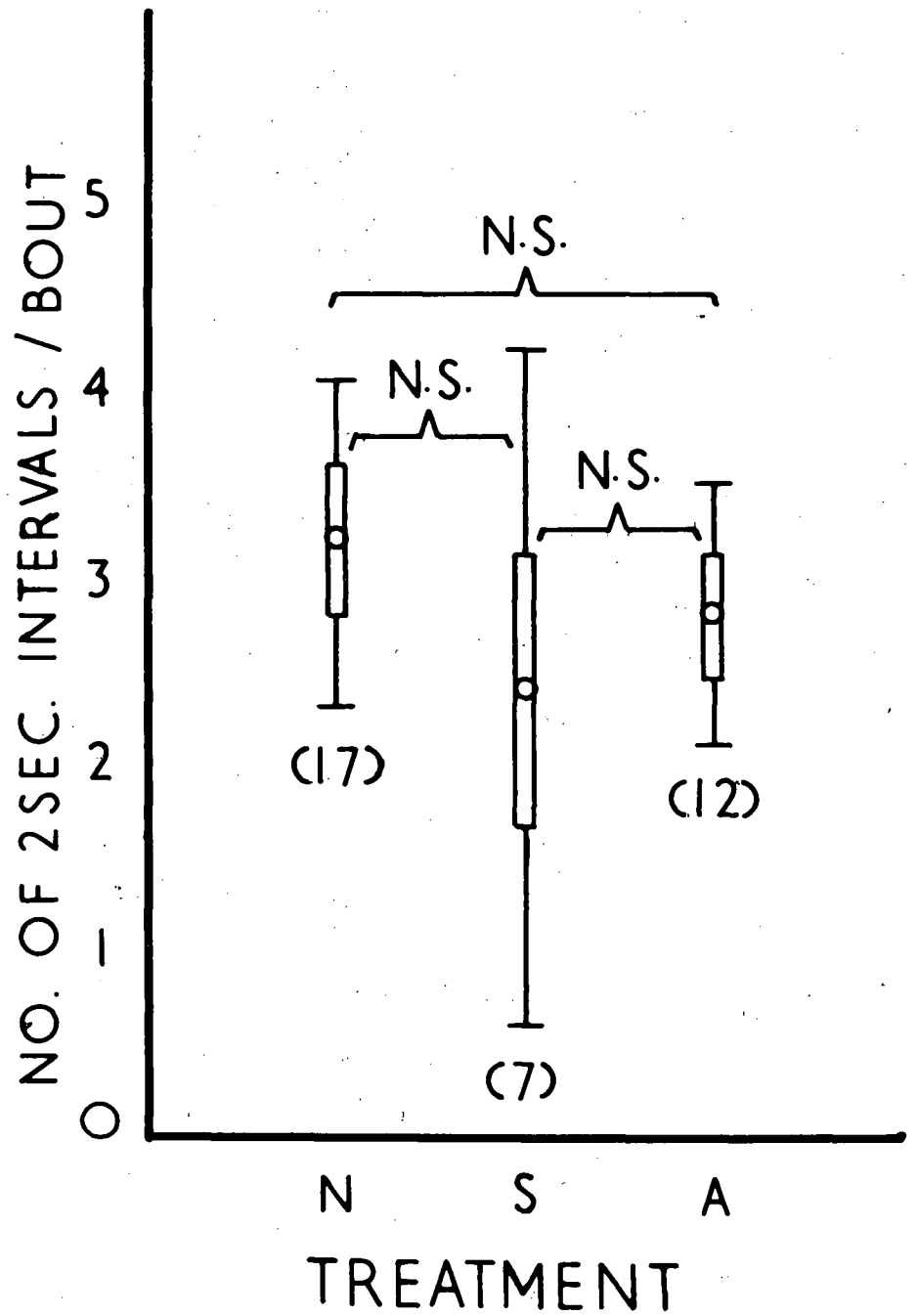


Figure 8.

