THE REGULATION OF SPAWNING BEHAVIOUR IN THE FEMALE GOLDFISH, CARASSIUS AURATUS

by

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ABSTRACT

This study of the regulation of spawning behaviour in the female goldfish (Carassius auratus) identified four endogenous factors believed to play major roles in spawning behaviour: (i) stimuli from ovulated eggs, (ii) ovarian steroids, (iii) pituitary hormones, and (iv) prostaglandins.

Spawning behaviour is synchronized with ovulation by the stimulus of an intraovarian mass of ovulated eggs. Normally, female goldfish begin to spawn on the morning of the day of ovulation and perform as many as several hundred spawning acts over a period of several hours; spawning behaviour ceases when all ovulated eggs have been shed. The duration of spawning behaviour was extended if oviposition (the release of eggs through the ovipore) was prevented by placing a plug in the ovipore. Spawning behaviour was terminated when ovulated eggs were removed by hand-stripping and restored when ovulated eggs were injected through the ovipore and into the ovarian lumen. This effect of eggs on spawning behaviour was not restricted to the day of ovulation but was seen in all fish with ovaries in any stage of vitellogenesis. Injection of several substitutes for ovulated eggs induced low levels of spawning behaviour.

Injection of ovulated eggs failed to induce spawning behaviour in female goldfish with regressed, nonvitellogenic ovaries. Pretreatment with a variety of gonadal steroids restored the spawning response to egg injection in these intact, regressed fish.

Hypophysectomized fish did not perform spawning behaviour when injected with ovulated eggs. Pretreatment of hypophysectomized fish with homogenized goldfish pituitaries or partially purified salmon gonadotropin (SG-G100) restored the response to egg injection. Aminoglutethimide, an inhibitor of steroid synthesis, blocked the effect of SG-G100 on spawning behaviour,

suggesting gonadotropin may exert its effect on behaviour by stimulating steroidogenesis. However, steroid treatments were totally ineffective in restoring the response to egg injection in hypophysectomized fish.

Prostaglandin (PG) appears to be involved in mediating the behavioural response to ovulated eggs. Indomethacin (IM), an inhibitor of PG synthesis, blocked the onset of spawning behaviour following egg injection. Injection of $PGF_{2\alpha}$ restored spawning behaviour in egg-injected, IM-treated fish; PGE_2 was less effective and PGE_1 was without effect. Injection of $PGF_{2\alpha}$ induced normal spawning behaviour in fish which had not been injected with ovulated eggs, suggesting that ovulated eggs induce spawning behaviour by stimulating synthesis of PG. The effects of PG on spawning behaviour of hypophysectomized fish treated with SG-G100 or steroids paralleled the effects of egg injection on fish receiving similar treatments; SG-G100 restored the spawning response to injection of PG, while steroid treatments were without effect.

Mechanisms by which ovarian and pituitary hormones and prostaglandins may influence spawning behaviour are discussed and a model of the regulation of spawning behaviour is proposed. In addition, an attempt is made to provide a theoretical basis for comparing the regulation of sexual behaviour in female vertebrates.

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CHAPTER I

GENERAL INTRODUCTION

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Research into many aspects of vertebrate reproduction has expanded 'explosively' in recent years. However, considering the numbers of ethologists, animal psychologists, neurophysiologists, and biochemists whose critical attentions now focus on the problems of hormonal control of female reproductive behaviour in many species of mammals, the research effort concerned with these phenomena in lower vertebrates is at best modest. Undoubtedly, this inequitable distribution of scientific labor stems from differences in the degree of relevance to problems of medicine and fertility in man and domestic animals. As greater emphasis is placed on the culture of edible fish species, greater emphasis also will be placed on the study of reproductive physiology and its relation to sexual behaviour in fish.

Remarkably little information concerning the hormonal regulation of sexual behaviour in female fish is available (Hoar, 1965; Liley, 1969) and much of this pertains to a reproductively specialized teleost, the viviparous guppy (Poecilia reticulata). In this species, the correlation of receptivity with gonadal state and the results of hypophysectomy, ovariectomy, and replacement therapies suggest that, as in higher vertebrates, ovarian and gonadotropic hormones participate in the regulation of sexual behaviour (Liley, 1968, 1969, 1972; Liley and Donaldson, 1969; Liley and Wishlow, 1974).

Apart from an early study (Noble and Kumpf, 1936; discussed in Liley, 1969) which reported partial restoration of sexual behaviour following treatment of female Hemichromis bimaculatus with an unidentified ovarian extract, replacement therapies (steroid hormones or ovarian extracts) have been ineffective in restoring spawning behaviour

of ovariectomized oviparous fish (Liley, 1969). Though the results of many studies acknowledge the 'close temporal relationship between ovulation and the spawning act' in female fish (Liley, 1969), the possible significance of this sequence in the regulation of spawning behaviour seems generally to have been overlooked. However, the observation that removal of ovulated eggs terminated the spawning behaviour of female goldfish led Yamazaki (1965) to hypothesize that 'ripe eggs in the ovarian lumen stimulate the spawning behaviour of females via some pathway'. Yamazaki's results further suggested that a role for ovulated eggs in spawning behaviour could explain why the technique of ovariectomy and steroid replacement therapy had yielded negative results in earlier studies of egg-laying fish (Liley, 1969).

In this thesis I have tested and found much support for Yamazaki's hypothesis concerning the role of ovulated eggs in spawning behaviour. In subsequent experiments, evidence is presented which indicates that pituitary and ovarian hormones influence the tendency of female goldfish to perform spawning behaviour when ovulated eggs are in the ovarian lumen. The mechanism by which eggs evoke spawning behaviour has been examined and it is suggested that protaglandins are involved. Emphasis has been placed on correlating sexual receptivity with ovarian histology in both treated and untreated fish. In summarizing the results of this study, comparative aspects of hormonal control of reproductive behaviour are emphasized and a basis for comparing reproductive behaviour in female vertebrates is proposed.

CHAPTER II

MATERIALS AND METHODS

A. General Maintenance

All fish used in this study were purchased from Hartz Mountain Pet Supplies Limited (Richmond, B.C.). To ensure an adequate supply of female goldfish, most shipments of fish were sexed at the warehouse; the remainder were shipped unsexed to the lab at the University of British Columbia. Common, comet, and intermediate types were used. Colors ranged from deep orange to silver and included many variations in mottled patterns, black tipped and white tipped fins, etc. Individuals with the wild, olive coloration were not used. The fish weighed from 15 to 60 g but the majority were in the range of 20 to 30 g.

To 'regulate' the reproductive state of both males and females, fish were maintained under two environmental regimes; cold water, long photoperiod (12 C; 16L:8D) and warm water, long photoperiod (20 C; 16L:8D). As reported by Yamamoto et al (1966), goldfish kept at 14 C or less develop to a prespawning stage but neither ovulate nor spermiate. When raised to 20 C, fish in this condition ovulate or spermiate within several days.

Usually, fish newly acquired from the warehouse (where they had been kept in running, dechlorinated, cold tapwater) were placed in large stock tanks of various sizes supplied with running dechlorinated tapwater thermostatically regulated at 10-13 C and illuminated for 16 hours per day with 20 - 40 watt fluorescent lights. In some cases, cold water stocks were kept outdoors in six-feet diameter fibreglass tanks under natural photoperiod but with some continuous low-level illumination. These tanks were not heated and sometimes were as cold as 5 C. To avoid introduction of disease, new shipments of cold water stock fish were kept separate from established stocks for at least several months. Cold water stock tanks contained no vegetation

or loose substrate. In some cases, cold water stocks were separated by sex.

Warm water stocks were kept 3 - 6 per 40 l tank in a fish room with air temperature control (water temperature 20 - 22 C) on a 16L:8D photoperiod. Each tank was supplied with 1" - 2" of quartz sand as bottom substrate, a floating mat of water sprite (Ceratopterus thalictroides), and an air stone. Filtration was used at the outset but quickly abandoned: outside box filters because of the maintenance involved, subgravel filters because of the acid water conditions produced when used in conjunction with Ceratopterus. A rapid fall in pH was by far the greatest cause of mortality in the warm water stock fish. In fish exposed to this condition, skin (and presumably gill) mucous apparently is denatured and becomes opaque; moribund individuals transferred to neutral water nearly always recovered within a few hours. Fortunately, an easily observable indicator, an increase in water clarity, always preceded the development of critically low pH by at least a day. Entrapment of much suspended detritus in the fine roots of Ceratopterus aids greatly in maintenance of water clarity, although this was likely of more importance to the experimenter than to the subjects.

All stocks were fed frozen brine shrimp ad libitum at least every other day. In addition, cold water stocks were fed Clark's New Age Trout Feed (Moore-Clark Company, Salt Lake City, Utah) on an irregular basis.

B. Hypophysectomy

Hypophysectomized fish were used in a number of experiments reported in this thesis. Mortalities directly due to the operation were low; however, disease and a relatively high incidence of incomplete

removal of the pituitary usually resulted in less than half the operated fish being used for experiments.

Hypophysectomy was performed as demonstrated by Yamazaki (personal communication). Fish which had been kept at 20 C for at least several days were chilled for 30 to 60 minutes in ice water to which had been added a small amount of tricaine methanesulphonate (MS-222, 0.01 %). Fish used directly from cold water stock tanks often failed to remain anaesthetized for the duration of the operation; addition of more MS-222 in these cases usually resulted in higher mortality. Anaesthetized fish were wrapped in moist paper towelling, positioned belly-up on a grooved wet sponge, and covered with crushed ice. The gular membrane on the left side was slit and a retractor inserted between the second and third gill arch to expose the roof of the buccal cavity. The dorsal buccal epithelium was then cut and folded back and the underlying parasphenoid bone drilled away with a dental burr. With practice, the location of the pituitary could be accurately determined by reference to conspicuous nerves on the surface of the bone; these nerves were cut in all hypophysectomies and sham operations. When the pituitary could be seen through the bone, probe and forceps were used to expose the pituitary which then was removed by aspiration. The cut edges of the buccal epithelium were repositioned but not sutured.

Following removal of the pituitary, fish were placed in 40 l aquaria filled with 25% sea-water ($7\overset{o}{/}_{oo}$) at 5 C and allowed to warm slowly to room temperature. Each tank had a subgravel filter and airstone but, in order to maintain salinity and avoid pH problems, no plants were added. No food was given until 3 or 4 days after the operation. All fish were kept in the dilute seawater throughout postoperative recovery, ovarian regression, hormone treatment, and behavioural testing.

A disease (or diseases) which virtually every hypophysectomized fish in my lab contracted was not identified. However, it developed with a readily identifiable set of symptoms and could be cured easily with low mortality provided treatment was begun early. The first indications of infection were a lowering of the dorsal fin, folding of the paired fins, slight loss of balance, and accelerated swimming near the surface. Within a day, areas of the body surface became reddened, apparently by enlargement and rupture of the blood vessels in the skin, and the initial symptoms were more pronounced. Treatment consists of the addition of 0.5 to 0.75 g $K_2Cr_2O_7$ to a 40 l tank. Fish undergoing treatment do not feed, apparently an effect of the drug, as healthy treated fish behave similarly. Several days following the disappearance of the reddening and the resumption of normal swimming, 50% of the aquarium water is replaced with 25% sea-water, and several days later the water is changed completely.

Preliminary tests showed that fish which had been completely hypophysectomized for more than one month (1) lost all body color, becoming a pale white, (2) had highly regressed ovaries, and (3) could not be induced to spawn in standard testing procedures.

Prior to assignment to experimental groups, prospective test females could be rejected for incomplete hypophysectomy on the basis of body coloration. This method is effective but not foolproof, presumably as pigmentation and reproduction are regulated by different populations of pituitary cells which may be removed separately in an incomplete ablation. Following behavioural testing, heads were checked for pituitary remnants under a dissecting microsope; heads were not examined histologically due to the time involved and a lack of confidence in the technique. Various aspects of ovarian histology were the final

criteria for accepting or rejecting behavioural data from fish judged on the basis of body coloration to be completely hypophysectomized. This procedure is discussed in the following section.

C. Histology of Regressed Ovaries in Intact and Hypophysectomized Fish

In interpreting the effects of various hormonal manipulations on the spawning behaviour of female goldfish, I have placed considerable emphasis on the ovarian histology of treated fish. The basic assumptions involved in this approach derive from the results of a number of studies of female teleost reproductive endocrinology which indicate that pituitary hormones stimulate growth of ovarian follicles and deposition of yolk. Correlations observed in the present study between the state of ovarian development and sexual receptivity have been used to develop histological criteria by which spawning behaviour data were accepted or discarded. The application of this procedure to various types of experiment is discussed below. A more detailed account of some aspects of the histology of regressed ovaries of intact and hypophysectomized fish is given in the Appendix.

In previous studies, the growth of goldfish oocytes has been divided into two basic stages. (Yamazaki, 1961), a first growth phase in which the oogonia develop into primary yolkless oocytes about 150μ in diameter, and a second growth phase characterized by the formation of two types of cytoplasmic inclusions, yolk vesicles (cortical granules) and yolk granules (proteinaceous yolk). Yolk vesicles first appear in oocytes about 150μ in diameter. The yolk granule stage, which does not begin until the oocyte is about 300μ in diameter, is divided into primary, secondary, and tertiary stages depending on the cytoplasmic pattern of granule distribution.

Following hypophysectomy of female goldfish, yolk-laden or second growth phase oocytes become atretic (Yamazaki, 1961, 1965). This

sensitivity of the yolky oocytes is related to stage of development. Oocytes in late yolk vesicle, primary and secondary yolk granule stages are the most sensitive and begin to degenerate within a few days of pituitary removal. Next to degenerate are the tertiary yolk stage oocytes. Least sensitive are the early yolk vesicle stage oocytes which often are present and apparently healthy several months after the operation. There is some evidence from this study that distinct changes in the distribution of the yolk vesicles precedes atresia in these oocytes. Eventually, all oocytes with yolk degenerate in the absence of the pituitary, no new yolk formation occurs, and the ovary remains in a regressed condition composed of various stages of first growth phase oocytes (Yamazaki, 1965). Published accounts suggest that in goldfish, as in other teleosts, previtellogenic oocytes are independent of the pituitary, at least to the extent that they do not degenerate following hypophysectomy. However, in the present study atresia of previtellogenic oocytes was observed many times in inteact and in hypophysectomized fish and their presence served as a valuable indicator of advanced ovarian regression. Details of previtellogenic atresia are discussed in the Appendix.

Treatment of hypophysectomized fish with gonadotropin preparations induces growth of oocytes and deposition of yolk vesicles and yolk granules (Yamazaki, 1965; Yamazaki and Donaldson, 1968). It appears that the mechanism by which the pituitary stimulates formation of yolk granules in fish is similar to that in other vertebrates (Chester Jones et al., 1972; Gallien, 1975). At the level of the ovarian follicle, gonadotropin stimulates both the production of estrogens (which induce the formation and mobilization of hepatic yolk proteins) and the uptake of yolk proteins from the blood

(Campbell and Idler, 1976; Emmersen and Petersen, 1976). Except for a study by Khoo (1974) in which estradiol, estrone, and estriol were reported to induce yolk vesicle formation in hypophysectomized female goldfish, there is no information concerning the effect of steroids on formation of this type of yolk.

Under the experimental conditions of this study, spawning behaviour could be induced without hormonal pretreatment in intact fish with ovaries in any stage of vitellogenesis: fish in which the ovaries contained only first growth phase oocytes were not receptive. For this reason, spawning behaviour data from all fish with ovaries containing any yolk are discarded from the results of experiments designed to test the ability of exogenous hormones to induce receptivity in intact fish. In this study, ovaries are described as nonvitellogenic if the oocytes contain neither yolk vesicles nor yolk granules.

