Parental Fanning Behavior and Prolactin Cell Activity in the Male Three-Spined Stickleback *Gasterosteus aculeatus* L.

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During the period that sexually mature male three-spined sticklebacks display parental fanning behavior, the rate of synthesis and release of the prolactin cells in the hypophysis, as estimated with quantitative electron microscopy, is enhanced considerably. This finding points to involvement of endogenous prolactin in the regulation of parental fanning behavior in this species.

Prolactin has become generally accepted as a hormone involved in the regulation of parental behavior in mammals and birds, even though the evidence often is indirect and contradictory (Zarrow *et al*., 1971; de Vlaming, 1979; Burke and Dennison, 1980). Notwithstanding this equivocal situation in the higher vertebrates, prolactin has also been mentioned as a parental hormone in those teleost species which care for their eggs and freshly emerged young. In a number of these species parental care expresses itself in fanning behavior, by which a stream of fresh water is propelled over the eggs by means of the pectoral fins. A stimulating influence of (mammalian) prolactin injections on fanning behavior has been reported for *Crenilabrus ocellatus* (Fiedler, 1962), *Symphysodon aequifasciata*, and *Pterophyllum scalare* (Blüm and Fiedler, 1965; Blüm, 1974). In a few other species prolactin has to be given in combination with gonadal steroids to induce higher levels of fanning behavior (e.g., *Leptomis gibbosus*, Kramer, (1973); *Aequidens pulcher*, Molenda, quoted by Fiedler, 1974). With respect to fanning behavior in the stickleback *Gasterosteus aculeatus* the literature contains conflicting reports. Thus, Smith and Hoar (1967) claimed that testosterone rather than prolactin stimulated male fanning behavior, whereas Molenda and Fiedler (1971) reported stimulation of fanning by means of prolactin injections.

Studies intended to substantiate the presumed role of prolactin as a parental hormone by means of determinations of plasma prolactin levels during different phases of the breeding cycle have recently been made in mammals (Terkel *et al*., 1979) and birds (Goldsmith, 1982). These studies generally showed that serum prolactin levels are higher during the period of maternal care in mammals and during the incubation period in birds. In teleost fishes as yet no plasma prolactin levels have been studied in relation to parental behavior. Metuzals *et al.* (1968) tried to obtain this kind of information indirectly by studying the relation between the (light microscopically determined) activity of prolactin cells and the occurrence of parental behavior in *Aequidens portalegrensis*. They found that the acidophilic α cells (located exclusively in the rostral pars distalis and thus most likely prolactin cells) showed signs of enhanced secretory activity when the fish had just spawned and thus had entered the period of parental care during which they fan their eggs. As at the time of our studies we had no means to determine plasma pro-
lactin levels, we also decided to study prolactin cell activity indirectly using quantitative electron microscopy.

The aim of our study was twofold. In the first place we wanted to establish whether or not prolactin cell activity in the stickleback is enhanced during the period of parental care for the eggs. In the second place, because all studies in fishes so far have been made with mammalian prolactin, we wanted to study the influence of species-specific prolactin on fanning behavior by means of implantation of extra prolactin-lobes into tail muscles. The present paper reports our findings on the first mentioned subject, while the results of the implantation experiment will be reported in a separate paper (de Ruiter et al., in prep.).

**MATERIALS AND METHODS**

The fish studied (*trachurus* form of *Gasterosteus aculeatus* L.) were obtained from laboratory stock. The fish were transferred to the laboratory and were kept on a light regime of 8L/16D at 20 ± 1 °C. Under these conditions hardly any fish will attain sexual maturity (Baggerman, 1972). In March of the following year, the fish (then measuring 52-60 mm) were kept on a light regime of 8L/16D at 20 ± 1 °C, a condition which induced maturation within about 30 days. During the experiment the males were exposed to a long daily photoperiod (16L/8D) and 20 ± 1 °C, a condition which induced maturation within about 30 days. Under these conditions hardly any fish will attain sexual maturity (Baggerman, 1972). In March of the following year, the fish (then measuring 52–60 mm) were kept on a long daily photoperiod (16L/8D) and 20 ± 1 °C, a condition which induced maturation within about 30 days. During the experiment the males were kept separately in 15-liter tanks filled with tap water at a temperature of 20 ± 1 °C. The daily photoperiod remained 16L/8D. The fish were fed *Tubifex* or *Daphnia* every other day.