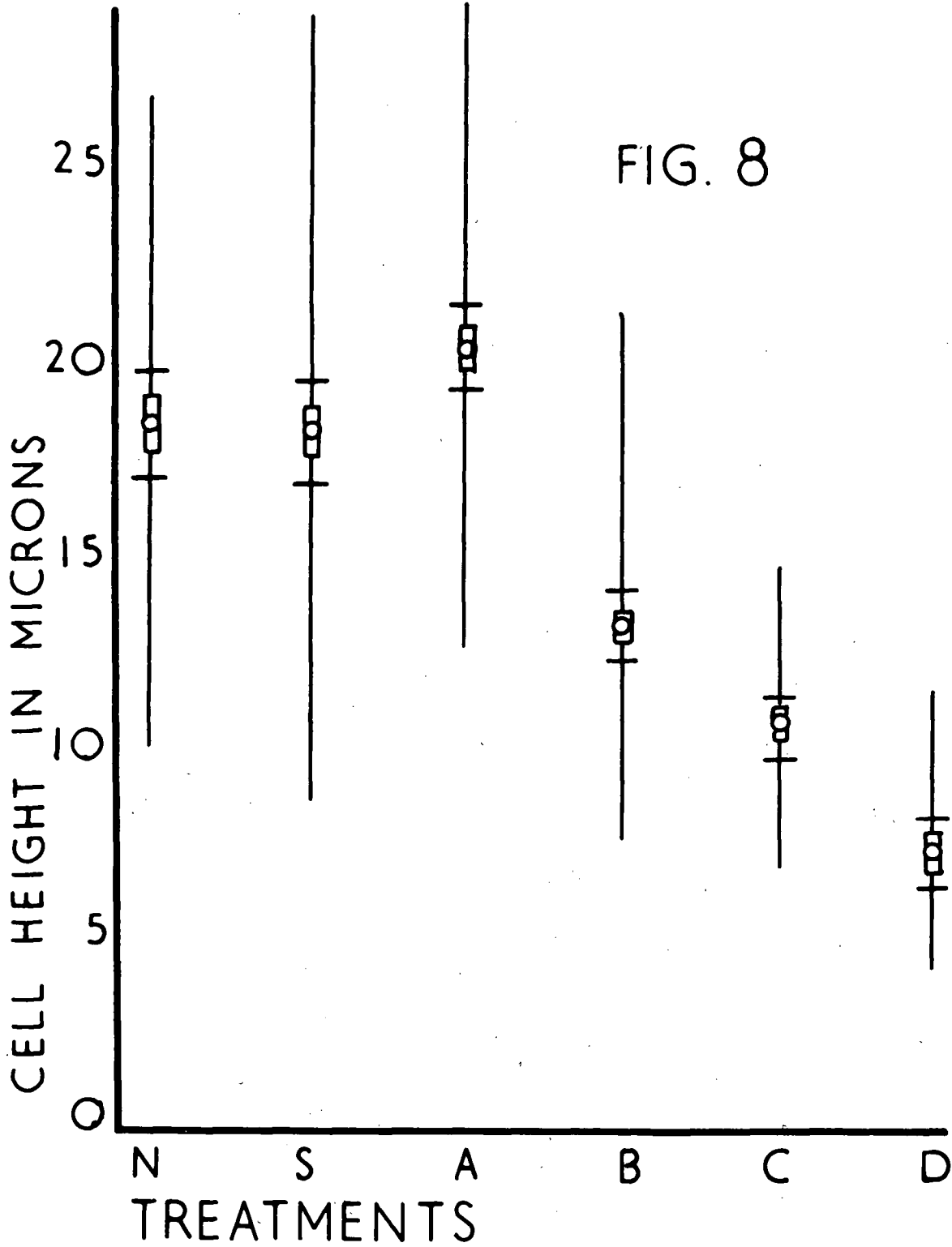
Average Heights of Granular Cells in Kidneys of Male
Stickleback Treated with Four Concentrations of
Testosterone, Normal and Sham Operated Fish

○ = mean

□ = standard deviation of the mean

⊥ = 95% confidence interval

| = range



3. Effects of Gonadectomy in the mature phase

Stickleback with eggs were gonadectomized or sham operated on the second or third day of their egg raising cycle, fish without eggs were observed then gonadectomized on one day then observed the next and following days. ↙

i) Occurance of fanning.

Baggerman (1957) quotes van Iersel and Burggraaf (personal communication) as saying that stickleback gonadectomized while raising eggs continue "normally though at a lower intensity" and that stickleback gonadectomized in the mature phase, with a nest built without eggs, do not fan. The occurrence of fanning in the current experiment is summarized below:

Out of 9 fish gonadectomized with eggs and living over 6 days, 8 removed eggs on day 3-4 from nest and essentially stopped fanning. 1 fanned at low level 5 days then stopped, eggs in nest but undeveloped.

Out of 3 fish with sham operation with eggs and living over 6 days, 3 fanned through to day 6 or 7.

Out of 7 fish gonadectomized without eggs and living over 6 days, 1 never fanned again; 4 stopped fanning after 2 days; 1 fanned for four days; 1 fanned for six days.

ii) Percent of time spent fanning.

The average amounts of time spent fanning under the different treatments are summarized in Table VIII and illustrated with confidence intervals in Figure 9. The main points to be noted are that: a) fish gonadectomized while raising eggs fanned much less than sham operated fish even during the second and third days when their eggs were still in the nest; b) from the fourth day on the fanning of fish gonadectomized, with eggs, was not significantly different from the very low level found in fish gonadectomized without eggs, and c) although fanning in the sham operated fish was much higher than in gonadectomized fish up until the seventh day (after the hatching date of the eggs) it was still well below that expected of a normal fish, from the second day on (van Iersel, 1953).

iii) Length of fanning bouts.