In experiments involving hypophysectomized fish, the problem of distinguishing acceptable behavioural data was more difficult. Basically, data from fish judged on the basis of body coloration to be completely hypophysectomized were considered acceptable if there was histological evidence of previous and ongoing atresia. However, as treatment of hypophysectomized fish either with pituitary homogenates or with gonadotropin preparations arrested atresia and induced yolk formation, completeness of hypophysectomy in these cases was determined on the basis of body coloration alone.

Unlike the situation in intact fish, the decision to accept or reject behavioural data was based not only on the presence or absence of yolk vesicles, which may persist for months after hypophysectomy, but also on the presence or absence of degenerating previtellogenic and vitellogenic oocytes. This was complicated by the fact that ovarian histology in hypophysectomized fish is influenced by the state of ovarian

development at the time of the operation. For example, ovaries from two females sampled one month after complete hypophysectomy may show very different patterns of degeneration. The ovaries of a female spontaneously regressed at the time of hypophysectomy will show no signs of second growth atresia but likely will contain various stages of degenerating previtellogenic oocytes. In contrast, a female hypophysectomized when the ovaries were in the early stages of yolk granule formation will have extensive second growth phase atresia, but normal yolk vesicle stage oocytes will probably be present and the degeneration of previtellogenic oocytes will not have commenced.

In general, information from previous studies (Yamazaki, 1961, 1965; Khoo, 1974) on the sequence of events and time course of atresia following hypophysectomy has aided in distinguishing ongoing but incomplete ovarian regression from incomplete hypophysectomy. However, the finding (Khoo, 1974) that estrogens induce yolk vesicle formation in hypophysectomized female goldfish suggested that in hypophysectomized fish receiving steroid treatment, completeness of pituitary removal could not be accurately determined on the basis of ovarian histology. In the present study, the results of Experiment 5, equivalent in design to that carried out by Khoo, appeared to show that estradiol and 5α-dihydrotestosterone induced formation of yolk vesicles. However, as degeneration of yolk vesicle stage oocytes may not be complete by the sixth week after hypophysectomy (Yamazaki, 1965; personal observation) and start-of-treatment controls were not included in Khoo's nor in the present study, it is equally reasonable that these steroids may simply inhibit the degeneration of early yolk vesicle stage oocytes. In fact, Khoo's statement that in some oocytes the 'induced' vesicles were scattered randomly in the cytoplasm, suggests that these oocytes were in the

early stages of atresia (see Appendix). In Experiment 11 of the present study, where steroid treatment was not begun until the third or fourth month after hypophysectomy, at which time degeneration of early yolk vesicle stage oocytes is complete, the ovaries of fish receiving estradiol showed no evidence of yolk vesicle formation.

D. Behavioural Testing Procedures

All behavioural tests were carried out in 60 l aquaria supplied with an undergravel filter, 2" of quartz sand, and a floating layer of Ceratopterus. Development of low pH from the use of undergravel filters was not a problem as these tanks were set up for only short periods.

All experiments involved the induction and maintenance of sexual activity in male goldfish and most experiments also involved the induction of ovulation in females. Both techniques are simple and dependable.

For the induction of ovulation, gravid female goldfish which had a distended abdomen (preferably soft and asymetrical) and expanded ovipore were selected from the cold water stock tanks about noon on day 0 and warmed to approximately 20 C over a period of several hours. Females treated in this way were never observed to ovulate on day 1, while a highly variable proportion (presumably dependent on the state of ovarian maturity) ovulated spontaneously the morning of day 2. Intraperitoneal (i.p.) injection of approximately 3 IU /g HCG(0.6% NaCl vehicle) on the afternoon of day 1 induced ovulation by 0600 h on day 2 in virtually all cases and this technique was used routinely (for a more thorough discussion of the timing of events in goldfish ovulation, see Stacey and Pandey [1975]).

In goldfish, the paired ovaries are enclosed dorsally by ovisacs

which are separate anteriorly and which join at the posterior of the ovaries to form a short oviduct. Following ovulation, many released oocytes move dorsally between the ovarian lamellae and fill the oviduct and ovisacs. The remainder of the ovulated eggs remain within the ovary until those in the ovisacs and oviduct are gradually released through the ovipore during oviposition. Ovulation is detected by applying slight pressure to the abdomen to release a stream of eggs from the ovipore.

The technique for inducing sexual activity in male goldfish is similar to that for the induction of ovulation in females. Stock males were usually separated from females at the time of purchase and kept under standard cold water stock conditions. Two or three days before they are required for behavioural testing, males which have well developed tubercles on the opercula or on the leading rays of the pectoral fins are injected i.p. with 3 IU/g HCG and then warmed to 20 C over several hours. Males in prespawning condition spermiate on the day after being warmed and injected and usually display sufficient sexual activity the following day. Most males remain highly active for at least a week; supplementary HCG injections or exposure to spawning females usually extend this period.

In earlier experiments, only one female at a time was tested in each observation tank as it was felt that the spawning behaviour of one female might influence the behaviour of another and that, as males given a choice of females usually show marked preference for certain individuals, unequal stimulation of the females would result. Observations confirmed neither of these suspicions. To increase the number of females which could be observed at one time, and in some cases to reduce the amount of courtship directed toward each female, as many as 5 females were tested simultaneously in a 60 l tank.

The initial method of behavioural testing was to place the female in the observation tank shortly before testing was to begin. Observation commenced with the introduction of an active male which had previously been kept in an all-male tank. It soon became evident that the sexual activity of males was much greater if the female was introduced to the male's tank and if more than one male was present. Thus, in later experiments, from 3 to 5 active males were kept in each observation tank from the time they had been warmed and injected with HCG. The general level of sexual activity in each tank was assessed on the morning of testing by placing an ovulated or gravid female into each observation tank and waiting until chasing had commenced. In tanks where male activity was only moderate, the 3 most active males were used for courting test females and the remaining males were removed for the duration of the test period. Only two males were used when male activity was high (the usual case), and only one male when the activity was extremely high. In a small aquarium, excessive chasing and butting can kill a female goldfish which is unable to escape.

Behavioural testing sessions were usually of 3 h duration. Most responding females commenced spawning within the first hour of testing and stopped spawning by the end of the third hour. It was felt that extending the test period would increase the incidence of egg-binding (see experiment 2). Spawning activity was recorded on grid charts as numbers of spawning acts in each 5 minute interval of the test period.

Following most tests, females were killed by over-anaesthetization in MS-222, and the ovaries fixed in Bouins Fluid, embedded in paraffin, sectioned and stained with Mallory connective tissue stain.

E. Description of Spawning Behaviour

When an ovulated female and a spermiated male goldfish are placed together in an aquarium provided with green vegetation, the usual course of events is as follows. The male approaches the female and may make contact (especially in the region of the ovipore) or may turn away and approach several times before making contact. Usually the female does not swim quickly at this stage and often appears to present the ovipore region to the male. This occurs as a turning away from the male, sometimes with the tail higher than the head. Especially when the male fails to investigate a receptive female, it may be observed that the female will approach the male, sometimes making contact, but usually swimming past the head of the male. Normally, males take the initiative in investigating the female, and the female appears to play a passive role.

The male soon begins to butt the female (pushing the female with his snout) in the region of the belly, ovipore and caudal peduncle if the female is swimming away, and in virtually any part of the body if the female remains stationary. A chase develops, the female swimming at a moderate speed, the male either swimming very close behind, or swimming beside and maintaining contact with the female. If green vegetation is not present, the female does not perform oviposition behaviour though some eggs may be dropped, apparently inadvertently due to the pressure of the male.

The female initiates spawning behaviour by performing a 'rise'. In this, she swims up toward floating vegetation, pushes the snout in (the head may or may not be covered), and occasionally mouthes the vegetation. Typically, rising is followed by a spawning act(oviposition). The female enters the vegetation and turns on her side, as the male parallels this motion from slightly behind and below. The pair

(female above, male below) then quickly swims up, breaking the water surface, and then down, leaving the vegetation. It is during this rapid, synchronized rising and arching, in which the male appears to attempt to push the female out of the water, that the eggs and sperm are released. Whether the female exercises any control over egg release during the spawning act is not known; however, even slight pressure against the belly such as occurs during prespawning chasing is sometimes sufficient to release some eggs. Netting ovulated females invariably causes egg release.

Following emergence from the vegetation, the female may resume swimming at a moderate speed, followed closely by the male. More often, the female will return to the vegetation and complete one or several spawning acts in quick succession. This 'clumping' of spawning acts interspersed with periods of chasing and butting agrees with Yamazaki's (1965) description.

Rising does not always lead to completion of a spawning act. Most females which have not begun to spawn will perform a number of rises before completing the first spawning act, after which the ratio of rises to spawning acts decreases. Occasionally, the male is responsible for the failure of rising to lead to spawning, either because the speed of his approach startles the female, or because of incorrect orientation to the female. In the cases where rising does not lead to spawning, the female either swims quickly away from the vegetation or remains motionless in the vegetation for as long as several minutes, not responding to the actions of the male.

Though female goldfish will sometimes spawn on submerged vegetation, and a minority of individuals seem to prefer this site (B.Partridge, personal communication), the majority of ovipositions occur on floating

vegetation both in the laboratory and under natural conditions (Innes, 1949). Due to the problem of distinguishing a spawning act on submerged vegetation from an attempt by the female to avoid the male, and because only floating vegetation was provided in warm water stock tanks, only floating vegetation was used in experimental situations.

Spawning may continue for several hours and involve several hundred spawning acts. Generally, termination of spawning is coincident with the shedding of all ovulated eggs, though on occasion small numbers of eggs have been removed from females which had ceased spawning behaviour (Yamazaki, 1965; personal observation). Though this was observed on only a few occasions and was not investigated further, it appeared that females which had finished spawning (i.e. had shed all ovulated eggs) inhibited the male's chasing and butting by increasing the swimming speed and becoming very effectively evasive for a short period of time.

Observations by myself, and by others (Innes, 1949) under more natural conditions, suggest that the courtship of male goldfish is a highly competitive, vigorous, and lengthy event. In experimental situations where 3 or 4 males were placed with an equivalent number of females in 60 l aquaria, one male invariably initiated courtship. Usually, this quickly stimulated courtship of the same female by one or more of the remaining males. In fact, the competitive stimulation of courtship between males was so reliable, that adding a new, active male to an experimental tank to stimulate courtship in inactive resident males became standard experimental procedure. There is evidence (Partridge, Liley, and Stacey, 1976) that male goldfish are attracted to and stimulated to court female goldfish by a pheromone, probably of ovarian origin.

This description of events in a normal spawning situation has ignored the question of whether variability in the stimulus provided by the male is a significant factor in the responsiveness of the female. This problem was not

investigated experimentally as several observations indicated that provided the male is sufficiently aroused to complete the spawning act, the intensity of male courtship has no obvious effect on the spawning behaviour of the female.

For example, on many occasions in which receptive females (either naturally ovulated or with experimentally induced receptivity) had been placed with relatively inactive males which had not begun to court, the female was seen to rise (a behaviour which usually elicits rising even in inactive males) and, after allowing the male to position, perform a normal spawning act without any of the usual preliminary investigation, butting, or chasing on the part of the male. This is not evidence that prespawning courtship has no stimulating effect on the female, but it does demonstrate that it is not prerequisite. Furthermore, in the multi-male, multi-female testing procedure used in many of my experiments, the males invariably showed a preference for certain of the females. While most initially 'unattractive' females became and remained attractive after they had begun to spawn, the usual situation was that certain females consistently elicited more courtship throughout the test, regardless of their spawning behaviour. In extreme cases, one or more females which never spawned throughout a test received almost constant courtship, while other spawning females were attended only at the times they entered the floating vegetation.

These observations have been interpreted as indicating that the occurrence and rate of spawning are functions of the physiology of the female goldfish. Obviously, the presence of the male is required at the time of the oviposition behaviour, but provided the male is sexually active enough to respond to the stimulus of a rising female and complete the spawning act, the intensity of the male's other courtship activities do not affect the spawning behaviour of the female. This view is at odds with that of Yamazaki (1965)

who suggests, without providing data, that prespawning courtship or chasing by males stimulates both ovulation and oviposition.

An important problem in the recording of female goldfish spawning behaviour is the choice of an appropriate parameter with which to measure sexual receptivity (the tendency to perform spawning behaviour). The apparent lack of female prespawning behaviours leave only two obvious indices of receptivity, rising behaviour and the spawning act.

Though rising behaviour might at first appear to be an acceptable measure of sexual receptivity, it has several serious drawbacks. The most important is that behaviour similar to rising occurs commonly in a nonsexual context in both males and females. Second, though each spawning act is necessarily preceded by a rise, the tendency for rising to be disrupted prior to the completion of a spawning act usually results in the performance of many more rises than spawning acts. This is particularly evident when spawning behaviour is beginning or is proceding at a low frequency. As noted previously (page 17), aspects of male spawning behaviour may alter the relation between the numbers of rises and spawning acts.

The spawning act is a distinctive, stereotyped, and easily quantified behaviour which is restricted to a sexual context; it is the only measure of sexual receptivity used in this study. The term receptivity is used strictly in a behavioural sense and implies nothing about underlying physiological mechanisms.

CHAPTER III

THE ROLE OF OVULATED EGGS IN THE SPAWNING BEHAVIOUR OF FEMALE GOLDFISH

A. Experiment 1. Effect of Removal and Replacement of Ovulated Eggs on the Spawning Behaviour of Ovulated Goldfish

1. Introduction

That ovulated eggs within the ovary are involved in the induction of spawning behaviour in female goldfish was suggested by Yamazaki (1965) whose observations on the effects of egg removal led him to speculate that 'ripe eggs in the ovarian lumen stimulate the spawning behaviour of females via some pathway'.

In the present study, preliminary examination of the role of ovulated eggs in stimulating spawning behaviour utilized several simple techniques. Yamazaki's results were repeated. Spawning ceased if all ovulated eggs were removed by hand-stripping (gently squeezing the area between the pectorals and the ovipore between moistened fingers). As stripping only a portion of the eggs did not terminate spawning, it was concluded that the effect of complete egg removal was not due to disturbance or injury, but rather to the absence of ovulated eggs, as Yamazaki had suggested.

It was found that the use of an 'oviduct plug' to prevent the release of ovulated eggs greatly extended the duration of spawning in ovulated fish. Whereas the normal duration of spawning is less than two hours, 'plugged' females would spawn throughout the day of ovulation and sometimes continue the following day.

The following experiment was carried out to determine whether placement of ovulated eggs in the ovarian lumen would restore rather than simply prolong spawning.

2. Materials and Methods

Females which had ovulated spontaneously (in response to warming but

without HCG injection) the morning of the test day were taken from holding tanks and placed singly in 60 l observation tanks. A spermiated male was introduced into each tank after 20 to 30 minutes. The latency to the first spawning act and the number of spawning acts in the following 20 minutes provided a record of the normal spawning activity of each fish. Immediately following this initial 20 minute spawning period, each fish was removed, anaesthetized in MS-222, and the eggs removed and stored in egg-injection syringes. For egg removal, females were dried gently with paper towelling and hand-held such that the ovipore was over (and the anal fin outside) a small plastic cup of about 5 ml capacity. It is essential to use plastic apparatus when handling ovulated eggs, as eggs will eventually adhere to glass. Furthermore, eggs must not be allowed to come into contact with water as this causes adhesion and hardening. Eggs are released into the cup by gentle pressure against the belly and drawn up into a plastic 1 ml syringe fitted with bevelled PE tubing.

Preliminary tests had shown that a single sequence of stripping removed only a portion of the ovulated eggs even if considerable pressure was exerted on the belly; when stripping was repeated in 10 or 15 minutes, additional eggs could usually be expressed with only slight pressure. It is believed that it is the eggs in the oviduct and in the posterior portions of the ovisacs which are removed during each stripping. Following removal of these accessible eggs, additional ovulated eggs move dorsally along the channels between the ovarian lamellae and into the ovisacs and oviduct, from which they can be removed by further stripping.

