The Effect of Prolactin on Fanning Behavior in the Male Three-Spined Stickleback, Gasterosteus aculeatus L.

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The effect of administration of homologous prolactin on fanning behavior, an important aspect of parental care in sticklebacks and many other teleost fish, was studied. Prolactin was administered by implantation of an additional pituitary prolactin lobe in the dorsal body musculature of males with a nest without eggs. The low but rather constant level of fanning behavior is significantly increased from Day 6 to Day 12 after administration of the prolactin lobe. The implantation has no noticeable effects on cell and nuclear size or on the ultrastructure of the \textit{in situ} prolactin cells of the recipient fish. Recovered prolactin lobe implants show two categories of prolactin cells. The major category consists of cells that are well developed and, similar to \textit{in situ} prolactin cells, show structural signs of high secretory activity. The other prolactin cell category in the implants shows signs of cellular involution. Ten days after implantation, the first category predominates, and 16 days after implantation the second category. Prolactin lobe implantation increases the number of mucocytes in the epithelium of the skin, in particular at Day 10, and to a lesser extent at Day 16. This is considered evidence for a transient rise in blood prolactin levels in the recipient fish. We conclude that prolactin stimulates fanning behavior in male sticklebacks. © 1986 Academic Press, Inc.

Fanning is an important aspect of parental care in many substrate brooding fish. It results in refreshment of the water around eggs and newly hatched young. In a previous paper (Slijkhuis et al., 1984) we reported that in male sticklebacks prolactin cell activity is enhanced during the period of about 7 days when the males take care of the eggs in the nests and spend much time fanning. We considered this as evidence that endogenous prolactin is involved in the regulation of parental fanning in sticklebacks, rather than testosterone, as has been suggested by others (Smith and Hoar, 1967).

It is well known that mammalian prolactin stimulates fanning behavior in several fish species (Blüm, 1966, 1974; Fiedler, 1974). Fish prolactin has not been tested so far. In this paper we report on the effect of administration of fish prolactin on fanning behavior in sticklebacks. We tried to stimulate fanning behavior by means of implantation of an additional rostral pars distalis of the pituitary gland (prolactin lobe) into the tail musculature. This was done on the basis of the finding of Leatherland (1970) that in the stickleback ectopic pituitary homotransplants will show structural signs of enhanced secretory activity. This may be due to lack of the inhibitory influence on prolactin cell activity exerted by the hypothalamus \textit{in situ}. Similar findings have been reported, e.g., for \textit{Gillichthys mirabilis} (Nagahama et al., 1974) and \textit{Oreochromis mossambicus} (Wendelaar Bonga and Meis, 1981). By implantation of an additional prolactin lobe we intended to supply the acceptor fish with an enhanced level of homologous prolactin. This approach was preferred above injection of prolactin preparations since multiple injections appeared to interfere with behavioral observations.

After fertilization male sticklebacks
spend increasingly more time fanning the eggs, up to 300–500 sec/15 min (Van Iersel, 1953; Baggerman, 1968; Slijkhuis et al., 1984). As we noticed that in males spending much but highly varying time on fanning, enhancement of the prolactin level by prolactin lobe implant was not expressed clearly in a further increase of fanning behavior, we decided to use males with a nest but without eggs. Such males regularly perform fanning behavior at a low but rather constant level (Van Iersel, 1953; Sevenster, 1961; Baggerman, 1968; Slijkhuis et al., 1984). It is referred to as irrelevant or displacement fanning. As to the causal factors underlying this type of fanning behavior in the stickleback, many hypotheses have been put forward (Sevenster, 1961; Wilz, 1970b; McFarland, 1974; Cohen and McFarland, 1979). Sevenster (1961) has presented arguments that displacement fanning, like parental fanning, is caused by activation of the parental system.

In this study the data on the effect of prolactin lobe implantation on fanning behavior are correlated with the light and electron microscopic examination of the structure of the ectopic as well as the in situ prolactin cells. Since in sticklebacks prolactin cell activity is related to the number of mucocytes in the skin (Wendelaar Bonga, 1978), this parameter is used as an indirect estimate of circulating prolactin levels in the experimental fish.

**MATERIALS AND METHODS**

The fish studied, the housing of the animals, the experimental setup, as well as the recording of the fanning behavior have been reported earlier (Slijkhuis et al., 1984). In contrast to the earlier study, the fish examined in this study had nests without eggs. Fanning behavior was recorded between 8.30 and 12.00 AM during a period of 8 days and started on the fifth day after completion of the nest. In the afternoon of the eighth recording day prolactin lobe transplantations were carried out.