There were ^t too few observations with fanning to ^o draw meaningful conclusions about differences in bout length. Scanning Table VIII indicates they were probably not different.

TABLE VIII

Average Percent of Time Spent Fanning and Average Lengths of
Fanning Bouts in Fish Gonadectomized or Sham Operated in

Mature Phase

Gonadectomy + performed
- not performed
Eggs + present
- not present

(eggs laid on day 0 in fish with eggs)

Day	Gonadect.	Eggs	No. of Observations	Mean % Fanning	No. of Observations	Mean Bout Length
1	-	+	25	9.21	25	5.2
1	-	-	9	4.82	9	5.6
2	-	+	3	23.48	3	5.5
2	+	+	9	4.49	7	7.5
2	+	-	7	0.67	4	3.4
2	S	+	3	23.97	3	6.9
3	+	+	9	1.08	6	4.8
3	+	-	7	1.14	5	4.8
3	S	+	3	21.73	3	9.7
4	+	+	9	0.51	3	5.7
4	+	-	7	0.83	2	12.9
4	S	+	3	17.52	3	10.4
5	+	+	9	0.17	1	9.0
5	+	-	7	0.45	2	9.5
5	S	+	3	17.19	3	6.0
6	+	+	9	0	0	0
6	+	-	7	0	0	0
6	S	+	3	16.61	3	5.5
7	+	+	9	0.02	1	0.9
7	+	-	7	0.14	1	5.7
7	S	+	3	1.46	1	6.5

Figure 9.

Average Percent of Time Spent Fanning on Consecutive
Days After Gonadectomy or Sham Operation by
Fish With and Without Eggs