Following egg removal the fish were revived and returned to the observation tanks where the behaviour of male and female was recorded for one

hour. During the first 30 minutes, the females were removed at 10 minute intervals and squeezed to remove remaining ovulated eggs; for the second 30 minutes, the pairs were left undisturbed. The purpose of this one hour observation period was to ensure that egg removal was complete and that spawning had terminated. In preliminary tests it had been found that low levels of spawning activity occurred if even a small number of ovulated eggs remained in the oviduct.

Following the observation period, the females were removed from the observation tanks, anaesthetized in MS-222, and given one of the following treatments:

Group I - a no-treatment handling control in which each fish was simply revived and returned to the observation tank,

Group II - an ovipore plug control in which each fish was fitted with an ovipore plug, revived, and returned to the observation tank with the male,

Group III - an egg-injection treatment in which each female was injected with its own ovulated eggs, fitted with an ovipore plug, revived, and returned to the spawning tank with the male.

Oviduct plugs consisted of PE tubing (various diameters) with a fire-polished glass plug in the proximal end and recurved barbs cut in the distal end (total length 4-8 mm). In inserting the plugs, it was often helpful to first insert a fine glass probe into the oviduct and stretch the anterior margin (the junction with the anus) forward. This precaution reduces the chance of placing the plug in the rectum. No attempt was made to replace all the eggs removed from each Group III female as preliminary tests had shown that this usually resulted in the rupture of the oviduct

and failure to induce spawning behaviour. Each female was injected with approximately 0.025 ml eggs per gram body weight.

Though the time between treatment of each fish and its return to the observation tank was not constant (as more than one fish was treated at a time), effort was made to minimize this interval. Thus, of the 33 fish tested, all but one were returned to the observation tanks within 10 minutes of treatment. Time of the first spawning act and all subsequent spawning activities were recorded for one hour.

3. Results and Discussion

There were no differences between the three treatment groups in the latency to the first pretreatment spawning act following initial introduction to the male, nor in the number of spawning acts in the 20 minute pretreatment period (Table I). Removal of ovulated eggs was highly effective in terminating spawning behaviour; only one of 11 control fish (Group I) spawned during the posttreatment period. The injection of ovulated eggs (Group III) restored spawning behaviour. This is obvious whether the number of fish responding or the spawning rate of responding fish is considered. Presence of an ovipore plug may have contributed to the spawning behaviour of Group III fish, as more fish spawned following treatment with an ovipore plug (Group II) than following the control treatment (Group I); however, the posttreatment responses of Group I and Group II were not significantly different (Mann-Whitney U-test).

A number of informal tests conducted concurrently with the present experiment showed that the ability of egg injection to induce spawning behavior is not restricted to the day of ovulation. Injection of eggs from

TABLE I

EFFECT OF REMOVAL AND REPLACEMENT OF OVULATED EGGS ON THE SPAWNING BEHAVIOUR OF OVULATED FEMALE GOLDFISH

Group	Treatment	Pre-Treatment Spawning: Latency (min)	Pre-Treatment Spawning: No. Spawning Acts/20 min.	Post-Treatment Spawning: No. Spawning Acts/20 min.*	Post-Treatment Spawning: % Restored
I	No Treatment	6	34	0	0
		6	60	0	0
(n=11)		5	21	0	0
		8	17	0	0
		6	25	0	0
		7	18	0	0
		50	41	7	17
		200	14	0	0
		6	56	0	0
		41	31	0	0
		4	33	0	0
		mean = 14.4	mean =31.8	(1/11)	
II	Ovipore Plug	19	44	0	0
		15	20	0	0
(n=11)		20	26	0	0
		2	55	0	0
		3	38	0	0
		10	54	10	18
		4	40	1	2
		5	25	0	0
		15	38	4	10
		2	40	11	27
		2	56	0	0
		mean = 8.8	mean= 39.6	(4/11)	
III	Ovulated Eggs	20	12	10	83
	and	6	57	37	65
	Ovipore Plug	4	60	57	95
		6	20	20	100
(n=11)		10	29	20	69
		5	30	7	23
		8	59	56	94
		4	26	46	177
		5	30	31	103
		3	40	8	20
		22	9	15	167
		mean = 8.4	mean= 33.8	(11/11)	

* = No. spawning acts in the most active 20 minute period of spawning in the test hour. No difference between groups in pretreatment spawning behaviour (Mann-Whitney U test).

ovulated donor females consistently induced normal spawning behaviour both in females which had ovulated as much as a month or more before being tested and in fish which had failed to ovulate after transfer to warm water.

These results both support Yamazaki's hypothesis concerning the stimulatory effect of ovulated eggs on spawning behaviour and demonstrate that the normally sequential processes of ovulation and oviposition can be easily dissociated. The simple technique of egg injection thus allows the endocrine regulation of sexual behaviour to be examined independently of the control of ovulation. This is critical when it is realized that the traditional approach of ovariectomy and replacement therapy is rather inappropriate in this case due to the dependence of the female's spawning behaviour on internal cues provided by eggs in the ovarian lumen.

B. Experiment 2. Induction of Spawning Behaviour by Injection of Egg Substitutes

1. Introduction

Although the technique of egg injection is a simple and reliable method for inducing spawning behaviour, it is associated with three obvious problems:

(i) the necessity, and occasional difficulty, of inducing ovulation in egg-donor females,

(ii) the possibility of differences in stimulus quality of eggs from donor females, and

(iii) the tendency for eggs to become adhesive (bound) on contact with water and thus to lose the ability to stimulate spawning.

Egg binding was a common problem and reduced final sample sizes in all experiments requiring egg injection. Binding sufficient to inhibit spawning usually occurs as hardened eggs attached to the withdrawan ovipore plug or as a small cluster of eggs which may be expressed with some difficulty from the ovipore by pressure on the belly. In some cases, binding may involve the hardening and adhesion of the entire mass of injected eggs. In cases where fish with bound eggs were not inhibited from spawning, it was assumed the binding had occurred after the onset of spawning behaviour. Data for all non-responding fish for which there was any suggestion of egg binding were discarded.

Finding an injectable egg substitute for the induction of spawning would eliminate the difficulties of using eggs and greatly expedite experiments involving the artificial induction of spawning behaviour (i.e., spawning without ovulation). To this end, three potential egg substitutes

have been tested; petroleum jelly, gelatin, and Dow Corning 200 Silicone Fluid.

2. Methods, Results, and Discussion

The discouraging results of the preliminary tests did not justify a full-scale experiment to test egg substitutes. Test conditions were not standardized and varied in such aspects as duration of the test period, the time elapsed since the previous ovulation of the test female, and whether or not an ovipore plug was used (Table II).

Petroleum jelly and Dow Corning 200 Silicone Fluid were simply drawn up in an egg injection syringe and injected. Gelatin was dissolved in warm dechlorinated tapwater at two concentrations, 0.3 g/ml and 0.6 g/ml, and stored overnight at 4 C. Injection was as for petroleum jelly and the Silicone Fluid.

As seen in Table II, both gelatin and petroleum jelly induced spawning behaviour in some fish, although the response to these substances was much less than that obtained with egg injection.

These limited results demonstrate that the ability to induce spawning behaviour is not a property unique to ovulated eggs and that in some fish physical cues alone may be sufficient to induce the response. The failure of the injected materials to duplicate the response to ovulated eggs is believed to result from their inability to duplicate the physical stimulus provided by eggs. The inability of physically altered (adhesive and hardened) bound eggs to induce spawning supports this contention.

An alternative explanation for the low stimulus quality of the tested egg substitutes is that they lack some chemical stimulant normally associated with ovulated eggs or fluid. The fact that binding of only a few eggs

TABLE II

EFFECT OF 'EGG SUBSTITUTES' (PETROLEUM JELLY, GELATIN, AND SILICONE FLUID) ON SPAWNING BEHAVIOUR OF RECEPTIVE FEMALE GOLDFISH

Treatment	Ovipore Plug Used	Condition of Fish	Duration of Observation Period (hours)	No. of Spawning Acts
Ovulated Eggs	+	1 week POV*	3	260
	-	"	1.5	65
Petroleum Jelly	+	"	2	0
	+	"	2	0
	-	"	3	0
	+	"	3	0
	+	"	3	3
	-	"	3	22
	+	"	3	108
Gelatin (low concentration)	-	"	2	0
	-	"	2	0
	-	"	2	0
Gelatin (high concentration)	-	"	2	35
	-	"	2	0
	-	"	2	0
	-	preovulatory	2	0
Silicone Fluid	-	1 week POV	1.5	0
	-	"	1.5	0

* Tested 1 week after ovulation

near the ovipore will often completely inhibit spawning even though the remainder of the injected eggs appears normal, would seem to argue against this alternative.

C. Summary of Chapter III

1. The duration of spawning behaviour of ovulated fish is extended by plugging the ovipore to prevent egg release.

2. In ovulated fish, spawning behaviour is terminated by removing ovulated eggs. Spawning behaviour is restored by injecting ovulated eggs through the ovipore and into the ovarian lumen.

3. Injection of ovulated eggs into preovulatory and postovulatory fish induces spawning behaviour within several hours.

4. On contact with water, ovulated eggs become hard and adhesive and lose the ability to induce spawning behaviour.

5. Several substitutes for ovulated eggs (gelatin, petroleum jelly) induce spawning behaviour when injected into the ovarian lumen; these substances are less effective than ovulated eggs.

CHAPTER IV

THE EFFECT OF STEROIDS ON THE SPAWNING BEHAVIOUR OF FEMALE GOLDFISH

A. Experiment 3. Steroid-Induced Spawning Behaviour in Intact, Regressed Female Goldfish

1. Introduction

Normally, gonadal maturation and ovulation in goldfish are accelerated by raising the water temperature to 20 C. However, the ovaries of spent or mature fish kept at 20 C for an extended period (2 to 3 months is usually sufficient) become regressed (Khoo, 1974); virtually all oocytes are in the previtellogenic stage although some may contain small yolk vesicles (Yamazaki, 1961, 1965). Since vitellogenesis resumes if regressed females are returned to cold water (12 C) soon after spawning, temperature regression appears not to result from an incapacity of the post-spawning reproductive system, but rather from an inhibition of the system by prolonged high temperature. The mechanism by which high temperature prevents ovarian recrudescence is not known; however, there is some evidence (Scruggs, 1951; Nagahama, 1973) that gonadotropin production is low in the post-spawning period. Examination of a similar phenomenon in another teleost, Gillichthys mirabilis, suggests the mechanism may operate by a decrease both in gonadotropin secretion and in the response of the gonad to gonadotropin (De Vlaming, 1972). Presumably, the functional significance of this temperature-induced regression is that the stimulatory effect of elevated temperature on gonadal maturation is confined to the early part of the warm season when rearing conditions for the fry are optimal.

Injection of ovulated eggs into temperature-regressed fish invariably failed to induce spawning behaviour. Khoo (personal communication) found that the activity of steroid dehydrogenases is very low in intact, regressed female goldfish and Liley (1972) has shown that estradiol is effective in

restoring the sexual receptivity of both hypophysectomized and ovariectomized female guppies. On the basis of these findings, it seemed a reasonable hypothesis that the lack of a spawning response to egg injection was due to reduced steroid, in particular to reduced estradiol, production.

To test this hypothesis, an experiment was carried out which showed that injection of estradiol restored the spawning of temperature-regressed fish in response to egg injection. As there was no reason to believe that estradiol is the only steroid capable of inducing spawning behaviour, the effects of other steroids were also examined; these additional experiments revealed an unexpected lack of specificity in the behavioural response to steroid treatment.

Ten steroids known to be synthesized by teleost ovaries (Eckstein, 1970; Eckstein and Katz, 1971; Lambert et al., 1971; Colombo and Belvedere, 1976; Ozon, 1972) were tested; cholesterol, pregnenolone, 17α -OH-pregnenolone, progesterone, 17α -OH-progesterone, dehydroepiandrosterone, androstenedione, testosterone, 11-ketotestosterone, and 17β -estradiol. The effects of estrone, estriol, 5α -dihydrotestosterone, and androsterone were also examined. Dehydroepiandrosterone, androstenedione, testosterone, estriol, estrone, and estradiol were chosen as they induce sexual behaviour in female mammals (Beyer et al., 1970, 1971) and because the latter three also stimulate receptive behaviour in female guppies (Liley, 1972). Cholesterol, pregnenolone, 17α -OH-pregnenolone, and 17α -OH-progesterone do not stimulate sexual behaviour in female mammals and were not expected to induce spawning behaviour. Similarly, it was thought that treatment with progesterone would not stimulate spawning behaviour. Facilitation by progesterone of sexual behaviour in female rodents requires estrogen pretreatment (e.g.,

Joslyn et al., 1971) and in ovariectomized female guppies progesterone fails to restore receptivity (Liley, 1972). The 5α-reduced androgens, androsterone and dihydrotestosterone, were tested to examine the possibility that androgens stimulate spawning behaviour following aromatization to estrogens; there is evidence that in mammals 5α-reduced steroids are not aromatized (Thompson et al., 1971).

B. Materials and Methods

Although this study is presented as a single experiment, it is in fact a series of small experiments carried out over a period of a year and a half. Tank facilities were such that it was not possible to obtain regressed fish and preovulatory egg-donor females in sufficient numbers to perform the entire experiment at one time. Each small experiment tested the effects of several steroids and a saline control injection. Test fish had been kept at 20 C for 3 to 9 months and were assumed to be regressed. Assignment to test groups was not random, but was arranged so that the 4-6 fish in each 40 l aquarium could be identified individually by morphological characters or color patterns. Only one treatment group was kept in each tank.

Steroids (Sigma) were ground to a fine powder in a Misco homogenizer, suspended at a concentration of 2.5 µg/µl in 0.6% NaCl containing 4 drops Tween 80/100 ml and injected at a dosage of 20 µg/g. Suspensions were made up 10 ml at a time and kept in sealed centrifuge tubes at 4 C. They were discarded when depleted to 4-5 ml as a precaution against degradation and changes in concentration.

Fish were lightly anaesthetized in MS-222 prior to injection and laid on a wet paper towel. The steroid suspension was agitated immediately prior

to injection, which was always intraperitoneal through the right body wall, slightly above and behind the base of the pelvic fins. The needle (25-30 gauge) was inserted at least 1/2" through the body wall to minimize the efflux of injected vehicle. Following injection, fish were immediately returned to the holding tank to recover. Each fish received 5 injections on alternate days over a 9 day period. On the morning of the day following the final injection (occasionally the following day if ovulated eggs were not available) fish were anesthetized in MS-222, injected with ovulated eggs (approximately 0.025 ml/g), fitted with an ovipore plug, and left to recover in 2 l beakers for 30 to 60 minutes. Fish were then placed in 60 l observation aquaria containing actively courting males and spawning behaviour was recorded for 3 hours. In some of the later tests in which more females than males were placed in each observation tank and the chance of injected eggs being expressed during chasing was low, ovipore plugs were not used.

Following the 3 hour test period, females were removed from the observation tanks and anaesthetized and the ovipore plugs were carefully removed. The fish were then checked for binding of the injected eggs by gently squeezing the belly near the ovipore with a moistened finger, and egg-bound, non-responding fish discarded from the sample. All fish were sacrificed and the ovaries fixed and prepared for histological examination.