Before the operation, randomly selected fish were anesthetized in a solution of 50 mg MS 222 per liter of 10% artificial seawater (3.65 g Wimex seasalt/liter tap water) to reduce osmotic stress.

Prolactin lobes were obtained from donor males in the same stage of sexual maturation as the recipient fish. The donor fish were killed by decapitation. Every pituitary gland was rapidly excised and its prolactin lobe was separated from the proximal pars distalis and quickly inserted in an incision in the tail musculature of the recipient male. The incision was made about 2 mm dorsocaudally of the anal opening. The prolactin lobe was carefully inserted with a fine glass rod (implantation group). The incision was closed with two or three sutures. In sham-operated fish a similar incision was made but no prolactin lobe was inserted.

After the operation the fish were replaced in their tanks for a recovery period of 2 days. Then recordings on fanning behavior were made for another 14 days. At the end of the experimental period the fish were killed. Their pituitary gland and prolactin lobe implant were dissected out and prepared for electron microscopy according to Slijkhuis et al., (1984).

In a separate series of experiments, following a similar experimental protocol, we collected pituitary glands, implants, and pieces of the skin, 4, 10, and 16 days after operation. The pituitary glands and implants were fixed for electron microscopy. Semithin sections (1-μm thickness) were stained with toluidine blue, and cell and nuclear volumes determined as described earlier (Wendelaar Bonga, 1978). The pieces of skin (5 x 5 mm) were dissected from the lateral body wall in the anal region. They were fixed for light microscopy for 24 hr in Bouin–Holland fluid, dehydrated, and embedded in paraffin. Cross sections of the skin (7-μm thickness) were stained with periodic acid–Schiff (PAS) and Mayer's hemalum. The density of the mucocytes was estimated by counting the number of cells in sections of the skin epithelium with a total length of 150 mm per animal. The thickness of the epithelium was determined at five places, at distances of 800 μm, in 25 sections per animal.

For statistical evaluation of the values for fanning before and after operation Wilcoxon's matched pairs rank sign test was used. The data on cell and nuclear volumes and on mucocytes were statistically analyzed using the Mann–Whitney U test.

**RESULTS**

**Fanning behavior.** Once a male has finished a nest, fanning behavior becomes noticeable. During fanning the male orients itself 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 fins. Time spent on fanning behavior...
Fanning activity in seconds per 15 min (mean daily values ± SD) of sexually mature male sticklebacks having a nest without eggs, before and after intramuscular implantation of a rostral pars distalis (RPD) or sham operation.

was estimated for individual males before and after implantation of a prolactin lobe or sham operation. The data are presented in Fig. 1. Notwithstanding the large individual and day-to-day variation in fanning activity (typical for fanning in sticklebacks), it is evident that time spent on fanning increases significantly after prolactin lobe implantation. The difference with the preimplantation level of fanning behavior is statistically significant ($P < 0.01$) from Day 6 to Day 12, when a high plateau is maintained, during which fanning activity is about three times higher than before implantation or than in sham-operated control fish. In the latter group the average fanning level is not significantly different from the preoperation level.

**Prolactin cells in sham-operated fish.** The prolactin cell ultrastructure of the sham-operated fish 10 or 16 days after operation resembles that of untreated sexually mature males having a nest without eggs, as has been described earlier (Slijkhuis et al., 1984). The large round or oval prolactin cells contain a nucleus with a prominent nucleolus and in the cytoplasm many long strands of granular endoplasmic reticulum, prominent Golgi areas, and many mitochondria are present. The Golgi areas are often surrounded by clear vesicles and presecretory granules, while the cell membrane often shows exocytotic phenomena. The prolactin cells are surrounded by stellate cells (Fig. 2).

**In situ prolactin cells in recipient fish.** The prolactin cells in the pituitary of the males receiving a prolactin lobe implant are similar in appearance to those of sham-operated males (Fig. 3). This is also reflected in the cell and nuclear volumes of the prolactin cells, which are unchanged in the recipient fish (Fig. 4).