The experimental set up was as follows: The tanks were arranged in pairs, one containing the experimental male and the other the so-called stimulus male, both with a nest. The tanks of each pair were placed side by side so that the animals were continuously in visual contact and could react toward each other, e.g., by showing agonistic behavior. As shown by Baggerman (1968) this arrangement can be used to imitate the natural situation in which males occupy adjacent territories and frequently display agonistic behavior. Males which are not allowed to court females tend to somewhat neglect their nests after a while. Therefore, ripe females were introduced into the tanks for a few minutes at least three times per week, a procedure which also somewhat resembles the natural situation and keeps the males active during a long period of time. The introduction of the females (which were not allowed to deposit eggs) always took place after the daily behavior record had been made. No females were introduced into the tanks of males which took care of eggs.

The reproductive and parental behavior patterns of the male three-spined stickleback have already been described extensively (van Iersel, 1953; Baggerman, 1968). A summary will be given here. In early spring sticklebacks, especially the *trachurus* form, migrate from the sea to freshwater to breed. Soon after arrival in freshwater the males start to defend a territory in which they subsequently build a nest.

**Parental fanning.** After the nest is finished the male will court sexually mature females. Once a female has deposited eggs in the nest, the male will fertilize them and chase her out of his territory. Then the parental phase of the reproductive cycle starts, in which fanning behavior is the most prominent behavioral element. During this fanning behavior the male orients himself in front of the nest and fans water over and through the nest by means of fast alternating movements of the left and right pectoral fin. The resulting backward movement is counteracted by fast tail movements and in this way the male remains in the same position in front of the nest. During the 6 or 7 days the eggs need to develop at 20°, the male spends increasingly more time on fanning, both with respect to the frequency and duration of the fanning bouts. Because of the association of fanning with the developing eggs in the nest, this fanning has been termed parental fanning behavior (van Iersel, 1953).

**Displacement fanning.** In addition fanning can also be performed by a male which has a nest only and no eggs. This fanning has been termed irrelevant fanning or displacement fanning, and its occurrence will be dealt with further under Discussion.

**Recording of fanning behavior.** Parental as well as displacement fanning is performed in bouts, which occur interspersed between other behavior components, such as agonistic and sexual behavior, nest building behavior, swimming and feeding. One fanning bout is defined as the period of time during which fanning is performed without interruption by a short pause, or by other behavioral elements. Beginning 5 days after the completion of the nest, fanning behavior of each male was recorded daily (between 8:30 and 12:00 AM) during 15 min. During this time the number of fanning bouts was recorded and the duration of each bout measured by means of a stopwatch. Fanning bouts lasting shorter than 1 s were recorded as lasting 1 s. In this way also the total amount of time spent on fanning per 15 min became known for each male, while the mean duration of each fanning bout could be calculated. For each experimental group the data of the males were averaged per day, thus yielding the daily mean number of bouts, the daily mean total duration of fanning, and the daily mean duration of a fanning bout. In addition mean fanning levels per group were calculated for the total period preceding fertilization of a clutch of eggs.
Experimental groups. The experimental males were divided into three groups of four males each. One group had nests but was not allowed to obtain eggs and therefore performed only displacement fanning. These males were regarded as controls (C group). The second group was allowed to fertilize one clutch of eggs and to take care of the eggs for 5 days. These males besides displacement fanning also showed parental fanning and were called the egg group (E group). This 5-day period was chosen because, as van Iersel (1953) showed, parental fanning reaches its maximum 4 days after hatching, which, at 20°, occurs 6–7 days after fertilization. Under natural conditions the young begin to leave the nest a few days after hatching; the males may subsequently build a new nest a few days later and start the cycle all over again. In this experiment all young were removed from the tanks on the fourth day after hatching. During the following two observation days the experimental situation of these males resembled to a certain extent that before egg deposition (this is the post-parental care (PPC) group). In this way the condition of the males of the three groups (C, E, and PPC) resembles that of male sticklebacks before, during, and after the completion of a parental cycle (including 4 days of brood care).