C. Results

Of 191 'regressed' fish which received steroid or saline injections in this experiment, only 41 individuals achieved the requirements of successful egg injection without binding, and complete absence of yolky

oocytes in the ovaries. The spawning data for these fish are presented in columns I and II of Table III. Spawning scores in columns III, IV, and V are from incompletely regressed fish with ovaries in the early stages of yolk vesicle formation. Ovaries of fish in column III contained some yolk vesicle stage oocytes; as they also contained early stages of degenerating yolk vesicle oocytes, it appeared that temperature regression had not yet been completed. In contrast, as the ovaries of the few fish in column IV contained some oocytes in the yolk vesicle stage, but had only advanced atretic yolk vesicle oocytes, it appeared either that temperature regression had been arrested prior to completion, or that vitellogenesis had recommenced. The fish in column V showed no signs of atresia and were in the early stages of yolk vesicle formation. The histological differences between vitellogenic (stages III, IV, and V) and nonvitellogenic ovaries (stages I and II) were much more obvious than were the differences between stages I and II or among stages III, IV, and V. In vitellogenic ovaries, oocytes containing yolk vesicles were usually quite numerous. On the other hand, the incidence of degenerating oocytes was nearly always low and it is quite likely that if larger portions of the ovary had been examined, some stage IV and V ovaries would have been reclassified as stage III. Data from egg-bound fish and from fish with advanced yolk formation are not included in Table III.

None of the 10 regressed control females injected with saline (columns I and II) performed any spawning acts during the 3 hour test period. Of the 5 saline-treated fish (column III) which had some early yolk vesicle stage oocytes plus signs of ongoing regression, only one individual performed one spawning act. In contrast, 4 of the 5 females in columns IV and V performed high levels of spawning during the test period.

TABLE III

EFFECT OF STEROID TREATMENTS ON SPAWNING BEHAVIOUR OF FEMALE GOLDFISH WITH TEMPERATURE-REGRESSED OVARIES

Treatment	No. of spawning acts of fish with ovaries in stage				
	I	II	III	IV	V
Saline	0	0	1	19	16
	0	0	0		0
	0	0	0		32
	0	0	0		95
	0	0	0		
Cholesterol			0		0
			0		0
			0		
			46		
			29		
Progesterone					0
					0
					0
					0
17∝-OH-Progesterone	0	0			
	0				
	0				
	0				
Pregnenolone	17		12	58	
			33		
17∝-OH-Pregnenolone	10	56	19	83	
		101			
Dehydroepiandrosterone			1		
			1		
			209		
			23		

Treatment	No. of spawning acts of fish with ovaries in stage				
	I	II	III	IV	V
Androstenedione	70	109	77		0
	77		44		
Testosterone	36		80		54
	7		176		
			314		
Estradiol	4	100	33	14	86
	119		0	38	40
			3		43
			131		6
			14		
			56		
			38		
Estriol	0		0		
			6		
			14		
Estrone	0	0	0		
			33		
			32		
Dihydrotestosterone		23	8		24
		32			16
					40
					36
11-Ketotestosterone	86		77		48
	15				
Androsterone	32	106	21		45
	7	52	17		82
	59		28		0
	18		83		
	55				

Stage I (no yolk vesicles; degenerating primary oocytes) and Stage II (no yolk vesicles; no degenerating primary oocytes) ovaries are completely regressed. Stage III (yolk vesicles; degenerating secondary oocytes), Stage IV (yolk vesicles; advanced degenerating secondary oocytes), and Stage V (yolk vesicles; no degenerating oocytes) ovaries are not completely regressed. Spawning scores are total number of spawning acts per 3 hour test period. Data from fish with advanced stages of yolk formation not included in table.

On the basis of the above histological interpretation, and of the relation between spawning behaviour and ovarian histology in saline-treated fish, it is suggested that only data from fish with nonvitellogenic ovaries (stages I and II) be accepted as evidence for the action of exogenous steroids on spawning behaviour. Many fish with ovaries in stages III, IV, and V probably were receptive prior to receiving steroid treatments.

In contrast to the lack of response of the control group, regressed individuals in groups treated with 17∝-OH-pregnenolone, androstenedione, testosterone, estradiol, dihydrotestosterone, 11-ketotestosterone, and androsterone performed high levels of spawning following egg injection. The regressed fish treated with pregnenolone responded to egg injection, suggesting that this steroid also affects spawning behaviour. Estriol and estrone appear to be ineffective, although the sample sizes are small.

17∝-OH-progesterone does not restore receptivity in regressed fish; none of 5 individuals tested showed any spawning response. There is no information as to the effectiveness of progesterone in restoring receptivity as none of the group receiving progesterone were completely regressed. However, it is of interest that none of these four marginally vitellogenic fish displayed any spawning behaviour following egg injection. This result may simply indicate a period of poor egg injection technique. Alternatively, it suggests an inhibitory effect of progesterone on responsiveness to egg injection in fish commencing ovarian maturation. Two other progesterone-treated fish with much more advanced yolk formation (not shown in Table III) spawned normally following egg injection.

No fish in the groups injected with cholesterol or dehydroepiandrosterone had completely regressed ovaries.

D. Discussion

The results of this experiment demonstrate that a variety of steroids restores the responsiveness of temperature-regressed fish to injection of ovulated eggs. The mechanism of action of the effective steroids is not known. They may stimulate central nervous structures regulating sexual behaviour, or may have some peripheral effect in sensitizing the fish to the stimulus of ovulated eggs in the oviduct. The only conclusion which can be drawn regarding the diversity of effective steroids is that under the conditions of this experiment the mechanism controlling sexual behaviour does not appear to be highly steroid specific. There are several obvious explanations which could account for this.

One explanation may simply be that the dosage of steroid used was pharmacologically high and thus specificity was masked. Certainly the dosage used in this study is much higher than those found effective in inducing receptivity in female mammals. However, in a study (Liley, 1972) which employed essentially the same dosage as in the present work, estradiol restored sexual behaviour of ovariectomized female guppies, and testosterone was ineffective.

Another plausible explanation is that the ovary of the temperature-regressed goldfish possesses an active steroidogenic pathway capable of converting exogenous steroids to one or more behaviourally active forms. Such an interpretation is not at variance with the reported steroidogenic potential of the previtellogenic ovary of the eel, Anguilla (Colombo and Belvedere, 1976).

The results of this experiment also emphasize that fish with ovaries in advanced stages of regression are responsive to the injection of eggs,

provided that the ovaries contain at least some yolk vesicle deposition. Thus it is evident that the mechanism regulating spawning behaviour is functional under endocrine conditions which likely are very different from those present at the time of ovulation.

The present experiment provides no evidence as to whether the exogenous steroids act directly to influence behaviour, or exert some action on or with the pituitary. The following experiments, using hypophysectomized fish, attempt to answer this question.

B. Experiment 4. Ineffectiveness of steroids on spawning behaviour of hypophysectomized female goldfish

1. Introduction

In Experiment 3 it was shown that a number of steroids restore spawning in response to injection of ovulated eggs in intact, nonreceptive, temperature-regressed female goldfish. Liley (1972) has shown that pituitary hormones are not necessary for the expression of sexual behaviour in the female guppy; estradiol injections alone restore the receptivity of hypophysectomized fish. To determine whether the pituitary is involved in the action of steroids on female goldfish spawning behaviour, an experiment similar to Experiment 3 was performed with hypophysectomized fish. Seven steroids were tested. Estradiol and testosterone were used as they were known to be effective in intact fish. Although some evidence suggests they are ineffective in intact female goldfish, estrone and estriol were used as they are effective in the female guppy (Liley, 1972). As deoxycorticosterone has been shown to induce ovulation and oviposition in hypophysectomized female Heteropneustes (Sundararaj and Goswami, 1966), this steroid was also tested. Progesterone was also tested and cholesterol was used as a control.

2. Materials and Methods

Females which had been kept at 20 C for 4 to 6 months and therefore were assumed to have regressed ovaries, were hypophysectomized and kept in 25% seawater for 4 to 9 weeks before beginning steroid treatment. Preparation, dosage, and frequency of injection of steroids was as described in Experiment 3. However, due to difficulties in inducing ovulation in donor females, the duration of treatment varied from 7 days (4 injections) to 19 days (10 injections). Collection and injection of ovulated eggs were as

described in Experiment 1 and testing procedures were as described in Experiment 3.

3. Results

As this experiment was carried out before discovery of the treatment for 'red disease', mortalities were high and the sample sizes are small.

Of 24 fish surviving the treatment, the data from six were discarded as the fish had bound eggs. None of the remaining 18 (estradiol - 7; estriol - 3; estrone - 2; testosterone - 1; progesterone - 3; cholesterol - 2) displayed any spawning activity during the three hour test period. No fish treated with deoxycorticosterone survived to be tested.

4. Discussion

It appears that hypophysectomy interferes in some way with the ability of exogenous steroids to potentiate the spawning response to injected eggs. This may indicate either that the effect of steroids on spawning behaviour of intact fish is mediated by the pituitary, or that a pituitary factor is required in addition to the steroid. It is also possible that the mechanism regulating spawning behaviour requires stimulation by a steroid or steroids other than those used in this experiment.

In the female rat, there is evidence that the lordosis response to exogenous steroids is decreased if the hormonal treatment is preceded by a prolonged period of hormonal deprivation (Damassa and Davidson, 1973; Beach and Orndoff, 1974). Thus the ineffectiveness of steroids in this experiment may have been due to a decline in sensitivity to steroids in the 4 to 9 week interval between hypophysectomy and the initiation of treatment (the period of hormone deprivation may have been longer as the fish most likely were regressed prior to hypophysectomy). The following experiment explores this latter possibility.

C. Experiment 5. Ineffectiveness of Long-Term Steroid Treatment on Spawning Behaviour of Hypophysectomized Female Goldfish

1. Introduction

In Experiment 4 it was suggested that the ineffectiveness of steroids in inducing sexual behaviour of hypophysectomized female goldfish might be due to a decrease in responsiveness to steroids following hypophysectomy. A similar phenomenon is reported to occur in female rats following ovariectomy (Beach and Orndoff, 1974; Damassa and Davidson, 1973).

In goldfish, where ovarian development is normally very prolonged, hormonal priming of sexual behaviour is likely to occur over an extended period. In addition, if the hypothesis that unreceptive, temperature-regressed fish are capable of in vivo steroid conversion is correct, it is likely that the hypophyseal-gonadal axis is active, although operating at a level insufficient to induce oocyte growth, vitellogenesis, and sexual receptivity. Thus, the degree of hormonal deprivation and the time required to restore receptivity by steroid treatment may be greater in hypophysectomized than in temperature-regressed fish. To examine this possibility, I carried out an experiment similar to Experiment 4, except that the interval between hypophysectomy and initiation of steroid treatment was shorter and the duration of steroid treatment was longer. Two steroids (estradiol and dihydrotestosterone) were tested, each known to be effective in restoring receptivity in intact, temperature-regressed fish (Experiment 3).

2. Materials and Methods

Female goldfish which had been warmed to 20 C several weeks earlier were hypophysectomized and kept in 25% seawater for a further 3 to 4 weeks

before being assigned to the steroid or saline treatment groups. Preparation and injection of steroids and procedures for handling and testing fish were as described in Experiment 4. However, the period over which fish received injections was increased to 40 days; injections were given every fourth day for the first 20 days and on alternate days for the next 20 days.

3. Results and Discussion

Of 21 fish which survived the injection schedule, one was too sick to test and 5 had bound eggs. None of the remaining 15 fish (saline - 5; estradiol - 5; dihydrotestosterone - 5) showed any spawning activity during the three hour observation period.

It has been postulated (Experiment 4) that the prolonged steroid deprivation following hypophysectomy decreased the sensitivity of goldfish target tissues to exogenous steroids; a decrease in the concentration of steroid receptor proteins may account for this proposed insensitivity. There is evidence that the concentration of estrogen receptor in the rodent uterus is influenced by estrogen (see review by Milgrom et al., 1973). However, it is doubtful whether the lack of effect of the 40-day treatment in the present experiment is the result of insufficient steroid priming, as the binding capacity of mammalian tissue increases within hours of exposure to estrogen (Milgrom et al., 1973). It is emphasized, however, that in the present experiment treatment was not begun until as much as a month after hypophysectomy; the possibility that responsiveness to egg injection might be maintained or restored by earlier treatment has not been examined.

The failure of exogenous steroids to restore sexual behaviour of hypophysectomized female goldfish differs from the effects of similar treatments

in female rats (Pfaff, 1970) and guppies (Liley, 1972). As steroids induce receptivity in intact but not in hypophysectomized goldfish, it is suggested that some pituitary factor(s) may be involved in the regulation of spawning behaviour. The experiments described in Chapter V were carried out to explore this possibility.

D. Summary of Chapter IV

1. In female goldfish with 'regressed' (nonvitellogenic) ovaries, injection of ovulated eggs does not induce spawning behaviour.

2. A number of steroids restore responsiveness to egg injection in regressed fish.

3. Hypophysectomy abolishes the spawning response to injection of ovulated eggs.

4. Steroid treatments do not restore responsiveness to egg injection in hypophysectomized fish.

CHAPTER V

ROLE OF THE PITUITARY

IN THE SPAWNING BEHAVIOUR OF FEMALE GOLDFISH

A. Experiment 6. Effect of Pituitary Replacement on the Spawning Behaviour of Hypophysectomized Female Goldfish

1. Introduction

In previous experiments, steroid injections restored the sexual behaviour of intact, temperature-regressed fish whereas similar treatment of hypophysectomized fish was without effect. These results suggested the involvement of the pituitary in the control of responsiveness to egg injection. To examine this possibility, hypophysectomized fish were injected either with estradiol or estradiol plus homogenized goldfish pituitaries, and tested for the ability to spawn following the injection of ovulated eggs.

2. Materials and Methods

Female goldfish were kept at 20 C for 1 to 2 weeks, hypophysectomized (or sham operated), kept a further 2 weeks in 25% seawater, and assigned to one of three treatment groups:

(1) pituitary replacement: Females in this group were injected i.p. with fresh macerated goldfish pituitaries (2/day) on alternate days over a 20 day period, during the last 8 of which they also received estradiol (20 µg/g i.p. on alternate days). Preparation of estradiol was as in previous experiments. Injected pituitaries were dissected from freshly killed donors (males and females; 10-15 g), ground in a small mortar and pestle, and mixed with a small volume of cold 0.6% NaCl to give an injection volume of 0.2-0.3 ml. As Experiments 4 and 5 showed that estradiol alone does not restore responsiveness to egg injection in hypophysectomized fish, all fish were primed with estradiol to eliminate the possibility that lack

of responsiveness could be due to insufficient estradiol, rather than to the absence of some pituitary factor.

(2) saline control: Treatment was as for group (1) except that roughly equivalent volumes of 0.6% NaCl were injected in place of macerated pituitaries.

(3) sham control: Females in this group were sham hypophysectomized and treated as in group (2).

On the morning of the day following the last injection, each fish was anaesthetized in MS-222, injected with ovulated eggs (0.025 ml/g), fitted with an ovipore plug, and left in a 2 l beaker of 25% seawater for 40 to 80 minutes before being placed with one or two active males in a 60 l observation tank. After a three hour observation period the fish were anaesthetized, checked for bound eggs, and checked by dissection for pituitary remnants. The ovaries were fixed in Bouin's Fluid and prepared for histological examination.

3. Results

Sham hypophysectomy had no obvious effect on female goldfish spawning behaviour. Five of seven sham fish spawned at a frequency of normal intact fish (Table IV). SH-3, which spawned only 3 times during the test period, had slightly bound injected eggs and regressed ovaries with very little yolk vesicle formation.

Even though estradiol was injected, hypophysectomy essentially abolished the response to injected eggs. Only 2 of the 8 females tested performed any spawning activity and in both fish the response was minimal.