○ Δ = mean

⊥ = 95% confidence intervals

With Eggs

- = normal
- ⊙ = sham operated
- = Gonadectomized

Without Eggs

- ▲ = normal
- △ = Gonadectomized

% OF TIME SPENT FANNING

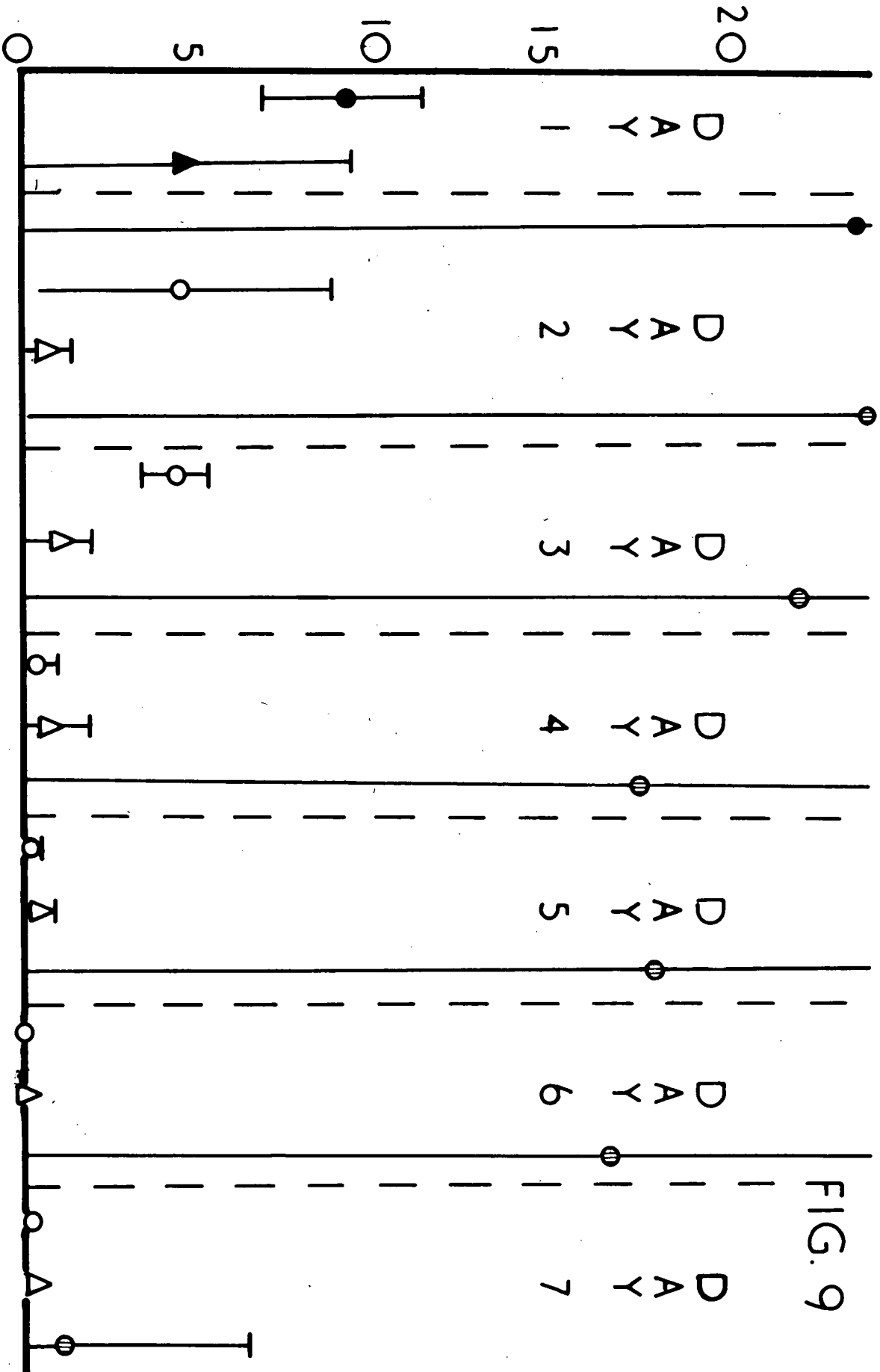


FIG. 9

DISCUSSION

I. PROLACTIN

There was good reason to think as did Baggerman (1957) that the fanning of the male stickleback might be influenced by Prolactin. This anterior pituitary hormone is associated with parental care and more specifically with maternal care throughout the vertebrates.

Prolactin (luteotrophic hormone, LTH) is a protein hormone secreted by the anterior pituitary in teleosts, birds and mammals (Beach, 1948). It has been linked with parental behaviour and physiology in several widely separate groups, as well as with an assortment of other factors e.g. migration to water in Diemyctilus viridescens (Grant and Grant, 1958), melanophore control in fish (Pickford and Kosto, 1957) or osmoregulation of Myxine (Chester-Jones et al, 1962). Prolactin's effect on parental phenomena seems to be elicited two ways:

1. inhibition of reproductive behaviour and physiology during the brooding phase;
2. stimulation of parental aspects of physiology and behaviour.

Aggression, controlled by gonadal hormones, is an integral part of the mating behaviour of many animals, usually it is directed against members of the same sex and juveniles of

the species. Thus when an animal is in its mating phase it may attack and kill young animals. This tendency must be suppressed after mating if the animal is to raise its own young successfully. A substance, like prolactin, which tends to suppress gonadotrophic activity will aid parental care. In the cock Nablvandov (1945) found that prolactin increased broodiness, but so did other gonadal inhibitors such as blindness and alcohol. Apparently prolactin merely inhibits F S H secretion, it does not seem to cause broodiness but to facilitate it by repressing sexual aggressiveness. Riddle (1938) found a similar FSH inhibiting action in pigeons but prolactin also led to increased parental behaviour. So too with the Jewel Fish where prolactin and gonadal hormones seem to work antagonistically. Castration greatly facilitates the induction of brooding behaviour by prolactin and this effect is nullified by injection of FSH into an intact fish (Noble et al, 1938).

Noble et al (1938) showed that prolactin was the most effective of several hormones in stimulating brooding behaviour in the Jewel Fish, Hemichromis bimaculatus. This fish builds a nest, fans the eggs to aerate them and herds its young for a while after hatching, failure to attack young fish was taken to indicate "broodiness". It is not clear whether other components of brooding behaviour were influenced or not. It must be noted that other substances including proluton, corpus luteum extract,

antuitrin-S, phenol, thyroxin, sodium hydroxide and alcohol also produced broodiness in some fish. Whether these act through prolactin release, an unknown hormonal factor, or the central nervous system was not investigated. No experiments on hypophysectomized fish were carried out. More recently Fiedler (1962) has shown that prolactin induces fanning even in the absence of a nest in the Wrasse Crenilabrus ocellatus. In still another teleost the Discus Fish, Symphysodon discus, a mucous secretion "discus milk" is produced by the skin and fed to the fry, this seems to be stimulated by prolactin (Egami and Ishii, 1962). An interesting effect is described by Ishii (1960, 1961). Apparently prolactin is important in maintaining gestation in viviparous teleosts, Ditrema and Neoditrema as well as in the top-minnow Gambusia. He also reported (in Egami, 1962) that prolactin showed no observable effect on the gonad or its "corpora lutea" in Lebistes reticulatus.