**Prolactin lobe implants.** In the prolactin lobe implants recovered from the tail musculature, two categories of prolactin cells can be distinguished. The first category consists of cells similar in appearance to those of the prolactin lobes *in situ* as described above. They show the normal signs of secretory activity, an extensive granular endoplasmic reticulum and Golgi areas with signs of granule formation. Some of the cells seem even more active than the *in situ* prolactin cells. The nucleus of these cells is very large and electron transparent, and often contains large nucleoli. The granular endoplasmic reticulum is very extensive and occasionally shows dilated cisterns. Free ribosomes are numerous. The Golgi areas are more extensive than in the *in situ* cells, whereas the number of secretory granules stored in the cytoplasm is reduced (Figs. 5, 6). The second category of prolactin cells consists of cells in different stages of degeneration. They usually contain a nucleus that is small and electron dense, with poorly developed or no nucleoli. In the cytoplasm normal cellular organelles are scarce and elements of the lysosomal system are prominent, namely, autophagous vacuoles and primary and secondary lysosomes. The autophagous vacuoles frequently contain secretory granules

![Fig. 1. Fanning activity in seconds per 15 min (mean daily values ± SD) of sexually mature male sticklebacks having a nest without eggs, before and after intramuscular implantation of a rostral pars distalis (RPD) or sham operation.](image-url)
and granular endoplasmic reticulum (Fig. 7). The implants show an extensive network of stellate cells between the prolactin cells that seems better developed than in the in situ lobes. The category of secretory active cells predominates in implants examined 10 days after implantation. Cells ($P > 0.001$) and nuclei ($P > 0.05$) are significantly enlarged when compared to the in situ prolactin cells of recipient fish or sham-operated fish. Degenerating cells are scarce, although their number differs between the implants. In implants recovered after 16 days, however, the latter category of cells predominates in the majority of the implants. The mean cell and nuclear volumes of the prolactin cells are significantly smaller than those of the implants at Day 10, and also smaller than those of the in situ prolactin cells (Fig. 4).

ACTH cells, which form a cell layer separating the prolactin lobe from the proximal pars distalis and from the neurohypophysis, were never observed in the prolactin lobe implants.

Skin epithelium. Four days after operation, no difference was noticeable between the number of mucocytes in the epidermis of sham-operated fish and the recipient fish (Fig. 8). Ten days after operation, the fish receiving a prolactin implant had significantly more mucocytes than the sham-operated group ($P < 0.001$). After 16 days the difference between both groups was smaller, but still statistically significant ($P < 0.05$).

DISCUSSION

Fanning behavior. Average time spent on displacement fanning before implantation or sham-operation varied from 29 to 42 sec/15 min, which is well within the range reported by Van Iersel (1953), Sevenster (1961), Baggerman (1968), and Slijkhuis et al., (1984). As sham operation did not notably influence fanning activity, whereas prolactin lobe implantation resulted in a significant increase of the average fanning level, this increase must be attributed to the effect exerted by the prolactin lobe implant. However, the highest average daily level reached by our experimental fish (91 sec/15 min), which had a nest without eggs, is still much lower than the average peak of parental fanning which males perform when caring for eggs. According to the above-mentioned authors peak parental fanning levels may vary from 300 to 500 sec/15 min. Van Iersel (1953) and Sevenster (1961) have presented evidence showing that the high levels of fanning during the parental period are due to a cooperation between internal parental motivation and external stimuli emanating from the developing eggs, particularly metabolic stimuli like CO$_2$ production. Thus the implantation of a prolactin lobe may have less effect on the circulating prolactin levels than the presence of eggs in the nest.

Prolactin cells. Ten days after implantation, the prolactin lobe implants contained many intact and apparently actively secreting prolactin cells. The cells were larger and seemed even more active than the prolactin cells in situ. Similar ultrastructural signs of secretory activity in prolactin cell lobes implanted in otherwise intact fish have been reported for other freshwater teleost species although the degree of activation seems to vary from species to species (Nagahama et al., 1975; Leatherland and Lin, 1976). For Gasterosteus acu-
Fig. 4. Cell volume (total height of bars) and nuclear volume (dotted part of bars) of prolactin cells of pituitary glands in situ of sham-operated fish (S) and of fish receiving a prolactin lobe implant (Pr-s), and of prolactin cells of the recovered pituitary lobe implants (Pr-i). The volumes were determined 10 days (left bars) and 16 days (right bars) after operation. Means ± SD of five fish per group.