Injection of a homogenate of goldfish pituitary restored the spawning behaviour of most of the test females. Furthermore, this treatment induced

TABLE IV

EFFECT OF INJECTION OF GOLDFISH PITUITARY HOMOGENATE AND ESTRADIOL ON THE SPAWNING RESPONSE TO INJECTION OF OVULATED EGGS IN HYPOPHYSECTOMIZED FEMALE GOLDFISH

Group	I	II	III
Treatment	Hypophysectomy 20 day pituitary treatment 8 day estradiol treatment	Hypophysectomy 20 day saline treatment 8 day estradiol treatment	Sham hypophysectomy 20 day saline treatment 8 day estradiol treatment
No. Spawning Acts/3 hour Test Period	56 2 72 57 19 0 12 2 11 111 123	2 0 0 0 0 0 1 0	25 155 3 67 25 0 137
	(mean = 42.3)		(mean = 58.5)

No significant difference between spawning behaviour of pituitary-injected (Group I) and sham hypophysectomized (Group III) fish (Mann-Whitney U-test).

yolk deposition ranging from early yolk vesicles to early yolk granules.

4. Discussion

The results of this experiment demonstrate a role for the pituitary in the induction of spawning in response to injected eggs, and support Yamazaki's (1965) finding concerning both the time course of second growth stage atresia following hypophysectomy and the induction of yolk formation following pituitary replacement.

In the present experiment, 5 of the 8 hypophysectomized controls contained no yolk in the ovaries. The slight yolk vesicle deposition seen in the other three fish (one of which spawned twice during the test period) is similar to that of some of Yamazaki's 9-week post-operative fish. As in my experiment, Yamazaki apparently did not carry out histological checks for completeness of hypophysectomy and thus there is a possibility that hypophysectomized fish with such minimal yolk deposition may have retained pituitary fragments. However, considering the high incidence of atresia of second growth phase oocytes in these ovaries and the post-hypophysectomy persistence of yolk vesicles seen in other experiments, it seems more likely that post-hypophysectomy regression in these fish was incomplete.

B. Experiment 7. Effect of Salmon Gonadotropin and Aminoglutethimide on Spawning Behaviour of Hypophysectomized Female Goldfish

1. Introduction

In the preceding experiment, injection of homogenized goldfish pituitary material into hypophysectomized fish demonstrated a role for the pituitary in spawning behaviour, but provided no information as to the nature or the mode of action of the pituitary factor(s) involved, In the present experiment an attempt was made to test the hypothesis that it is gonadotropin which is involved in the regulation of spawning behaviour by treating hypophysectomized females with a partially purified spring salmon (Oncorhynchus tshawytscha) gonadotropin preparation, SG-G100 (gift of Dr. E.M. Donaldson). To examine the possibility that gonadotropin might exert its effect through stimulation of steroidogenesis, some gonadotropin-treated fish also were injected with the steroid enzyme inhibitor, aminoglutethimide (AG, Ciba). As AG inhibits side-chain cleavage of cholesterol (Gaunt et al., 1968; Gower, 1974), injection of this chemical should restrict or prevent entry of precursors into the ovarian steroidogenic pathway.

If gonadotropin induces spawning behaviour simply by stimulating ovarian steroid production, then concomitant treatment with AG should block or at least reduce the response; but if gonadotropin influences behaviour by some mechanism not requiring steroids, then AG should have no inhibitory effect. However, it is possible that the mechanism regulating spawning behaviour requires both gonadotropin and steroid. In this case, treatment with AG would also block spawning. To control for the possibility that spawning behaviour is induced by a combination of gonadotropin and estrogen,

all fish receiving gonadotropin and AG also were injected with estradiol, which previously had been found not to induce receptivity in hypophysectomized fish (Experiments 4, 5, and 6).

The major fault in the design of this experiment is that if only gonadotropin and estradiol are required to restore responsiveness to egg injection, treatment with AG would appear to have noe effect. If however, a steroid other than estradiol is required, treatment with AG should block the effect of gonadotropin even though estradiol is present.

2. Materials and Methods

Females were transferred from 12 C to 20 C for several days prior to hypophysectomy, after which they were kept in 25% seawater for 23 to 27 days before beginning treatment. Six treatment groups were used (see Table V):

(i) high dose gonadotropin (SG-G100; 15 µg/g) plus estradiol (E_2; 20 µg/g)

(ii) low dose SG-G100 (3 µg/g) plus E_2

(iii) high dose SG-G100 plus AG (100 µg/g) plus E_2

(iv) low dose SG-G100 plus AG plus E_2

(v) control saline (0.6% NaCl) plus E_2

(vi) control E_2

All groups received injections (either SG, AG, saline, or E_2) on alternate days over a 20 day period. In addition, groups i to v were given E_2 on alternate days for the last 9 days of treatment.

SG-G100 was administered in two dosages to establish a dose-response relationship for groups i and ii; it was thought that this dose effect might also be evidence in groups iii and iv. Group v served as control for the E_2 injections given to groups i to iv and group vi provided an additional

TABLE V

VEHICLE VOLUMES (PER INJECTION DAY AND TOTAL)

INJECTED IN EXPERIMENT 7

Volumes of vehicles (ml) injected per 20 g fish

Treatment Group	on each of first 5 injection days					on each of second 5 injection days					Over 10 injection days
	SG	AG	SAL	E_2	Total	SG	AG	SAL	E_2	Total	
i, ii	.10				.10	.10			.08	.18	1.40
iii, iv	.10	.06			.16	.10	.06		.08	.24	2.00
v			.10		.10			.10	.08	.18	1.40
vi				.16	.16				.16	.16	1.60

SG = salmon gonadotropin (SG-G100)

AG = aminoglutethimide

Sal = saline

E_2 = estradiol

control for the extended E_2 exposure assumed to result from SG-G100 stimulation of steroidogenesis in groups i and ii.

Preparation and injection of E_2 was as described previously (Experiment 3). SG-G100 was prepared immediately before injection by dissolving the lyophilized hormone in cold 0.6% NaCl to a concentration of 0.6 µg/µl (low dose) or 3.0 µg/µl (high dose). AG was ground to a fine powder, suspended at a concentration 30 µg/µl in 0.6% NaCl containing 4 drops Tween 80/100 ml and injected i.p. at a dosage of 100 µg/g.

To reduce handling to a minimum, no attempt was made to give equal numbers of injections to all groups. However, concentrations of E_2 suspensions were adjusted so that groups receiving three chemicals at a time (groups iii and iv) received only 50% more vehicle than group vi which was given only single injections. As indicated in Table V, the differences in total vehicle volumes injected over the duration of the experiment were even smaller.

On day 20, each fish was anaesthetized in MS-222, injected with ovulated eggs (0.02 ml/g), fitted with an ovipore plug, and left in a 2 l beaker of 25% seawater for 60-75 minutes before being placed with one or two active males (one female per 60 l observation tank). The fish were observed continuously for three hours, anaesthetized, checked for bound eggs, and sacrificed. The heads were checked by dissection for completeness of hypophysectomy, and ovaries were fixed and prepared for histological examination.

3. Results

As a result of mortality and binding of injected eggs, the final

sample sizes in this experiment were small. The data are presented in Table VI.

As in previous experiments, estradiol failed to restore receptivity in hypophysectomized fish (Group v); one of four fish showed a low level of response. It is not clear whether the response of this fish was due to residual receptivity or to the estrogen treatment. No completely hypophysectomized group vi (estradiol-treated) fish survived to be tested.

At both the high and low dosages (Group i and ii), salmon gonadotropin was effective in restoring the response to injected eggs, all 7 fish responding in the behavioural tests. In addition, the gonadotropin had a marked effect on vitellogenesis, ranging from the induction of the early yolk vesicle stage in most fish, to the early yolk granule stage in one female. These effects on oocyte development are consistent with those reported by Yamazaki and Donaldson (1968); the greater degree of ovarian response in their study likely was due to higher gonadotropin dosage and healthier fish. Except for one degenerating oocyte in one fish, gonadotropin treatment also prevented the degeneration of first and second growth stage oocytes. This is in contrast to the results of estradiol treatment (groups v and vi) where 8 of 10 fish had degenerating oocytes.

Administration of aminoglutethimide was highly effective in inhibiting spawning in response to egg injection in hypophysectomized, gonadotropin treated fish (Group iii and iv); only one of six fish responded in the behavioural tests. However, the ovarian histology of fish treated with gonadotropin and aminoglutethimide was similar to that of the fish receiving gonadotropin alone; yolk vesicles were present in all ovaries and atresia of oocytes was inhibited completely.

TABLE VI

EFFECT OF SALMON GONADOTROPIN (SG-G100) AND AMINOGLUTETHIMIDE ON THE SPAWNING RESPONSE TO EGG INJECTION IN HYPOPHYSECTOMIZED FEMALE GOLDFISH

Group	i	ii	iii	iv	v	vi
Treatment First 10 days	SG - 15 µg/g	SG - 3 µg/g	SG - 15 µg/g AG - 100 µg/g	SG - 3 µg/g AG - 100 µg/g	saline	E_2 - 20 µg/g
Second 10 days	SG - 15 µg/g E_2 - 20 µg/g	SG - 3 µg/g E_2 - 20 µg/g	SG - 15 µg/g AG - 100 µg/g E_2- 20 µg/g	SG - 3 µg/g AG - 100 µg/g E_2 - 20 µg/g	saline E_2 - 20µg/g	E_2 - 20 µg/g
No. spawning	116	72	0	44	0	no
acts/3 hour	61	36	0	0	0	fish
test period	6	18	0	0	5	tested
	38					

SG = salmon gonadotrpin (SG-G100) AG = aminoglutethimide E_2 = estradiol

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4. Discussion

The results of this experiment demonstrate that in hypophysectomized female goldfish, treatment with a partially purified salmon gonadotropin preparation (SG-G100) combined with estradiol (E_2) is effective in restoring spawning in response to the injection of ovulated eggs. As in previous experiments, treatment with E_2 alone was ineffective. These findings suggest that gonadotropin is the pituitary factor responsible for restoring receptivity in the hypophysectomized fish injected with goldfish pituitary homogenate in Experiment 6. However, as the gonadotropin preparation used in this experiment likely contained some thyrotropic activity (Donaldson and McBride, 1974), it is possible that it was thyrotropin rather than, or in addition to, gonadotropin which restored receptivity.

When hypophysectomized fish receiving SG-G100 and E_2 also were injected with the steroid enzyme inhibitor, aminoglutethimide (AG), the response to injections of eggs was strongly inhibited. This inhibition of spawning behaviour by AG may simply have been pharmacological and independent of any effects on steroidogenesis; however, neither the non-spawning behaviours nor the general condition of AG-treated fish were obviously different from those of the other groups. If AG affected spawning by inhibiting steroidogenesis, then it appears the effect was due to inhibition of the production of an essential steroid (or steroids) other than estradiol. Whether or not AG was administered, SG-G100 stimulated yolk production. This is to be expected, as gonadotropin and estradiol were present in all cases.

C. Summary of Chapter V

1. Injection of homogenized goldfish pituitaries or partially purified salmon gonadotropin into hypophysectomized female goldfish restores responsiveness to injection of ovulated eggs.

2. In hypophysectomized fish treated with salmon gonadotropin and estradiol, injection of the steroid enzyme inhibitor, aminoglutethimide, inhibits responsiveness to injection of ovulated eggs, suggesting a steroid other than estradiol may play a role in spawning behaviour.

CHAPTER VI

THE ROLE OF PROSTAGLANDINS IN THE SPAWNING BEHAVIOUR OF FEMALE GOLDFISH

A. Introduction

The majority of the preceding experiments were designed to examine the effects of various hormones on the spawning responses of female goldfish to the injection of ovulated eggs. These studies have been concerned with relatively long-term effects of hormonal deprivation and replacement over periods of days or weeks. In this section, the short-term regulation of sexual behaviour is examined in experiments involving the mechanism by which egg injection triggers spawning.

In his study of the spawning behaviour of goldfish, Yamazaki (1965) noted that even though a female had ovulated, spawning would not occur unless both aquatic vegetation and a sexually active male were present. Removal of the vegetation inhibited spawning immediately, while replacement restored it within minutes. My observations also show that if an ovulated female, isolated from other fish for days, is placed with a sexually active male, spawning can occur within a minute. However, a consistent feature of spawning induced by egg injection is that there is a variable latent period (as much as several hours) between the time eggs are injected and the time spawning begins. Although this latent period may be nothing more than an inhibition induced by anaesthetization and handling during egg injection, some preliminary data suggest this is not so.

Seven highly receptive fish with second growth phase oocytes were anaesthetized and injected with ovulated eggs: two females (#1,2) were placed with active males immediately, four (#3-6) were placed with the males after 200 minutes 'incubation' in isolation from other fish, and one (#7) was placed with males following a second anaesthetization at the end of the 200 minute incubation (Table VII). In the fish placed with males

TABLE VII

EFFECT OF INCUBATION TIME (DELAY BETWEEN EGG INJECTION AND PLACEMENT OF FEMALE WITH MALE) ON LATENCY TO FIRST SPAWNING ACT IN RECENTLY OVULATED (3-to 7-DAY POSTOVULATORY) FEMALES.

Fish	Incubation (min)	Latency to first spawning act	No. spawning acts in first 40 min of spawning
1 - POV-7	10	37	52
2 - POV-4	4	55	25
3 - POV-7	200	3	74
4 - POV-7	200	9	19
5 - POV-3	200	2	118
6 - POV-4	200	9	52
7 - POV-3	200*	12	66

*Fish #7 received a second anaesthetization of 200 minutes.

immediately after recovery from anaesthetic, the latency to the first spawning act was considerably greater than that in fish incubated for 200 minutes. This was true even when an incubated fish was anaesthetized a second time.

These limited results suggest that eggs in the ovarian lumen stimulate spawning by a different mechanism than do the presence of male goldfish or aquatic vegetation. Whereas presentation of the latter stimuli elicits spawning within a few minutes in fish which have had ovulated eggs in the ovarian lumen for some time, injection of ovulated eggs is followed by a latent period which apparently is not due simply to anaesthetization. Of course, the second anaesthetization at 200 minutes is a poor control as it indicates only that MS-222 has little effect on the behaviour of a fish which is already receptive.

It was thought that the latent period might represent the time required for oviduct stimulation to effect changes in central neural structures controlling sexual behaviour. As a number of workers have reported that injection of neurohypophyseal hormones induces a 'spawning reflex', oviposition, or parturition in a variety of teleost species (Liley, 1969; Macey et al., 1974), it seemed possible that these hormones might be involved in mediating the effects of egg injection on the spawning behaviour of female goldfish. No formal experiments were carried out to test this hypothesis as in all preliminary tests oxytocin was completely ineffective in inducing spawning behaviour in female goldfish with vitellogenic oocytes or in increasing the rate of spawning in ovulated fish. Similar results have apparently been obtained by Pickford (unpublished results cited in Macey et al., 1974).

In female mammals, physical stimulation (stretch) of the reproductive tract and other smooth muscles (situations roughly comparable to ovulation or egg injection in goldfish) is associated with the release of prostaglandins (Poyser et al., 1971; Piper and Vane, 1971; Csapo, 1973). Prostaglandins (PG), the 'intrinsic uterine stimulant' (Csapo, 1973), have been implicated in the oviposition of birds (Hertelendy, 1972, 1973; Hertelendy et al., 1974, 1975; Wechsung and Houvenaghel, 1976), in the parturition of mammals (Liggins et al., 1973; Labhsetwar, 1974; Aiken, 1974; Flint et al., 1974; Currie, 1975; Umo et al., 1976), and in the mechanism of action of some IUDs (Spilman and Duby, 1972; Chaudhuri, 1975). The findings that steroids may alter the release of PG from the genital tract (Roberts et al., 1975), that the response of the genital tract to PG may be modified by steroids (Csapo, 1973; Spilman, 1974), and that PG may play a role in the action of LH on ovarian tissue (Kuehl et al., 1970; Marsh et al., 1974) all suggested that investigation of PG function in the female goldfish might shed light on the problem of the induction of spawning behaviour by egg injection. The following experiments present the results of these investigations.