So, we have a hormone which, if we accept the idea that the same basic hormones have been adapted to different functions in the course of evolution (Medawar, 1953), has controlled functions facilitating care of the young in a wide area of the vertebrate subphylum (Eisner, 1961, Lehrman, 1961). It has also been related to other functions such as osmoregulation or water need in Fundulus (Pickford and Atz, 1957) and Diemyctilus (Grant and Grant, 1958) and melanophore contraction in fish (Pickford and Atz, 1957). Once a hormone has acquired some

control of one phase of life e.g. parental reactions, it seems logical that later developments in the physiology and behaviour of this phase might fall under its control e.g. corpus luteum and mammary gland. Even the sketchy reports of osmoregulatory and chromatophore effects might be correlated to this when we remember the importance of osmotic medium and colour change in the reproduction of many fish and amphibians. Although prolactin-like substances have been found in many lower vertebrates e.g. fish (Pickford and Atz, 1957), relatively few investigations of its function have been carried out. And, in many of these investigations mammalian prolactin was used though it is not clear whether or not the hormone is chemically similar in all groups (Gerschwind, 1958).

Prolactin has been related to parental behaviour in two fish (see above); in fact, it is the only hormone which has actually been linked to a parental behaviour pattern such as fanning in a teleost. The relationship between prolactin and parental behaviour is, however, a tenuous one. In the Jewel Fish where the effect seems to be antagonistic, many other substances will elicit very nearly the same effect. In the wrasse, the only other fish in which a behaviour change associated with prolactin has been found, it seems fairly clear-cut although the dosage level used, 1 to 6mg. for fish with an

average length of 8.4 cm. was high. Dosages of this order proved fatal to stickleback. The high dosage required may, of course, reflect an insensitivity of the wrasse to mammalian prolactin.

It must also be noted that prolactin, like other pituitary proteins, is not a pure substance. Feidler's (1962) hormone may have acted through the effect of a contaminant, or through feed-back from another gland.

It seemed that prolactin was the most likely endocrine candidate for control of the fanning in the male stickleback. Baggerman (1957) quotes van Iersel and Burggraaf (personal communication) as finding that when males castrated in the sexual phase were given eggs they started a normal fanning cycle though at a somewhat lower level, thus showing the gonads were not essential for parental fanning. Baggerman's own results confirmed this, and she speculated that parental behaviour might be "induced and maintained by prolactin secreted by the pituitary." The finding of van Iersel (1953) that a peak in fanning was noted on the normal hatching date even when the eggs were removed from the nest of the male stickleback, indicated the presence of an "internal factor" controlling in part, the expression of the fanning cycle. This also encouraged the view

that a hormone controlled fanning. On these bases, experiments were undertaken to examine the effects of prolactin on fanning in the male stickleback. Male fish with eggs were given a series of injections of prolactin at what seemed to be a reasonable, physiological dosage based on studies on small mammals. If prolactin maintained the fanning cycle there would be a higher level of fanning shown in those fish receiving prolactin than in uninjected fish or those injected with saline. Mortality of eggs and fish invalidated the results of this experiment (only one prolactin and one normal fish yielded results, the normal fish fanning more than the prolactin injected fish). Next, stickleback without eggs were given a series of prolactin injections. If prolactin induced the parental cycle then it should cause an increase in the fanning of fish without eggs to a level above the normal "displacement" fanning. This time the experiment went well but again prolactin showed no excitatory effect on fanning. The hormone injected fish fanned less than solvent injected fish (Figure 1). It was thought that this might be due to a deleterious effect of frequent injection, too low a dosage or the need of some "priming" effect of the eggs (Sevenster-Bol, 1962). Therefore fish were given a higher dosage in a single injection after they had begun to raise eggs. This did cause increased fanning

but not as much of an increase as injection of the solvent used to dissolve the hormone alone (Table V). Later in the course of an experiment designed to test several factors, prolactin was given in a series of injections to fish under 8-hour days which were brought into breeding condition with testosterone. It was hoped that with the pituitary suppressed by the short photoperiod, any effects of the injected prolactin which might have been masked by the active pituitary in earlier experiments would show up. As before, prolactin injected fish fanned at a lower level than control fish. If one were forced to form a conclusion from these experiments on the effect of prolactin on fanning, it could only be that prolactin has a slightly inhibitory action on parental and "displacement" fanning the male stickleback. The work of Feidler (1962) on the wrasse Crenilabrus ocellatus, of course, shows that this conclusion can not be generalized to include other fish which show parental fanning. It can also be pointed out however that Noble et al (1938) report no effect of prolactin on the fanning of the Jewel Fish, although prolactin did seem to influence the "broodiness" of the fish towards its young. Possibly in the stickleback too prolactin is related to the short period of care for the newly hatched young, rather than to the fanning behaviour.

II. PITUITARY AND GONADAL HORMONES

The results of the experiments with prolactin raised several questions about the hormonal control of parental behaviour in the male stickleback. Is fanning under the control of the pituitary? Are gonadal hormones necessary for its expression? Or, does removal or inhibition of the gonads lead to an increase in parental behaviour as in the Jewel Fish (Noble et al 1938) or the rooster (Nalvandov, 1945)?