leatus. Leatherland (1970) has demonstrated that the prolactin cells of pituitary glands implanted in the tail musculature for 1 and 2 weeks are significantly enlarged, as are their nuclei. A similar enlargement we observed for the cells of the prolactin lobes implanted for 10 days. In the implants recovered after 16 days, the large, apparently actively secreting cells were predominated by degenerating cells, which resulted in a decrease of the mean cell and nuclear volumes. Since autophagous vacuoles predominated over secondary lysosomes the process of degeneration may have started only a few days before recovery of the implants. This could explain why the fanning activity dropped suddenly after Day 13. Degeneration of grafted prolactin cells in fish is uncommon. Autotransplantation of the pituitary gland of the goby Gillichthys mirabilis for 1 month (Nagahama et al., 1974), of Anguilla anguilla for 2 months (Olivereau and Dimovska, 1968), or of Gambusia sp. for 12 months (Kah et al., 1979) resulted in viable and apparently active prolactin cells. Similarly, homotransplantation of pituitary glands does not affect or even stimulate the secretory activity of the transplanted prolactin cells of, e.g., hypophysectomized Poecilia formosa (Olivereau and Ball, 1966) or intact Gillichthys mirabilis (Nagahama et al., 1974) for at least a month. In hypophysectomized P. formosa the pituitary grafts enabled the fish to survive transfer from seawater to fresh water for several months after implantation. In this species freshwater survival is fully dependent on prolactin (Ball et al., 1965). In our experiments not the complete pituitary gland but only its rostral pars distalis was transplanted, after careful removal of most of the ACTH cells. It is possible that this dissection procedure, intended to ensure that the transplants release only prolactin and, at the most, insignificant amounts of other hormones, has contributed to the involution of the prolactin cells that we observed after 2 weeks.

The size and ultrastructure of the in situ prolactin cells were not noticeably changed by the prolactin lobe implants. Thus, there is no structural evidence for reduced activity due to negative feedback caused by the implants. This is in line with observations of Leatherland (1970), who reported for the same species as used in our study, Gasterosteus aculeatus, that cell and nuclear sizes of the in situ prolactin cells were

**FIGS. 5-7.** Prolactin cells of prolactin lobes implanted for 16 days in male sticklebacks. Figures 5 and 6 show parts of well-developed prolactin cells, having extensive granular endoplasmic reticulum (ger) as well as many presecretory granules (arrows) that are budded off from the Golgi membranes (G). Figure 7 shows a detail of a degenerative prolactin cell, exhibiting many elements of the lysosomal system, like autophagous vacuoles (av) containing secretory granules and granular endoplasmic reticulum and secondary lysosomes (sl). Fig. 5. ×14,000. Figs. 6 and 7. ×18,000.
unchanged during the first 2 weeks after the fish received a pituitary homotransplant. In *Gillichthys mirabilis*, homotransplantation did not lead to appreciable cytological changes of the in situ prolactin cells in the first 2 weeks, although indications of reduced activity were observed after 40 to 50 days (Nagahama et al., 1974).

Structure of skin epithelium. Prolactin is known to increase the density of mucocytes in the epithelium of the skin of several fish species (Blüm, 1966, 1974; Blüm and Fiedler, 1972, Schwerdtfeger, 1979; Wendelaar Bonga and Meis, 1981). The stimulation of the number of mucocytes of teleost epidermis by prolactin has not been observed in all fish species examined so far (Wendelaar Bonga and Meis, 1981). However, in three-spined and nine-spined sticklebacks mucocyte density is positively correlated with the number and activity of the prolactin cells (Wendelaar Bonga, 1978; Benjamin, 1980). We therefore feel justified in using the mucocyte density of the skin epidermis in sticklebacks as a parameter for blood prolactin levels. In sexually mature male sticklebacks in the nest-building phase of their reproductive period, prolactin levels are elevated. We came to this conclusion after studying the ultrastructure of the prolactin cells of such males (Slijkhuis et al., 1984). The present data show that if males in this stage of the reproductive cycle were supplied with an additional prolactin lobe, mucocyte density increased significantly. We consider this to be evidence that circulating prolactin levels were indeed increased in the recipient fish during most of the observation period. The number of epidermal mucocytes of the fish examined 10 days after implantation of the lobes was much higher than after 16 days. The reduction at Day 16 indicates that blood prolactin levels decreased at the end of the second week of implantation. Such a decrease explains the drop in fanning activity that occurred after Day 12. Whereas fanning activity was back to normal values at Day 16, the number of mucocytes was still above the control level. Since growth, differentiation, and disappearance of mucocytes are processes that take at least several days, it is very possible that the blood prolactin levels were already back to normal levels at Day 16.