B. Experiment 8. Inhibition by Indomethacin of Spawning Behaviour Induced by Injection of Ovulated Eggs

1. Introduction

It was postulated that if PGs were involved in goldfish spawning behaviour, their synthesis or release may be increased by the stimulus provided by egg injection. In female mammals, endogenous PG production can be inhibited by injection of indomethacin (IM), a potent inhibitor of PG synthesis which apparently affects a complex of enzymes referred to as prostaglandin synthetase (Vane, 1971). As a first step in demonstrating a role for PG in goldfish spawning behaviour, IM injection was used to inhibit endogenous PG production. To examine the speed and duration of action of IM, injections were given at various times in relation to the injection of eggs and the onset of spawning behaviour.

2. Materials and Methods

All female goldfish used in this experiment had ovaries in various stages of vitellogenesis and therefore could have been expected to spawn when injected with ovulated eggs. Four treatment groups were used (see Table VIII). Indomethacin (IM, Sigma) was injected i.p. as a saline suspension (0.6% NaCl; 4 drops Tween 80/100 ml) at a dosage of 10 µg/g (5µl/g). This dosage had been effective in a study of goldfish ovulation (Stacey and Pandey, 1975).

At 1000 h on the test day, fish were anaesthetized in MS-222, weighed and injected with ovulated eggs (0.02 ml/g). They were then left for 1 hour, placed in 60 l observation tanks with actively courting males, and observed continuously for three hours. Females in Group i had received

TABLE VIII

EFFECT OF INDOMETHACIN ON SPAWNING IN RESPONSE TO INJECTION OF OVULATED EGGS

Group	Treatment	N	No. Responding	Mean Acts*	No. Spawning (range)
i	IM 10 h before eggs	7	0	0+	
ii	IM with eggs	5	0	0+	
iii	saline with eggs	9	9	6++	(2-11)
	IM after 20 minutes spawning	9	1	(1)+++	
iv	saline with eggs	8	8	5++	(1-9)
	saline after 20 minutes spawning		8	19+++	(5-37)

* Based on responding individuals.

\+ Mean no. per 3 h test period
++ Mean no. per 20 min
+++ Mean no. after 2nd injection
IM = indomethacin

IM at 2400 h the previous evening, 10 hours before egg injection, while those in Group ii had received IM while anaesthetized for egg injection. Groups iii and iv received saline injections coincident with egg injections as a control for the IM injection given to Group ii. In addition, Group iii received an IM injection and Group iv a second saline injection 20 minutes after spawning had commenced. For this second injection fish were not anaesthetized, but simply netted from the observation tank, injected while hand held, and replaced immediately.

3. Results and Discussion

As shown in Table VIII, none of the females injected with IM 10 hours prior to, or coincident with, injection of ovulated eggs spawned during the 3 hour test period; in contrast, all females injected with saline at the time of egg injection (Groups iii and iv) spawned in the test period. Following IM injection, one Group iii female performed one spawning act (5 minutes post-injection), whereas all group iv females continued to spawn following the second control saline injection.

Injection of an IM suspension is thus seen to have a rapid and relatively long-lasting inhibitory effect on the spawning response to injected eggs. (In contrast, courting males, which were observed for up to three hours post-injection, showed no obvious behavioural response to the injection of IM; however, no quantitative measures were taken. It is not known whether treatment with IM inhibits the release of sperm).

C. Experiment 9. Effect of Prostaglandins on the Spawning Behaviour of Indomethacin-Treated Female Goldfish

1. Introduction

This experiment tested the abilities of injected prostaglandins (PGE_1, PGE_2, $PGF_{2\alpha}$) to overcome the indomethacin-induced inhibition of spawning following egg injection. These three PGs were chosen as they were found to be effective in overcoming the indomethacin blockade of ovulation in female goldfish (Stacey and Pandey, 1975).

2. Materials and Methods

Five treatment groups were used and the initial treatment for all was as for Group ii in the preceding experiment. Fish were anaesthetized, weighed, and injected with ovulated eggs (0.02 ml/g) and IM (10 µg/g) between 900 and 1000 h on the test day. All fish were then left for one hour to recover, netted, injected i.p. with the saline vehicle (Group i) or one of the PGs (Groups ii-v), placed in the observation tanks with active males, and observed continuously for 2 hours. PGE_1 (Group ii) and PGE_2 (Group iii) were prepared by dissolving in 95% ethanol (1 mg/0.1 ml) and diluting 9X with buffered saline (20 mg Na_2CO_3/100 ml 0.6% NaCl). $PGF_{2\alpha}$ (Group iv) was simply dissolved in buffered saline (1 mg/ml). Group v received $PGF_{2\alpha}$ prepared in the same manner as PGE_1 and PGE_2 as a control for possible behavioural effects of the ethanol solvent. All PGs (Upjohn) were injected i.p. at a dose of 5 µg/g (5 µl/g).

3. Results and Discussion

None of the Group i fish treated with the saline vehicle spawned during the 2 hour test period (Table IX). Though the sample size was

TABLE IX

EFFECT OF PROSTAGLANDINS ON THE SPAWNING RESPONSE TO INJECTION OF OVULATED EGGS IN FEMALE GOLDFISH TREATED WITH INDOMETHACIN

Group	Treatment	N	No. responding	Mean No. Acts*	Spawning (range)
i	IM, eggs and saline	6	0	0	
ii	IM, eggs and PGE_1	5	0	0	
iii	IM, eggs and PGE_2	7	2	5	(4,6)
iv	IM, eggs and $PGF_{2\alpha}$	7	5	27	(7-62)
v	IM, eggs and $PGF_{2\alpha}$ (alcohol vehicle)	4	3	52	(5-115)

* Based on individuals responding in 2 h test period.

Indomethacin (IM) and eggs injected 1 h before test,

PGs injected at start of test

small, PGE_1 (5 fish) had no effect in overcoming the indomethacin blockade, and PGE_2 was only slightly active, inducing low levels of spawning in 2 of the 7 fish tested. In contrast, $PGF_{2\alpha}$ was quite effective in inducing spawning behaviour in IM-treated fish; presence of ethanol in the vehicle does not seem to account for the ineffectiveness of PGE_1 and PGE_2, as 3 of the 4 Group V fish spawned normally.

The latencies from the injection of PG to the onset of spawning were quite variable (5-100 minutes; mean 40 minutes). There were no significant differences among the latencies of the responding fish in groups iii, iv, and v. Of the 8 fish which spawned following $PGF_{2\alpha}$ injection, all but one continued to spawn for the remainder of the observation period.

D. Experiment 10. Effect of Prostaglandins Alone (Without Egg Injection) on the Spawning Behaviour of Female Goldfish

1. Introduction

Based on the findings that indomethacin (IM) blocked the spawning response to injected eggs, and that PGs (especially $PGF_{2\alpha}$) were effective in overcoming this blockade, this experiment was conducted to determine whether PG injection alone (i.e., without prior treatment with ovulated eggs) was sufficient to induce spawning behaviour, and whether the order of PG potencies was as found in Experiment 9 ($PGF_{2\alpha} > PGE_2 > PGE_1$).

2. Methods, Results, and Discussion

Female goldfish were injected i.p. (no anaesthetization) with either $PGF_{2\alpha}$, PGE_1, or PGE_2 at 5 µg/g, placed immediately with active males, and observed continuously for 3 hours. No control group was used.

As shown in Table X, $PGF_{2\alpha}$ was highly effective in inducing spawning behaviour even though ovulated eggs were not present in the ovarian lumen. Several of the fish in this group were injected with IM 20 minutes after spawning had commenced; there was no apparent effect on spawning behaviour, PGE_2 was marginally effective in inducing spawning behaviour (2 of 10 fish responding) and PGE_1 was without effect.

As in Experiment 9, the latencies to the onset of spawning were quite variable (5-135 minutes; mean 45 minutes). Also, the duration of spawning in the fish responding to $PGF_{2\alpha}$ was considerable, several individuals responding regularly for longer than 2 hours.

These results suggest that the effect which ovulated eggs in the ovarian lumen have on spawning behaviour is mediated through the release of

TABLE X

EFFECT OF PROSTAGLANDINS ON THE SPAWNING BEHAVIOUR OF FEMALE GOLDFISH WITHOUT OVULATED EGGS IN THE OVARIAN LUMEN

Group	Treatment	N	No. Responding	Mean No. Acts*	Spawning (Range)
i	PGE_1	10	0	0	
ii	PGE_2	10	2	11	(7,15)
iii	$PGF_{2\alpha}$	13	13	59	(11-208)

* Based on individuals responding in 3 h test period.

PGs injected at start of test.

prostaglandins, and that it is some action of prostaglandins, independent of the presence of eggs, which induces spawning in response to male courtship.

E. Experiment 11. Effect of Hypophysectomy and Gonadotropin Replacement on Prostaglandin-Induced Spawning Behaviour in Female Goldfish

1. Introduction

In experiments presented in Chapters IV and V, treatments with pituitary and ovarian hormones were found to affect the responsiveness of female goldfish to egg injection. A variety of steroid hormones were effective in inducing receptivity in intact, temperature-regressed fish, whereas in hypophysectomized fish, all steroids tested were found to be totally ineffective. Treatment with goldfish pituitary homogenate or a partially purified salmon gonadotropin preparation (SG-G100) was highly effective in restoring spawning behaviour in hypophysectomized fish. As the injection of the steroid enzyme inhibitor, aminoglutethimide, inhibited the response to SG-G100, it was suggested that the mode of action of gonadotropin on spawning behaviour may be through the stimulation of ovarian steroidogenesis. A number of interpretations of these results is possible.

In many mammalian studies, prostaglandin release has been shown to be correlated with reproductive cycles and to be influenced by gonadotropin and steroids (see references in section A, this chapter). As PG is apparently essential for spawning behaviour to occur, it seemed a plausible explanation that the inability of steroids to restore receptivity in hypophysectomized fish is due to the absence of PG synthesis following hypophysectomy. The following experiment examined this hypothesis by comparing the response to $PGF_{2\alpha}$ injection in hypophysectomized fish treated either with steroids, salmon gonadotropin, or a saline vehicle.

2. Materials and Methods

Females which had been hypophysectomized 3 to 4 months earlier were injected i.p. (no anaesthetization) as follows:

Group i - saline vehicle (0.6% NaCl; 4 drops Tween 80/100 ml),

Group ii - 10 µg/g spring salmon (Oncorhynchus tshawytscha) gonadotropin (SG-G100, lot #BCR-3) dissolved in saline,

Group iii - 20 µg/g 17β-estradiol suspended in saline.

All treatment groups received injections at 5 µl/g on alternate days for a 15 day period. On the morning of the day following the last injection, fish were injected i.p. with 5 µg/g $PGF_{2\alpha}$, placed immediately with actively courting males, and observed continuously for 3 hours. Fish in groups i and ii were then sacrificed and the heads examined by dissection for pituitary remnants. Ovaries were fixed and prepared for histological examination.

To examine the possibility that gonadotropin-induced receptivity was due to steroids other than estradiol, Group iii was divided into two groups; Group iiiA received estradiol injections on alternate days for a further 15 day period, while Group iiiB received injections of a mixture of steroids (estradiol, 20 µg/g; testosterone, 5α-dihydrotestosterone, 11-keto-testosterone, progesterone, and deoxycorticosterone all at 5 µg/g) for the same period of time. Testing and related procedures following this second injection period were the same as those following the first.

3. Results

Injection of $PGF_{2\alpha}$ failed to induce spawning behaviour in female goldfish which had been hypophysectomized for 3 to 4 months; a 2 week

pretreatment with salmon gonadotropin restored PG-induced spawning behaviour in 8 of 13 hypophysectomized fish tested (Table XI). Treatment with estradiol or a combination of steroids was without effect in restoring the responsiveness to PG, only 1 of 12 fish showing a low level of spawning in each testing session.

As in the two preceding experiments, the latency to spawning was variable (20-160 minutes; mean 60 minutes) and the duration of spawning was often considerable, 4 of the 8 responding fish in Group ii spawning for longer than 2 hours.

Histological examination of the ovaries revealed no yolk deposition in any Group i or Group iii fish. The ovaries of the 6 most active spawners in Group ii contained small numbers of oocytes in the early yolk vesicle stage. Of the 5 non-responding Group ii fish, one had ovaries with early yolk vesicles, three had no yolk vesicle stage oocytes, and the ovaries of one contained only σ-stage corpora lutea (Khoo, 1975). No oocytes containing yolk granules were observed. The greater ovarian response to SG-G100 reported for hypophysectomized goldfish by Yamazaki and Donaldson (1968) may have been due either to a longer injection schedule (3 weeks rather than 2) or a shorter period following hypophysectomy (2 months rather than 3 or 4).

SG-G100 was also effective in preventing oocyte degeneration: whereas 13 of 16 Group i and 8 of 12 Group iii fish had ovaries containing degenerating previtellogenic oocytes, these were found in only 3 of 13 Group ii fish.

4. Discussion

In hypophysectomized fish, salmon gonadotropin restores the spawning

TABLE XI

EFFECT OF SALMON GONADOTROPIN (SG-G100) AND STEROIDS ON SPAWNING BEHAVIOUR IN RESPONSE TO INJECTION OF PROSTAGLANDIN $F_{2\alpha}$ IN HYPOPHYSECTOMIZED FEMALE GOLDFISH

Group	Treatment	N	No. responding	Mean No. Acts*	Spawning (range)
i	saline	16	0	0	
ii	salmon gonadotropin	13	8	26	(5-53)
iii	estradiol	12	1	(3)	
iiiA	estradiol	6	1	(5)	
iiiB	steroid mixture	6	0	0	

* Based on individuals responding in 3 h test period

PGs injected at start of test

response to prostaglandin ($PGF_{2\alpha}$) injection, while treatment with estradiol or a mixture of steroids has no effect. The lack of effect of estradiol is not likely to be due to an inhibitory effect of high steroid dosage, as the same dosage given to intact regressed fish (Experiment 3) and with salmon gonadotropin to hypophysectomized fish (Experiment 7) did not inhibit spawning.

The results of this experiment demonstrate that responsiveness to PG is modulated by gonadotropin; however, they do not support the hypothesis that the ineffectiveness of steroids on the responsiveness of hypophysectomized fish to egg injection is due to the absence of PG. In hypophysectomized fish treated with salmon gonadotropin or steroids, the effects of PG on spawning behaviour are similar to the effects of egg injection, suggesting that fish which fail to respond to eggs simply may not respond to endogenous PG.

F. Summary of Chapter VI

1. Injection of indomethacin, an inhibitor of prostaglandin synthesis, blocks the effect of ovulated eggs on spawning behaviour.

2. Injection of prostaglandins restores spawning behaviour in egg-injected fish treated with indomethacin and induces spawning in fish which have not been injected with ovulated eggs.

3. The spawning response to prostaglandin injection is abolished by hypophysectomy and restored in hypophysectomized fish by treatment with salmon gonadotropin. Steroid treatment is ineffective in restoring the response to prostaglandin injection in hypophysectomized fish.

CHAPTER VII

GENERAL DISCUSSION

A. Introduction

This study not only raises a number of questions as to the nature of the roles of the hormones examined; it also suggests fascinating problems concerning the evolution of the regulation of sexual behaviour in female vertebrates in general.

There is much scope for speculation on the mechanism which regulates spawning behaviour in female goldfish. However, it must be acknowledged that all conclusions derived from this study are biased to the extent that they are based on results obtained in an experimental situation designed to measure only oviposition behaviour. As detailed by Beach (1976), hormones may influence three basic aspects of female reproductive behaviour; (i) attractivity - measured in terms of the appetitive sexual responses evoked in conspecific males, (ii) proceptivity - appetitive sexual behaviours evoked in females by males, and (iii) receptivity - the consummatory phase of the mating sequence. As mentioned above (page 16), in situations where the male goldfish is relatively sexually inactive, females may exhibit proceptive behaviour including components which would fall into the categories Beach (1976) refers to as affiliative, solicitational, approach-withdrawal, and contact responses. One aspect of non-behavioural stimuli contributing to the attractivity of female goldfish consists of unidentified substances which apparently are released by the ovaries of preovulatory and ovulated fish and function as olfactory stimulants of appetitive sexual responses in males (Partridge et al.,1976).