The first question could be answered most satisfactorily with hypophysectomized animals. Hypophysectomy of male stickleback could not, however, be performed with sufficient recovery to permit observations. Therefore photoperiod was used to achieve "physiological hypophysectomy" by keeping fish under alternating periods of 8-hours of light and 16-hours of darkness. This photoperiod has been found by Baggerman (1957) and Wai and Hoar (1963) to suppress development of mating behaviour and aggression in male stickleback, presumably by suppressing the secretion of pituitary gonadotrophins. This was further confirmed in the current experiment by the failure of fish with intact gonads (both unoperated and sham operated) to reach maturity under 8-hour days. Gonadectomized fish under 8-hour days were treated with methyl testosterone to bring them into breeding condition, their levels of "displacement"

fanning were then compared to those of fish gonadectomized and treated with testosterone in the same way, but held under 16-hour days. These fish were gonadectomized to prevent the effect of pituitary activity on the gonads from influencing the results. In this way both groups of fish received the same amount of testosterone presumably only the activity of the pituitary gland itself was changed. The results (Figure 3.) show that fanning was performed less (though not significantly) in the fish under 16-hour days. Apparently without the benefit of the intact gonad, the pituitary does not stimulate increased fanning. This is indicated too in the same figure by the fact that three groups of 8-hour fish, which differed from the two above groups only in that they received injections of pituitary fractions, crude anterior pituitary extract, luteinizing hormone (thought to increase aggression in sticklebacks; Wai and Hoar, 1963) or prolactin, also showed generally lower levels of fanning. The higher amount of time spent fanning by normal fish under 16-hour days may indicate an output of hormones by the pituitary stimulated gonads that was higher than the amount of testosterone picked up from the medium by treated fish, or it may indicate a difference in activity between the hormones of the intact gonad and methyl testosterone.

From the above experiment it seemed logical to investigate the relationship between fanning and the gonads. Unfortunately, the next experiment was planned with a pituitary effect rather than a gonadal one in mind. Gonadectomized fish under 16-hour photoperiods were placed in a range methyl testosterone concentrations from $1:4 \times 10^6$, the same as the previous experiment, down to $1:4 \times 10^9$. This experiment was intended to produce two or more groups of fish which built nests but had different levels of gonadal hormone. If, as in the case of the Jewel Fish, (Noble et al 1938) the gonadal hormones were antagonistic to parental care then there should be more parental care shown in the group with the lower testosterone dosage.

Two such groups were produced. As would be expected from the results of previous experiments, no increase in fanning due to unmasking of a pituitary effect was found. Instead the group in the lower concentration showed no fanning while those in the higher concentration did show fanning, though less than that shown by 16-hour normal fish (Figure 6.). If testosterone dosage above, as well as below those used previously had been used, a clearer trend might have been found in the relationship between testosterone and fanning.

One interesting result of experiment was the fact that the lowest concentration (1/1000th of the highest) still led to a high degree of colouration and to some sand digging; the next

highest concentration led to more sand digging, the higher one than that to some rough nest building and the highest level to well formed nests and "displacement" fanning. This suggests that the temporal relationship of some components of the sexual and breeding behaviour i.e. colouration, sand digging, and nest building and displacement parental behaviour, may be due to a gradual increase in production of gonadal hormones as the season progresses.

The results of the experiment above indicated that fanning might be under the influence of testosterone, despite sketchy reports to the contrary (van Iersel and Burggraaf, Baggerman, 1957) which give no indication of the number of fish used, the conditions of the experiment or the levels of "parental behaviour" obtained.

Males with and without eggs were gonadectomized in the mature phase and compared with sham operated fish (Figure 9). It was found that removal of the gonads led to a very marked decrease in both parental and "displacement" fanning, and to removal of eggs from the nest by a majority of the fish gonadectomized with eggs in the nest. Fanning occurred occasionally even in fish without eggs, as differing from the results of Hoar (1962) though it was very low, 1.0 percent or less, and decreased gradually over the seven days or less. The longer

observation period (20 minutes rather than 10 minutes used by Hoar, 1962) probably accounts for the observation of this very low frequency of fanning during the first few days after gonadectomy. The decline in fanning after the third day in fish which initially had eggs may be in part due to the lack of stimulus from the eggs after the fish had removed them from the nest. This is indicated by the one gonadectomized fish which did not remove its eggs; it continued to fan at a low level for the full seven days. The removal of the eggs in the first place may be directly due to the absence or reduction of gonadal hormones, or a secondary effect of death of the eggs due to insufficient aeration. Under the conditions of this experiment, eggs left in a nest after the male had died failed to develop. In any case it is plain the gonadal hormones are required for a normal level of fanning, although the behaviour pattern itself can occur in the absence of gonads. This result does not necessarily show that testosterone has a direct effect on fanning, it may well be that fanning does not normally occur unless the fish is confronted with the stimulus of the nest, nest entrance or eggs. If testosterone controls nest building activity, (Wain and Hoar, 1963) it is possible that with less testosterone the fish approaches the nest less often thus finding itself in the "releasing situation" for fanning less

frequently. In the case of egg rearing fish, the lower level of fanning would retard or prevent development of the eggs, this would decrease the effectiveness of the releasing situation and further decrease fanning. It is plain only that under normal circumstances the gonadal hormones are necessary for a normal fanning cycle. There is no indication of a separate controlling mechanism for parental and "displacement" fanning.