Relationship between parental fanning behavior and prolactin. In a previous paper (Slijkhuis et al., 1984) we presented evidence indicating that parental fanning behavior in the male stickleback depends on a high level of prolactin secretion. Here we present support for this conclusion by showing that administration of homologous prolactin leads to a higher level of fanning behavior. However, for practical reasons (see introductory paragraphs) we used displacement fanning rather than parental fanning as criterion for fanning behavior and thus the question arises whether prolactin also stimulates parental behavior. The distinction between displacement (or irrelevant) fanning and parental fanning is based on functional grounds: parental fanning serves to ventilate the eggs, whereas displacement fanning, which is carried out in front of an empty nest, obviously cannot serve the same function and hence seems out of context or irrelevant (Sevenster, 1961). In the more recent literature several different functions for displacement fanning have been proposed (Wilz, 1970a, b; McFarland, 1974; Rohwer, 1978; Cohen and McFarland, 1979; Ridley and Rechten, 1981). Also with respect to the causal factors underlying the performance of displacement fanning, factors with which the present paper is concerned, the literature contains a number of controversial hypotheses. Sevenster (1961) presented evidence indicating that a number of external factors which are able to stimulate parental fanning (such as the concentration of CO₂ in the nest) are also able to stimulate displacement fanning. He concluded that parental motivation is probably one of the causal factors involved in the performance of dis-
placement fanning. Blüm (1974), working with the cichlid fish *Symphysodon aequifasciata axelrodi*, also found that increasing amounts of CO₂ were able to stimulate fanning behavior in the absence of eggs. Although several authors have formulated different hypotheses concerning the possible causal factors underlying displacement fanning (Wilz, 1970b; McFarland, 1974; Cohen and McFarland, 1979) none of them seems to have challenged Sevenster's (1961) conclusion that parental motivation is at least one of the causal factors involved in this type of fanning. On the basis of the above considerations we conclude that there are close relationships between displacement fanning and parental fanning, although it does not seem fully justified to use displacement fanning as a measure of parental fanning. A similar conclusion was reached by Noakes (1979). Nevertheless, our finding that enhancement of the circulating prolactin level in sticklebacks results in an increase of displacement fanning lends support to our previous conclusion that prolactin is a causal factor involved in the regulation of parentally motivated fanning behavior in this species (Slijkhuis et al., 1984). This conclusion was based on the observation that the prolactin cells are activated during the period of intense parental fanning displayed by male sticklebacks tending a nest with eggs.

Our present observations are the first that demonstrate an effect of teleost prolactin on fanning in fish. Mammalian prolactin has been shown to stimulate fanning behavior in a number of teleost species (Blüm, 1966, 1974; Blüm et al., 1972; Fiedler, 1974). Further support for prolactin as a hormone controlling fanning behavior in teleosts comes from studies of Fiedler and Blüm (1972) and Blüm and Fiedler (1974). They have reported specific activation of populations of neurons in the area dorsalis of the telencephalon in the centrarchid fish *Lepomis gibbosus* and the cichlid fishes *Asteronotus ocellatus* and *Tilapia marinae*. These three species show fanning behavior. It is significant that administration of prolactin had no such effect in species that do not show fanning behavior, like the mouthbreeding cichlids *Tilapia leucosticta*, *T. nilotica*, and the cyprinids *Carassius auratus gibelio* and *Idus idus* (Blüm and Fiedler, 1974). Preliminary results on the mouthbreeding cichlid *Oreochromis mossambicus* have shown that prolactin secretion is unchanged during the different stages of the mouthbreeding cycle (Wendelaar Bonga et al., 1983). Thus prolactin is probably involved in the control of fanning behavior and possibly in other aspects of parental care (Fiedler, 1974), but not in the control of parental care in general.

Smith and Hoar (1967) have reported that injections of mammalian prolactin were ineffective in stimulating fanning behavior in sticklebacks. We have argued before that the high concentrations used by these authors are in general ineffective in fish (Slijkhuis et al., 1984).

Although prolactin may have a specific action on fanning, this type of behavior occurs only in fish that are sexually mature. Thus, gonadal hormones are certainly
involved in the control of fanning. This is supported by observations of Kramer (1973) and Molenda (Fiedler, 1974) that prolactin injections are only stimulating displacement fanning in *Lepomis gibbosus* and *Aequidens pulcher* when given in combination with gonadal steroids. However, whereas castration of sticklebacks on the first or second day after operation results in cessation of fanning (Smith and Hoar, 1967), castration on the third or fourth day has no effect (Baggerman, 1968). This indicates that the role of gonadal steroids is of a permissive nature.

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