Ultrastucture and morphometry. At the end of the experiment the males were decapitated and the pituitary gland was excised and prepared for electron microscopy. The glands were prefixed in a cacodylate-buffered (0.1 M; pH 7.2) glutaraldehyde solution (3%) for 15 min at room temperature. Then the rostral pars distalis (containing mainly prolactin cells) was separated from the gland and fixed in a similarly buffered mixture of 3% gluteraldehyde, 1% osmium tetroxide, and 5% potassium dichromate (1:1:1) for 1 hr at 0°. Postfixation was performed in a 1% solution of uranyl acetate in distilled water and was followed by dehydration in ethanol and embedding in Spurr's epoxy resin. Ultrathin sections were examined in a Philips EM 201 electron microscope.

In order to determine the activity of the prolactin cells quantitatively, lineal integrative analysis according to Loud et al. (1965) was applied. To this end a square grid of sampling lines (distance of the lines 10 mm) was projected onto the electron micrographs (final magnification 16,000 to 18,000 x). The nuclei were excluded from the sampling area. A length of 1000 μm per animal was sampled. The extent of the rough endoplasmic reticulum (RER) was determined by counting the number of intersections of its membranes with the sampling lines, and substituting the figure obtained in Eq. 5, as given by Loud et al. (1965), which yields the length of the membranes of the RER per surface unit of cytoplasm. Volumetric determinations of mitochondria and of Golgi areas were performed by counting the fraction of intersections formed by the sampling lines projected on these organelles. According to Delesse’s theorem this fraction is equivalent to the volume fraction of these organelles in the cytoplasm (Weibel and Gomez, 1962). In addition areas of 2000 μm² of cytoplasm per animal were determined using Kontron MOP AM 01 equipment and the number of exocytotic profiles in these areas was counted. Wilcoxon’s test was applied for statistical evaluation. All tests were two-sided at the 5% significance level.

RESULTS

Fanning behavior

C group. In this group only displacement fanning occurred. The number of fanning bouts, as well as the total duration of fanning per 15 min was low throughout the 19 days of observation; this resulted in a short mean duration of each fanning bout (Table 1; Fig. 1). As is common for displacement fanning, as well as for parental fanning (van Iersel, 1953; Sevenster, 1961; Baggerman, 1968), large variations may occur between individual animals and also within one animal from day to day and even from one time of the day to another.

E group. During the 14 days prior to fertilization, the number of fanning bouts was comparable to that of the C group, although the total duration of displacement fanning was much longer; this is a result of a considerably longer mean bout length than in the C group (Table 1). However, due to the even larger individual differences in displacement fanning among the E males than among the C males (Table 1, Fig. 1), the differences in time spent on displacement fanning between the C and E males were not statistically significant. We have no explanation for the high mean bout length and the large variations in displacement fanning occurring in the males of the E group.

After fertilization the males entered the parental period and fanning behavior performed during this period is, by definition, parental fanning. As Fig. 1 shows, the total duration of parental fanning per 15 min in-
TABLE I
MEANS ± SD NUMBER OF FANNING BOUTS, LENGTH OF FANNING BOUTS, AND TOTAL DURATION OF FANNING IN SEXUALLY MATURE MALE STICKLEBACKS

<table>
<thead>
<tr>
<th></th>
<th>Number of bouts/15 min</th>
<th>Bout length (seconds/15 min)</th>
<th>Total duration of fanning (seconds/15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Displacement fanning(^a^)</td>
<td>2.0 ± 1.2</td>
<td>5.6 ± 1.4</td>
<td>11.3 ± 5.0</td>
</tr>
<tr>
<td>E group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Displacement fanning(^b^)</td>
<td>2.8 ± 1.8</td>
<td>11.0 ± 6.7</td>
<td>31.4 ± 24.0</td>
</tr>
<tr>
<td>Parental fanning(^c^)</td>
<td>19.0 ± 4.5</td>
<td>13.5 ± 10.8</td>
<td>310.0 ± 120.8</td>
</tr>
<tr>
<td>PPC group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Displacement fanning(^d^)</td>
<td>3.1 ± 0.7</td>
<td>5.1 ± 2.3</td>
<td>15.3 ± 9.2</td>
</tr>
<tr>
<td>Parental fanning(^e^)</td>
<td>26.5 ± 6.5</td>
<td>16.3 ± 2.8</td>
<td>336.0 ± 83.5</td>
</tr>
<tr>
<td>Displacement fanning(^f^)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note. For details about the groups of males see text and Fig. 1.
\(^a^\) Mean of 19 days.
\(^b^\) Mean of 14 days.
\(^c^\) Data of the fifth day only.
\(^d^\) Mean of 7 days.
\(^e^\) Data of the sixth day only.
\(^f^\) Data of the 11th and 12th day only.