One line of reasoning may indicate that testosterone is more likely to increase parental behaviour in teleosts, and particularly in the stickleback, than it is in birds or mammals. Testosterone is a male hormone. In a majority of birds and to an even greater extent in mammals parental care is the prerogative of the female (Lehrman, 1961). The male seldom helps to care for the young and in some cases in birds where the male does help, this is accompanied by decreased development of the testis at the time of parental activity (Lehrman, 1961). In teleosts, on the other hand, parental behaviour is most often carried out by the male. Barlow (1964) suggests that this tendency may have evolved from a situation in which a territorial male continued to defend his territory for the purposes of further breeding, after the fertilized eggs of the first mating were present in the territory. These eggs might later become the focus of the territory, any increase in care

or protection of the brood giving a selective advantage. In stickleback in particular, aggression which often must be suppressed in male animals before they can show parental behaviour, is under the influence of the pituitary (Hoar, 1962) rather than the gonad. A relationship between testis and parental care seems more likely where parental behaviour is the prerogative of the male and aggression is related to pituitary rather than gonadal activity as in the stickleback.

It has already been shown (Hoar 1962) that the gonadal hormone is probably the major factor involved in nest building and sexual behaviour. However Sevenster-Bol has shown that the sight of eggs leads to a short temporary decrease in sexual activity (zig zagging) in the male stickleback and van Iersel (1953) reported long range decline in sexual activity with increasing number of broods. The relationship between sexual behaviour and fanning seems to be mutual inhibition (van Iersel 1953). Wai and Hoar (1962) showed that both testosterone and male genetic background were necessary for sexual behaviour, although they did not demonstrate a quantitative relationship between testosterone dosage and intensity of sexual behaviour. The experiments reported above in this paper seem to show that testosterone is required for normal expression of fanning. The only explanation which seems to reconcile these two results

with the mutual inhibition found by van Iersel (1953) would be that testosterone raised the general reproductive activity of the fish. This activity would then take the form most appropriate to the stimulus situation in which the fish found itself. If there were no eggs present sexual activity would occur, increasing stimulus from eggs would lead to higher parental activity and lower sexual activity in a relationship resembling competitive inhibition.

SUMMARY

The relationships between fanning and prolactin and testosterone in the male stickleback were investigated. The results are summarized below.

1) Prolactin administered in a series of injections to fish in the "displacement" fanning phase did not lead to an increase in fanning but rather to a slight decrease in percent of time spent fanning and in the length of fanning bouts, indicating prolactin alone does not initiate "parental" fanning.

2) Prolactin administered in a single injection during the parental fanning cycle did not lead to an increase in fanning greater than that caused by injection of the solvent in which the prolactin dissolved, indicating that the level of parental fanning is not directly related to the level of prolactin in the fish.

3) Fish gonadectomized under 8-hour photoperiod fanned more than gonadectomized fish under 16-hour photoperiod which received the same concentration of testosterone. Injection of pituitary extracts into fish under 8-hour photoperiod reduced fanning to levels comparable to those under 16-hours, indicating that the effect of the pituitary on fanning may be slightly inhibitory.

4) In stickleback which were placed in a wide range of testosterone concentrations under 16-hours only fish in the two highest concentrations built nests. Of these only those in the highest concentration fanned, although less than normal fish, indicating that a relatively high concentration of testosterone is required for normal fanning. Measurement of height of kidney cells showed that fish in the highest testosterone concentration had higher mean cell height than the normal fish in this experiment indicating that methyl testosterone is not as effective in replacing the behavioural effects of the gonad as it is in replacing the effect on cell height in the kidney.

5) Gonadectomizing male stickleback in the "displacement" fanning and parental fanning stages led to a substantial decrease in fanning as compared with sham operated fish, although in neither group was fanning completely eliminated. Gonadal hormone may be necessary for normal fanning levels, but not for the expression of fanning as such.