The present study has dealt solely with the endocrine control of the spawning act, the consummatory phase of female goldfish sexual behaviour;

it is possible that had data been gathered on the effect of hormones on the stimulus quality of females or on the tendency of females to approach males or engage in other types of proceptive behaviour, the results and conclusions might have been different.

The results of experiments presented in this thesis identify four endogenous factors believed to play major roles in spawning behaviour; (i) pituitary hormones (apparently gonadotropin), (ii) ovarian steroids, (iii) stimuli from ovulated eggs in the oviduct, and (iv) prostaglandins. As so little is known of the endocrine regulation of female reproductive behaviour in other teleostean species, speculation concerning the function of these four factors in the control of spawning behaviour in goldfish is based largely on information from studies of reproduction and sexual behaviour in higher vertebrates, particularly mammals.

B. The Role of the Pituitary

The results of hypophysectomy and pituitary replacement therapy demonstrate that the pituitary plays at least an indirect role in regulating spawning behaviour in female goldfish. That pituitary gonadotropin is involved in this regulation is suggested by the effectiveness of salmon gonadotropin in restoring receptivity in hypophysectomized fish.

It is possible that the effect of salmon gonadotropin on spawning behaviour was due to, or enhanced by, contamination of the gonadotropin preparation with thyrotropin. Donaldson and McBride (1974) found evidence that salmon gonadotropin injection in adult salmon led to increased thyroid activity, an effect which they attributed to thyrotropic activity in the gonadotropin preparation. It is not clear how thyrotropin or thyroxin might be involved in stimulating receptivity. If these hormones were

required to mediate the effect of steroids on behaviour, then the lack of effect of steroids in hypophysectomized fish would be understandable. However, there apparently is no evidence that thyrotropin or thyroxin enhances the action of steroids on target issues.

It is not known how gonadotropin restores responsiveness to egg injection in hypophysectomized fish. However, it is suggested that either or both of two basic mechanisms may be involved:

1. gonadotropin may directly affect central neural structures controlling sexual behaviour, or
2. gonadotropin may exert an indirect control over behaviour by stimulating the formation of steroid hormones which in turn act directly on the mechanism regulating sexual responsiveness.

There is no direct evidence from this or other studies of teleosts that gonadotropin exerts a direct control over female sexual behaviour. However, it has been suggested that in male Gasterosteus aculeatus (Hoar, 1962; Baggerman, 1966) and Cymatogaster aggregata (Wiebe, 1967) gonadotropin may affect behaviour associated with reproduction through a mechanism independent of the gonads. Gonadotropin has been suggested to exert a direct control over sexual behaviour in the female guppy (Liley, 1968); more recent evidence (Liley and Donaldson, 1969; Liley, 1972) fails to provide support for this hypothesis.

There appears to be no evidence that gonadotropins enhance sexual behaviour in female mammals except through stimulation of steroidogenesis. In ovariectomized rats, hypophysectomy does not affect lordosis induced by high estrogen dosage (Pfaff, 1970) and facilitates female sexual behaviour when threshold dosages of estrogen are employed (Crowley et al.,1976).

As injection of luteinizing hormone supresses lordosis responses in hypophysectomized-ovariectomized individuals, Crowley et al. (1976) suggest that this gonadotropin may affect sexual behaviour by inhibiting the release of luteinizing hormone-releasing hormone (LH-RH). LH-RH stimulates lordosis in the female rat (Pfaff, 1973; Moss and Foreman,1976).

There is some evidence that gonadotropin induces receptivity in female goldfish by stimulating steroidogenesis. As estrogen enhances yolk formation in other teleosts (Campbell and Idler, 1976; Emmersen and Petersen, 1976), it is likely that salmon gonadotropin, which induces yolk formation in hypophysectomized goldfish (Yamazaki and Donaldson, 1968), also stimulates the production of estrogen and other steroids. Thus, the ability of aminoglutethimide to inhibit spawning behaviour of salmon gonadotropin-treated goldfish suggests that gonadotropin influences receptivity by stimulating steroidogenesis. A similar mechanism has been proposed to explain the effect of salmon gonadotropin on sexual behaviour in the female guppy (Liley and Donaldson, 1969).

In addition to inhibiting side-chain cleavage of cholesterol (Gaunt et al., 1968; Gower, 1974), aminoglutethimide has been shown to inhibit aromatization (Thompson and Siiteri, 1973) and to block the stimulatory effect of testosterone on sexual behaviour in the male rat (Beyer et al., 1976). It is not likely that aminoglutethimide suppressed spawning behaviour by inhibiting aromatization, as all fish receiving gonadotropin and aminoglutethimide were also injected with estradiol. The fact that estradiol did not overcome the inhibitory effect of aminoglutethimide suggests that spawning behaviour may be regulated by steroids other than (or in addition to) estradiol.

The possibility that the effect of aminoglutethimide on spawning behaviour is pharmacological, and not related to an effect on steroidogenesis, cannot be excluded. In mammals, aminoglutethimide affects the synthesis of thyroid (Rallison et al., 1967) and adrenocortical hormones (Philbert et al., 1968) and depresses brain activity (Elazar and Blum, 1971). However, neither in the present study nor in that of Beyer et al. (1976) did aminoglutethimide treatment produce any obvious changes in non-reproductive behaviour.

Salmon gonadotropin restores receptivity in hypophysectomized female guppies but not in fish which have also been ovariectomized (Liley and Donaldson, 1969), suggesting that the hormone exerts its effect on behaviour by stimulating ovarian steroidogenesis. The subsequent finding that estrogen treatment induces receptivity in hypophysectomized female guppies (Liley, 1972) demonstrates that in this teleost gonadotropin is not essential for the expression of sexual behaviour. As in the guppy, steroid hormone restores receptivity in the hypophysectomized female rat (Pfaff, 1970); in fact, hypophysectomy has been found to enhance behavioural responsiveness to estrogen treatment in the rat (Crowley et al., 1976), possibly by removing short-loop feedback inhibition of LH-RH release (see Kuhl and Taubert, 1975).

In female goldfish, the results of steroid treatment of intact, regressed fish and of aminoglutethimide injections in hypophysectomized, gonadotropin treated individuals indicate that steroids may be involved in regulating spawning behaviour. However, the failure of steroid treatments alone to restore receptivity of hypophysectomized fish suggests that, in contrast to the situation in rats and guppies, gonadotropin may play an

indispensable role in the sexual behaviour of female goldfish. Whether this apparent requirement for gonadotropin is of physiological significance, or simply results from inappropriate steroid therapy applied to hypophysectomized fish, cannot be determined without further study.

C. The Role of Steroids

1. Introduction

This study provides the first demonstration that steroids stimulate spawning behaviour in an oviparous female fish. In intact goldfish with regressed, nonvitellogenic ovaries, a variety of steroids restores spawning behaviour in response to injection of ovulated eggs; hypophysectomy abolishes this effect of steroids on behaviour. These findings suggest that the pituitary is involved in the effects of steroids in intact fish, but do not clarify the relative contribution of steroid and pituitary hormones to the regulation of spawning behaviour. On the basis of the results presented in this study, it is not possible to determine whether the induction of spawning behaviour results from the combined action of pituitary and ovarian hormones, or requires endocrine input from only one of these organs.

In the following discussion, speculations concerning the role of steroids in goldfish spawning behaviour are based on the effects of steroids on reproductive physiology and behaviour of other female vertebrates. Therefore, it is emphasized at the outset that the relationship between ovarian development and the onset of sexual receptivity in goldfish appears to be unique.

The onset of estrous behaviour in mammals has been attributed to

periovulatory fluctuations in steroid hormones (Davidson and Levine, 1972); also in the guppy, female sexual behaviour normally occurs when the ovary is in an advanced stage of development (Liley, 1968). In female goldfish, however, spawning behaviour may be induced in the absence of endocrine stimuli associated with the later stages of ovarian development; fish with partially regressed ovaries (containing minimal yolk vesicle formation) perform normal spawning behaviour when injected with ovulated eggs. It could be argued that this finding in goldfish is analogous to the results of experiments in female rats in which high levels of sensory stimulation (manual palpation of flanks and perineum) induce lordosis responses in the absence of or with low doses of estrogen (see discussion by Crowley et al., 1976). However, the fact that normal sensory stimuli (presence of ovulated eggs in the genital tract, courtship of male goldfish) induce spawning behaviour at any time during an extended period of receptivity (when yolk vesicles or granules are present in the oocytes) raises the possibility that, if steroids are involved in regulating spawning behaviour, the role of these hormones may be fundamentally different from the role they play in reproductive behaviour of other female vertebrates.

2. Effects of Steroids in Intact Female Goldfish

Two obvious explanations could account for the diversity of steroids which induce spawning behaviour in intact, regressed goldfish. The mechanism controlling receptivity may respond to a variety of steroids. Alternatively, steroid conversion may occur in regressed fish; some of the steroids shown to restore responsiveness to egg injection may be metabolized to one or more active forms which influence behaviour.

There is no published information to indicate whether mechanisms controlling female sexual behaviour in other species of oviparous fish are sensitive only to certain steroids. However, in the viviparous female guppy, only estrogens have been shown to induce receptivity (Liley, 1972). In this species, treatment with estradiol, estrone, estriol, or diethylstilboestrol restores sexual behaviour while cortisol, corticosterone and progesterone are without effect; females treated with testosterone exhibit male sexual behaviour.

The results of mammalian studies indicate that, where conversion of androgens to estrogens can be ruled out, sexual behaviour in females is induced only by estrogens (progesterone is also required in some species to facilitate the priming effects of estrogen: Ciaccio and Lisk, 1967; Joslyn et al., 1971). Dihydrotestosterone, which cannot be aromatized, fails to induce estrous behaviour in female rabbits (Beyer et al., 1970) or to increase sexual motivation in female rats (McDonald and Meyerson, 1973). On the other hand, a number of estrogens (estradiol, estrone, estriol) are capable of inducing receptivity in rats (Beyer et al., 1971) and guinea pigs (Feder and Silver, 1974). As estriol apparently is not converted to estradiol (Ruh et al., 1973), it appears that more than one naturally occurring estrogen may directly influence sexual behaviour in female mammals.

Sexual behaviour in female mammals and in the female guppy appear to be induced specifically by estrogens. Therefore it is unlikely that the effectiveness of the wide variety of steroids (estradiol, androgens, 17α-OH-pregnenolone, and possibly pregnenolone) which restores responsiveness to egg injection in female goldfish is due to a lack of specificity in the response to steroids. Rather, the effects of steroids in goldfish are

more consistent with the concept that many of the exogenous steroids are converted *in vivo* prior to affecting behaviour.

In some mammalian studies (e.g. Beyer *et al.*, 1970; Whalen *et al.*, 1972), the effects of non-estrogen steroids on female sexual behaviour have been attributed to *in vivo* conversion of the exogenous steroids to estrogens. The capacity for extragonadal conversion of behaviourally active exogenous steroids has been demonstrated in several species (review by Ryan *et al.*, 1972) and much recent work has attempted to correlate the metabolism of androgens in central neural tissue with the effects of androgens and estrogens on sexual behaviour (Perez-Palacios *et al.*, 1975; Naftolin and Ryan, 1975). Recent demonstrations (Christensen and Clemens, 1976; Beyer *et al.*, 1976) that inhibition of aromatizing enzymes blocks the stimulatory effect of testosterone on sexual behaviour of male rats further support the concept that the effects of some steroid treatments on behaviour may be mediated by *in vivo* steroid conversion.

Although there is no evidence that *in vivo* steroid conversion occurred under the conditions of my experiments, there is evidence that the capacity for conversion is present in goldfish (Khoo, 1974, 1975) and other teleosts (Ozon, 1972). Of relevance is a study by Colombo and Belvedere (1976) who examined ovarian steroid synthesis in sexually immature *Anguilla* in which the oocytes were about to commence vitellogenesis. *In vitro* incubation of the previtellogenic ovarian tissue with pregnenolone or progesterone yielded testosterone plus several intermediate metabolites. If the steroidogenic properties of the immature *Anguilla* ovary are similar to those of the temperature-regressed goldfish, then the effects of pregnenolone and

17α -OH-pregnenolone on spawning behaviour could be explained as the result of conversion of these steroids to androgens or estrogens.

Difficulties arise when the concept of *in vivo* conversion is used to interpret the behavioural effects of androgens and estradiol. For example, if it is assumed that estradiol stimulates the mechanism controlling spawning behaviour, then the effects of androstenedione and testosterone could be explained as the result of aromatization. However, if the goldfish is similar to mammals in being unable to aromatize 5α -reduced steroids (Thompson *et al.*, 1971), the effects of androsterone and dihydrotestosterone could not be explained as the result of conversion of these steroids to estrogen (it is not known whether 11-ketotestosterone can be converted to estrogen). On the other hand, if it is assumed that androgens regulate receptivity in the female goldfish, then the results of androgen treatments could be interpreted as evidence that the mechanism controlling spawning behaviour responds to a variety of androgens; in female guppies (Liley, 1972) and mammals (Beyer *et al.*, 1971; Feder and Silver, 1974), sexual receiptivity is induced by more than one estrogen. This latter interpretation fails to account for the behavioural activity of estradiol.

That estrogen is likely to be directly involved in stimulating spawning behaviour is suggested by the effects of estrogen on sexual behaviour of female guppies (Liley, 1972), lizards (Crews, 1975), birds (Noble, 1972), and many mammals (Young, 1961; Beach, 1964; Davidson and Levine, 1972). However, androgen appears to control receptivity in the female rhesus monkey (Everitt and Herbert, 1975). Therefore, considering both the

behavioural effects of the androgens used in this study and the fact that plasma androgens increase prior to spawning in female goldfish (Schreck and Hopwood, 1974) and in other female teleosts (Schmidt and Idler, 1962; Schreck et al., 1972; Katz and Eckstein, 1974; Campbell et al., 1976), the possibility that androgens may regulate spawning behaviour should not be ignored.

As female sexual behaviour in the guppy and in many mammals is induced only by estrogens (and androgens which can be metabolized to estrogens) it is unlikely that both estrogens and non-aromatizable androgens act directly to stimulate receptivity in female goldfish. A more reasonable explanation of the effects of estrogens and androgens on spawning behaviour of intact, regressed goldfish is that either estrogen or androgen (perhaps both) acts indirectly by stimulating gonadotropin release. This possibility is considered in the following section of the discussion.

In summary, no single explanation is likely to account for the diversity of steroids which induce responsiveness to egg injection in intact, regressed goldfish. Pregnenolone and 17$^{\alpha}$ -OH-pregnenolone probably are converted in vivo to behaviourally active metabolites. Similarly, androstenedione and testosterone may be metabolized prior to affecting behaviour. However, the effectiveness of estradiol, and of androsterone and dihydrotestosterone (which likely cannot be aromatized), indicates that both androgen and estrogen restore receptivity in intact fish. These steroids may act directly on the mechanism controlling spawning behaviour. Alternatively, androgen and / or estrogen may induce receptivity indirectly by stimulating gonadotropin release.

3. Possible Mechanisms of Steroid Action

Exogenous steroids may restore responsiveness to egg injection indirectly, by stimulating gonadotropin secretion, or directly, by acting on central neural or peripheral structures controlling spawning behaviour.