As Fig. 1 shows, parental fanning begins to decline around the day of hatching (as is common in sticklebacks), reaching a very low level on Days 9 and 10. After the young had been removed, no more displacement fanning was observed during the last two observation days before autopsy. Had the animals been allowed to remain in their aquaria, they would have built a new nest within a few days and would have resumed displacement fanning at a low level.

The Activity of the Prolactin Cells

In sticklebacks, as in many other teleost fishes, the prolactin cells are situated as a compact mass of secretory cells in the rostral pars distalis of the pituitary gland, interspersed by irregular strands of small chromophobic stellate cells (Leatherland, 1970; Wendelaar Bonga, 1978).

C group. In the fish of the C group the prolactin cells are large round or oval cells with a large nucleus; often a prominent nucleolus is present. The cytoplasm of the...
cells contains many long strands of rough endoplasmic reticulum (Fig. 2). These strands are mostly found at the periphery of the cells and around the nucleus. The Golgi areas are prominent and located in a central region close to the nucleus. The Golgi sacculi usually are slightly dilated and elongated. They are often surrounded by small vesicles and presecretory granules (Fig. 2). The presence of presecretory granules reflects synthesis by the Golgi area (Farquhar and Palade, 1981; Wendelaar Bonga, 1978). Many well-developed mitochondria are found throughout the cytoplasm. Indentations of the cell membrane, with or without a granule-like inclusion are regularly observed (Fig. 3). Such indentations are thought to indicate places of hormone release by exocytosis (Leatherland, 1970; Batten et al., 1976). The results of the quantitative analysis of the prolactin cells of the C group are presented in Fig. 5.

**E group.** The males in group E were used to determine the relation between parental fanning and prolactin cell activity on Day 5 of the parental cycle, when fanning is about to reach its maximum value.

In fish performing parental fanning, the Golgi areas are considerably enlarged and the sacculi more dilated, while much more presecretory granules are found than in the control fish (Fig. 4). These features indicate a higher secretory activity of the prolactin cells. The quantitative data show that compared to the C males, no difference could be found in extent of the rough endoplasmic reticulum (Fig. 5). However, the volume fraction of the Golgi areas in the E males was 40% higher than that in the C males \( (P < 0.001) \). Moreover, the number of exocytotic profiles showed a more than twofold increase \( (P < 0.001) \). The mean volume fraction of the mitochondria was also elevated considerably, but not significantly.

**PPC group.** The PPC males were used to study prolactin cell activity after completion of the parental phase, including a period of 2 days following removal of the young from the tanks. The structure of the prolactin cells of the PPC males was very much like that of the C males. The values for the extent of the RER, the volume of Golgi areas, the volume fraction of the mitochondria as well as for the number of exocytotic profiles all closely resemble those of the C males (Fig. 5) and the slight dif-
Figs. 2–4. Prolactin cells of sexually mature male sticklebacks in freshwater with a nest containing no eggs (C group; Figs. 2 and 3), or with a nest and fertilized eggs on the fifth day of the parental cycle (E group; Fig. 4). The organelles involved in synthesis of hormone granules (gr), like nucleus (n), mitochondria (m), rough endoplasmic reticulum (rer), and Golgi apparatus (Go), are shown in Fig. 2. Figures 3 and 4 show exocytotic release of a secretory granule (arrows) into the intercellular space. Figure 4 shows a detail of a prolactin cell of a male of the E group. Newly formed presecretory granules are budded off (arrowheads) from the dilated Golgi (Go) sacculi; nu, nucleolus; pr, prolactin cell; des, desmosome; is, intercellular space; st, stellate cell surrounding the prolactin cells. Fig. 2, ×14,200; Fig. 3, ×34,800; Fig. 4, ×22,100.
With respect to the height of parental fanning between fertilization and hatching, our data and those in the literature (van Iersel, 1953; Baggerman, 1968) are in good agreement. Since the level of displacement fanning before fertilization was lower in our groups than that found by the authors mentioned above, the percentage increase of parental fanning was much higher in our groups (E and PPC). The duration of 6 days for the eggs to hatch at 20° is in agreement with the findings of van Iersel (1953).