BIBLIOGRAPHY

- Baerends, G. P. and J. Baerends van Roon (1950). An introduction to the ethology of Cichlid fishes. Behaviour, Suppl., 1:1-243.
- Baggerman, B. (1957). An experimental study on the timing of breeding and migration in the three-spined stickleback (Gasterosteus aculeatus L.) Arch. Neerl. Zool. 12:105-317.
- Barlow, G. W. (1964). Ethology of an Asian Teleost Badis badis.V. Dynamics of Fanning and other Parental Activities with Comments on the Behaviour of the Larvae and Postlarvae. Zeit. fur Tierpsychol. 21(1):99-123.
- Beach, F. A. (1948). Hormones and Behaviour, New York, Hoeber Inc.
- Bol, A. C. Angela (1959). A consummatory situation the effect of eggs on the sexual behaviour of the male three-spined stickleback (Gasterosteus aculeatus L.) Experimentia 15:115-116.
- Chester-Jones, I., J. G. Phillips and D. Bellamy (1962). Hormones affecting environmental adjustment. General and Comparative Endocrinology, Supplement 1, 36-47.

- Egami, N. and S. Ishii (1962). Hypophyseal control of reproductive functions in teleost fishes. General and Comparative Endocrinology, Supplement 1, 248-253.
- Fiedler, Kurt (1962). Die Wirkung von Prolactin auf das Verhalten des Lippfisches Crenilabrus ocellatus (Forskol) Zool. Jb. Physiol. Bd. 69, s, 609-20.
- Grant, W. C. and J. A. Grant (1958). Water drive studies on hypophysectomized efts of Diemyctylus viridescens. Biol. Bull. 114, 1-9.
- Gerschwind, I.I. (1958). Species variation in protein and polypeptide hormones. In: Gorbman (1958), pp. 412-443.
- Heuts, M. J. (1947). Experimental studies on adaptive evolution in Gasterosteus aculeatus. Evolution, 1:89-102.
- Hoar, W. S. (1962). Hormones and the reproductive behaviour of the male three-spined stickleback (Gasterosteus aculeatus). Animal Behaviour, 10 (3-4):247-266.
- Iersel, J. J. A. van (1953). An analysis of the parental behaviour of the male three-spined stickleback (Gasterosteus aculeatus L.). Behaviour, Suppl. 3, 1-159.
- Ikeda, K. (1933). Effect of castration on the secondary sexual characters of anadromous three-spined stickleback, Gasterosteus aculeatus (L.). Japan J. Zool. 5:135-157.

- Ishii, S. (1960). Effects of estrone and progesterone on the ovary of the viviparous teleost, Neoditrema ransonneti, during gestation. J. Fac. Sci. Univ. Tokyo Ser. IV 9, 101-109. From: Egami and Ishii, 1962.
- Ishii, S. (1961). Effects of hormones on the gestation of the top-minnow. J. Fac. Sci. Univ. Tokyo, Ser. IV 9, From: Egami and Ishii, 1962.
- Lehrman, D. S. (1961). Hormones and parental behaviour in birds and infra-human mammals. In W. C. Young ed., Sex and Internal Secretions, Baltimore, Williams and Wilkins vol. 2 (F) Ch. 21:268-1382.
- Medawar, P. B. (1953). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. In: Evolution, Sympos. Soc. Exp. Biol., No. 7, Cambridge, Engl, Cambridge U. Press, pp. 320-338.
- Morris, D. (1954). The reproductive behaviour of the River Bullhead (Cottus gobio L.) with special reference to the fanning activity. Behaviour 7, 1-32.
- Morris, D. (1958). The reproductive behaviour of the ten-spined stickleback (Pygostius pungitius L.). Behaviour, Suppl. 6:1-154.
- Nalvandov, A. V. (1945). A study of the effect of prolactin on broodiness and on cock testis. Endocrinology 36, 251-258.

- Noble, G. K., K. F. Kumpf and V. N. Billings (1938). Induction of brooding behaviour in the Jewel Fish. *Endocrinology* 23, 353-359.
- Pickford, G. E. and J. W. Atz (1957). *The Physiology of the Pituitary Gland of Fishes*. New York Zool. Soc., N.Y.
- Pickford, G. E. and B. Kosto (1957). New melanocytes in Fundulus by M.S.H. and prolactin. *Endocrinology*, 61, 177-196.
From: Prosser and Brown (1961).
- Riddle, O. (1938). Prolactin, a product of the anterior pituitary and the part it plays in vital processes. *Scientific Monthly*, 47, 97-113.
- Smith, R.J.F. (1962). Mortality of anadromous Stickleback (Gasterosteus aculeatus L.) in fresh water, B.A. Thesis, Dept. of Zool. U.B.C.
- Steel, R.G.D. and J. H. Torrie (1960). Principles and Procedures of Statistics. Toronto, McGraw-Hill.
- Tinbergen, N. (1951). The Study of Instinct. Oxford, Clarendon Press, 228 pp.
- Wain, Evelyn Hui and W. S. Hoar (1963). The secondary sex characters and reproductive behaviour of gonadectomized sticklebacks treated with methyl testosterone. *Can. J. Zool.* 41:611-628