**Prolactin Cell Activity**

Quantitative analysis at the ultrastructural level has been accepted as a technique to establish the secretory activity of glandular cells. The extent of the membranes of the rough endoplasmic reticulum and the fractional volume of the Golgi areas and mitochondria have been considered parameters that reflect the biosynthetic activity of the prolactin cells (Batten *et al*., 1976; Wandelhaar Bonga, 1978). In the present study the occurrence of exocytotic profiles has been quantified additionally as a parameter for prolactin release. As judged by most of these structural parameters it can be concluded that prolactin cell secretory activity is markedly enhanced during the period of parental fanning. An enhanced rate of prolactin synthesis in this period is further indicated by the increased number of presecretory granules budded off from the Golgi apparatus. As concluded from the rise of the number of exocytotic profiles, prolactin release has increased almost threefold during parental fanning. After the parental period prolactin synthesis and release apparently return to control levels.

**DISCUSSION**

**Fanning Behavior**

Our data show that the level of fanning behavior is rather low before fertilization, and that it rises quickly after fertilization to reach a peak value (10–20 times higher than the level before fertilization) around Day 6 when the young begin to hatch. From that time fanning decreases sharply to reach the prefertilization level within about 4–5 days after hatching of the eggs.

In general, our data on fanning behavior are in agreement with those given in the literature (van Iersel, 1953; Sevenster, 1961; Baggerman, 1968; Molenda and Fiedler, 1971).
tent of rough endoplasmic reticulum (Roubos and Moorer-van Delft, 1976). It is possible that the period of 5 days of parental care, as in the present experiment, was too short to result in notable changes in the extent of the rough endoplasmic reticulum. However, from other comparable experiments of one of us (S. E. W. B.), we know that in fish under similar conditions as those of the E group both cell and nuclear volume of the prolactin cells increase about 25% when compared to those of control fish under similar conditions as those of the C group. This may point to an (from our data undetectable) absolute increase of the extent of rough endoplasmic reticulum in fish displaying parental fanning. Although prolactin cell activity was greatly increased during the period of parental care, the cells nevertheless also show signs of secretory activity before and after this period. For in euryhaline fish, including sticklebacks, prolactin cells are involved in hydromineral regulation under freshwater conditions (Nagahama et al., 1973; Schreibman et al., 1973; Wendelaar Bonga, 1978).

Correlation between Fanning Behavior and Prolactin

Our data show that the low levels of displacement fanning before and after the period of parental care are concurrent with relatively low levels of prolactin cell activity. In addition, the greatly enhanced parental fanning activity on Day 5 of the parental period is accompanied by a high secretory activity of the prolactin cells. This correlation strongly suggests that parental fanning depends on a high level of prolactin in the blood. Metuzals et al. (1968) also found a clear correlation between an enhanced activity of the presumptive prolactin cells at a time when the fish (A. portalegrensis) had just spawned and thus had entered the period of parental care.

Comparison of our data on the relation between prolactin and parental fanning with data in the literature are complicated by the fact that most authors have studied the relations between prolactin and displacement fanning rather than parental fanning. These two types of fanning behavior are distinguished on functional grounds: parental fanning serves to provide the eggs with a stream of fresh water, whereas the function of fanning in front of a nest without eggs, as occurs in the case of displacement (irrelevant) fanning is not yet understood. Strictly speaking, the use of displacement fanning as parameter for parental behavior is only allowed after it has been established that its occurrence, like that of parental fanning, is based on the same parental motivation. So far, only Sevenerster (1961) has given some experimental evidence indicating that parental motivation may be at least one of the factors involved in the causation of displacement fanning in the stickleback.

A stimulating influence of ovine prolactin injections on displacement fanning has been reported in C. ocellatus (Fiedler, 1962), in S. aequifasciata, and in P. scalare (Blüm and Fiedler, 1965; Blüm, 1974). In the stickleback, Smith and Hoar (1967) reported that they could stimulate neither displacement nor parental fanning by injections of ovine prolactin and concluded that prolactin is not involved in the regulation of fanning behavior in this species. However, the failure of Smith and Hoar (1967) to affect fanning with ovine prolactin was likely due to the excessively high dose used, since Molenda and Fiedler (1971) showed that only low doses of prolactin stimulate displacement fanning, whereas higher doses inhibit the occurrence of this behavior.

Smith and Hoar (1967) also reported that treatment of gonadectomized male sticklebacks with androgen induced the performance of displacement fanning (although only in males which had started nest building as result of this treatment) and concluded that androgen rather than pro-
lactin activates fanning behavior. However, this conclusion is not warranted without further experimentation, since (as Baggerman (1968) suggested) the androgen injections in this case most likely affected displacement fanning indirectly rather than directly, by stimulating the performance of nest building and enhancing the aggressive and sexual motivation. It is well known (1) that male hormone is able to induce the performance of nest building and aggressive and sexual behavior (Hoar, 1962; Baggerman, 1968; Liley, 1969; Fiedler, 1974), while (2) displacement fanning only occurs in sticklebacks with a nest and is greatly stimulated by the activation of the aggressive and sexual motivation (Baggerman, 1968).

In all species mentioned so far in this Discussion, fanning behavior only occurs in individuals that are sexually mature, which may imply that gonadal hormones play some role in prolactin controlled fanning behavior. This idea seems to be supported by the findings that in *L. gibbosus* (Kramer, 1973) and *A. pulcher* (Molenda, quoted by Fiedler (1974)) prolactin injections were only able to stimulate displacement fanning when given in combination with gonadal steroids. Moreover, Smith and Hoar (1967) found that castration of male sticklebacks on the first or second day after fertilization of a clutch of eggs resulted in an almost immediate cessation of all fanning behavior. However, the suggested relation between prolactin and gonadal hormones is certainly more complex, since de Jongh-van der Heij (results given by Baggerman (1968)) found that castration on the third and fourth day after fertilization had no adverse effect on parental fanning, since the fanning cycle followed a course comparable to that of the sham-operated controls. Evidently, further experimentation is needed to elucidate the possible role of gonadal steroids in prolactin controlled fanning behavior.

The ultimate aim of investigations like the present one is to understand the way the parental fanning cycle (Fig. 1, lower panel) is regulated. This cycle begins after fertilization of freshly laid eggs. van Iersel (1953) showed that the amount of fanning behavior increases with increasing age of the eggs and it is likely that fanning is regulated by the metabolic activities of the developing eggs, although he suggested that visual and tactile stimuli from the eggs may play an additional role. The question then arises how prolactin would fit into this picture. One possibility is that the act of fertilization or the perception of freshly laid eggs triggers an enhanced secretion of prolactin, resulting in a higher level of fanning behavior. As the eggs develop, their increased metabolic activities could stimulate the secretion of increasing amounts of prolactin, resulting in increasing amounts of fanning. Another possibility is that the enhanced level of prolactin secretion triggered by the factors mentioned above would play only a permissive role, in that it enables the male to react to the increasing stimuli emanating from the developing eggs by increasing amounts of fanning behavior. We shall return to these two possibilities in our second paper (de Ruiter et al., in prep.). Another question pertains to the factors controlling the decline of fanning on the day following hatching. As van Iersel (1953) showed, this is caused by stimuli from the hatching eggs, as well as by internal factors. These external and internal factors could induce a reduction of the amount of prolactin secreted, resulting in a decrease of fanning behavior. However, the hatching process could also induce a reduction of prolactin secretion as well as of fanning behavior, without the two processes being causally related.

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