Implicit in the hypothesis that steroids induce receptivity by stimulating gonadotropin release is the assumption that in regressed, unreceptive fish plasma gonadotropin is absent, or present in low concentrations. Plasma gonadotropin has been measured in preovulatory (Breton et al., 1972) but not in regressed goldfish; however, Nagahama (1973) described signs of nuclear and cytoplasmic degeneration in pituitary gonadotrophs from female goldfish with regressed ovaries. Also in Gillichthys mirabilis, which undergoes a temperature-induced gonadal regression similar to that of goldfish (De Vlaming, 1972), ultrastructural examination of gonadotrophs indicates that secretory activity of these cells is depressed in regressed fish (Zambrano, 1972). In female brook trout and sockeye salmon with ovaries in early stages of development, plasma gonadotropin levels are low or undetectable (Crim et al., 1975). As partially regressed goldfish with minimal yolk vesicle deposition perform spawning behaviour when injected with ovulated eggs, low plasma gonadotropin titres may be sufficient to induce sexual receptivity. Thus, injection of steroids may restore responsiveness in regressed fish by stimulating only small increases in gonadotropin release.

In female rats, steroids have been shown both to inhibit and to facilitate release of gonadotropin (Davidson; 1969; Everett, 1969). Although some of these effects are exerted at the level of the hypothalamus, estrogen (Cooper and McCann, 1975) and testosterone (Perez-Palacios et al., 1976)

also increase the response of the pituitary to luteinizing hormone-releasing hormone. Steroids may act on the teleost hypothalamus and pituitary to regulate gonadotropin secretion; in the male sunfish (Lepomis cyanellus), Pfaff et al., (unpublished results cited in Morrell et al., 1975) demonstrated retention of labelled testosterone in the anterior pituitary and nucleus lateralis tuberis (an infundibular region suggested to regulate gonadotropin secretion in goldfish [Peter, 1970]).

Generally, exogenous steroids exert inhibitory effects on the ovaries of intact fish (see review by Pickford and Atz, 1957). In the catfish, Heteropneustes fossilis, estradiol or testosterone treatments reduce both the size and number of pituitary basophils, suggesting that ovarian atresia following administration of these steroids results from inhibition of gonadotropin secretion (Sundararaj and Goswami, 1968). Similar indirect evidence indicates that exogenous steroids inhibit gonadotropin secretion in female goldfish; injection of estrogens or testosterone induces atresia of vitellogenic oocytes (Khoo, 1974) and estrogen treatment produces degenerative changes in gonadotrophs (Nagahama, 1973). The results of these studies suggest that, in fish with maturing ovaries, estrogen and testosterone lower gonadotropin output to levels insufficient for maintenance of yolky oocytes; however, these findings do not eliminate the possibility that in regressed fish these steroids may stimulate a low rate of gonadotropin release. Thus whether such a mechanism can account for the effects of steroids on spawning behaviour of regressed fish remains an open question.

As noted above, if steroids are directly involved in the regulation of spawning behaviour, they may act on central neural structures or may affect the responsiveness of the genital tract to the presence of ovulated eggs.

Estrogen stimulates sexual behaviour in female mammals by actions on specific areas of the brain, in particular the region of the preoptic nucleus - anterior hypothalamus. There is evidence that the preoptic area is involved in the control of reproductive behaviour in teleosts. Electrical stimulation of the preoptic region evokes courtship behaviour in male bluegill sunfish, Lepomis macrochirus (Demski and Knigge, 1971), release of milt in male sunfish and goldfish, and release of eggs in ovulated female goldfish (Demski et al., 1975). Furthermore, electrolytic lesions of the preoptic area in Fundulus abolish the spawning reflex response to injection of neurohypophyseal hormones (Macey et al., 1974). There is no evidence in teleosts that steroids are involved in the function of the preoptic area or of other brain areas which may regulate reproductive behaviour. Although Pfaff et al. (unpublished results cited in Morrell et al. 1975) demonstrated binding of testosterone in the nucleus lateralis tuberis of the male sunfish, Lepomis cyanellus, the functional significance of this finding is not known.

Steroids may influence spawning behaviour by an action on the genital tract. In the female rat, physical stimulation of the perigenital area plays a role in sexual behaviour (Pfaff et al., 1973; Kow and Pfaff, 1976) and estrogen increases the sensitivity of this region (Kow and Pfaff, 1973-4). Similarly, steroids may affect spawning behaviour in goldfish by sensitizing the oviduct to the stimulus provided by ovulated eggs. In discussion to follow it is suggested that ovulated oocytes are transported to the ovipore by ciliary action and that it is the portion of the oviduct near the ovipore which is sensitive to ovulated eggs. Steroids may regulate this proposed ciliary ova transport; treatment of goldfish fry with ethinylestradiol or

methyltestosterone induces hypertrophy and extensive ciliation of oviduct epithelium, processes which are normally associated with vitellogenesis (Takahashi and Takano, 1971). In female mammals, the effects of estradiol on proliferation of cilia in the genital tract are well known (More and Masterton, 1976).

In summary, evidence that exogenous steroids inhibit gonadotropin secretion in goldfish and other teleosts suggests that steroids stimulate spawning behaviour in intact, regressed female goldfish by an action on the genital tract or on the central nervous system. Steroids may influence spawning behaviour solely through an action on the genital tract, activating a mechanism which detects ovulation and relays this information to the central nervous system. It is suggested that if steroids influence spawning behaviour by an action on the central nervous system, the mechanism involved may be different from that in female mammals. For example, in female mammals, elevated plasma estrogen levels induce a brief period of receptivity near the time of ovulation; endocrine stimuli associated with ovulation are not necessary to induce receptivity in goldfish, as females are responsive to injection of ovulated eggs at any time during vitellogenesis. Thus, steroids in mammals function as chemical messengers from the ovaries to the brain, signalling that the ovaries are prepared for ovulation. In female goldfish, stimuli generated by intraovarian ovulated eggs synchronize spawning behaviour with ovulation; steroids (if they are involved) appear only to prime the mechanism regulating receptivity, and not to transmit specific information regarding the state of ovarian development.

D. The Role of Ovulated Eggs.

Spawning behaviour of female goldfish is temporally linked with ovulation

by the stimulus of ovulated eggs in the genital tract. The ability of ovulated eggs to induce spawning behaviour is not restricted to the day of ovulation but is seen in all fish with ovaries in any stage of vitellogenesis. As discussed in detail above, both pituitary and ovarian hormones may affect responsiveness to ovulated eggs.

The site of action of ovulated eggs in inducing spawning behaviour is not known; however, several observations suggest that the terminal portion of the oviduct may be involved. For example, whereas stripping of all easily removed eggs (believed to be those in the oviduct and posterior ovisac) usually terminates spawning for 10 to 15 minutes, removing only a portion of these eggs, and thus leaving additional eggs at the ovipore, has little effect on spawning behaviour. It was also observed that in females from which all accessible eggs had been removed, resumption of spawning generally was restricted to individuals which, on further stripping, were found to have eggs at the ovipore. Furthermore, in some cases where spawning behaviour following egg injection is inhibited by egg binding (hardening and adhesion of eggs in contact with water), only a small number of eggs at the ovipore are bound and the remainder of the injected eggs in the oviduct and ovisacs are apparently normal.

It is not known how ovulated eggs are transported to the ovipore. Eggs may be moved by the activity of smooth muscles or, more likely, by coordinated ciliary motion as has been suggested to occur in ovum transport in the rabbit oviduct (Halbert et al., 1976) and in the frog coelom (Suvarnalatha and Sarkar, 1972). In goldfish, both the ovarian lamellae and the ovisacs possess conspicuously ciliated epithelium.

The effect of ovulated eggs on behaviour is thought to be mediated at

least partially by physical cues, as substitutes for eggs (gelatin, petroleum jelly)are marginally effective. The decreased ability of bound eggs to induce spawning may be due either to inappropriate physical characteristics or to localized loss of a chemical stimulant.

There is evidence that stimulation of the genital tract influences female sexual behaviour not only in teleosts but also in other vertebrate classes. The close temporal correlation between ovulation and spawning (Liley, 1969) suggests that a mechanism whereby ovulated eggs stimulates spawning behaviour may be widespread in this group. In previous studies of oviparous fish (see review by Liley, 1969),failure to induce female sexual behaviour by steroid therapy may have been due to the absence of the requisite stimuli from internal sexual structures. Stimuli from ovulated eggs may function in the reproductive behaviour of some female Anura. Intraperitoneal saline injections (10-30 ml) induce oviposition behaviour in preovulatory and recently spent Rana pipiens (Noble and Aronson, 1942) indicating that abdominal distension may serve a function similar to oviduct distension in goldfish. Noble and Aronson (1942) suggest that, as saline injection fails to induce oviposition behaviour in females treated more than thirteen days after ovulation, the behavioural response to abdominal distension may be influenced by hormones. Although physical stimulation of the vagina and cervix facilitates lordosis responses in the female rat (Rodriguez-Sierra et al., 1975), this phenomenon differs in several aspects from the effects of ovulated eggs on spawning behaviour in goldfish. For example, the facilitatory effect of cervical probing occurs within a minute, persists for several hours after withdrawal of the stimulus, and is neither facilitated by estrogen treatment nor reduced by hypophysectomy.

E. The Role of Prostaglandins

Prostaglandins (PGs) appear to play a role in the spawning behaviour of female goldfish. The results of the present study suggest that stimulation of the oviduct following ovulation (or injection of ovulated eggs or egg substitutes causes the release of PGs (most likely $PGF_{2\alpha}$) which then act directly or indirectly to induce spawning behaviour.

The fact that indomethacin (an inhibitor of PG synthesis) eliminates all spawning within minutes suggests that endogenous PG is utilized rapidly and that spawning behaviour following ovulation or egg injection results from PG released over extended periods. The often considerable duration of spawning following i.p. PG injection indicates a slow uptake from the peritoneal cavity. The variability in the latency to the onset of the behavioural response probably reflects the imprecision of this route of administration. The fact that some fish begin to spawn within a few minutes of receiving PG injection suggests that the latent period preceding spawning behaviour induced by egg injection may be the time required for the stimulus of eggs in the oviduct to elevate PG to effective levels.

The capacity for PG biosynthesis has been demonstrated in several tissues of the carp (Christ and Van Dorp, 1972) and PGE and PGF have been isolated from the testes of several teleost species (Nomura et al., 1973). The source of endogenous PG which induces spawning behaviour in goldfish is not known. PG may be released from the oviduct following physical stimulation, as has been shown to occur in mammalian uteri (Poyser et al., 1971), or it may be released in the brain in response to afferent signals generated in the oviduct.

It is also possible that PG is released by ovarian macrophages in

response to egg injection. Macrophages have been implicated in the action of IUDs (Myatt et al., 1975) and it has been suggested (Higgs and Youlten, 1975) that PG production by leukocytes might regulate the emigration of leukocytes from blood vessels during acute inflammation. If similar mechanisms were activated by egg injection in goldfish, the latency from egg injection to onset of spawning might represent the time required for sufficient numbers of cells to aggregate at the injected eggs. Macrophage aggregations in the vicinity of injected eggs are observed frequently in histological preparations (Fig.29 in the Appendix). The fact that there is much evidence that the number and phagocytic activities of macrophages are greatly increased by estrogen and fluctuate with the female reproductive cycle (Vernon-Roberts, 1969) suggests that the possibility of macrophage involvement in prostaglandin production and spawning behaviour merits investigation.

Recent unpublisheddstudies of female spawning behaviour in two other oviparous teleosts, (Jordanella floridae (Crawford, 1975) and Gasterosteus (Lam, Chan and Pandey,1976), support the present findings that PGs are involved in oviposition behaviour. Indomethacin injection abolished the responses of ovulated female sticklebacks; following $PGF_{2\alpha}$ injection, these responses were at least partially restored and in some cases oviposition occurred. Though injection of $PGF_{2\alpha}$ into female Jordanella failed to induce oviposition or even behavioural coordination with courting males, spawning reflex responses similar to those seen in the spawning behaviour of this and related cyprinodonts were usually elicited.

Both in Fundulus (Pickford, 1952; Wilhelmi et al., 1955)and in female Jordanella (Crawford, 1975), injections of neurohypophyseal hormones induce

a spawning reflex response similar to that observed in female Jordanella following administration of $PGF_{2\alpha}$. In contrast, treatment with neurohypophyseal hormones fails to stimulate spawning behaviour in goldfish (present study; Pickford, unpublished results cited in Macey et al., 1974) and in several other teleosts (see Macey et al., 1974). On the basis of the earlier work on Fundulus (Pickford, 1952; Wilhelmi et al., 1955), which demonstrated that pharmacologically high doses of neurohypophyseal hormones (injected intraperitoneally) are required to induce the spawning reflex response and that these hormone treatments are equally effective in intact and gonadectomized fish, Macey et al.(1974) proposed that neurohypophyseal hormones may act on some brain centre to elicit the behavioural response; the finding (Macey et al., 1974) that electrolytic lesions of the nucleus preopticus impaired or abolished the spawning reflex response to hormone injection provided support for this hypothesis. However, it has recently been shown (Peter and Knight, unpublished results cited in Peter, 1976) that, even when neurohypophyseal hormones are administered by intraventricular injection, the dosage required to elicit the spawning reflex response is similar to that required intraperitoneally. Peter (1976) interprets these results as evidence of a peripheral rather than a central effect of these hormones and further suggests that a peripheral action of 'neurohypophyseal hormones is probably not a part of the normal mechanism for triggering spawning behaviour in teleosts'.

The response of Fundulus to neurohypophyseal hormones is similar to that of female Jordanella injected with either oxytocin of $PGF_{2\alpha}$; in neither species do treated fish show behavioural coordination with the opposite sex. Thus it is questionable whether the behavioural effect of

$PGF_{2\alpha}$ in female Jordanella is physiological. Furthermore, it is stressed that the effect of neurohypophyseal hormones in Fundulus, where both gonadectomized (Wilhelmi et al., 1955) and hypophysectomized fish (Pickford, 1952) show a spawning response without coordination of the sexes, contrasts sharply with the ability of $PGF_{2\alpha}$ to induce all aspects of spawning behaviour in female goldfish, but only in the normal spawning environment (in the presence of males and aquatic vegetation) and under pituitary stimulation.

The mode of action of PG in inducing spawning behaviour in female goldfish is not known. Hall et al. (1975) showed that injection of PGE_2 into the third ventricle of ovariectomized, estrogen-primed female rats induced receptivity similar to that following treatment with estradiol and progesterone. As LH-RH, shown to induce or increase lordosis in hypophysectomized, estradiol-primed female rats (Pfaff, 1973), may be released under the stimulation of PGE_2 (Eskay et al., 1975; Ojeda et al., 1975), Hall et al. suggest that the mechanism by which PGE_2 increases sexual behaviour in female rats may involve the release of LH-RH. Hypothalamic implants of LH-RH have been shown by Dyer and Dyball (1974) to affect electrical activity of hypothalamic neurons, and these workers stress the possibility that if this hypothalamic peptide functions both as a neurotransmitter and as a releasing factor, a single neuron or group of neurons could influence both behaviour and pituitary function. There is evidence that the goldfish hypothalamus contains the teleost equivalent to mammalian LH-RH (Peter, 1973; Crim et al., 1976); release of this hypothalamic factor may be involved in the action of PG on goldfish spawning behaviour.

In the only report of an effect of PG on the sexual behaviour of male

mammals, twice-daily injections of $PGF_{2\alpha}$ (PGE_1 was without effect) induced a 70% increase in mean number of ejaculations of male rabbits after 9 days of treatment (Agmo, 1975). The long latency of this response suggests a less direct effect on behaviour than is likely to occur in the female goldfish.

PG (and, indirectly, ovulation and egg injection) may exert some effect on ovarian or oviductal smooth muscle which is monitored centrally through afferent stimulation. Portions of the female goldfish reproductive tract may be similar to mammalian uteri which, as well as responding to PG (Aiken, 1974), will also release PG when physically stimulated by stretch (Poyser et al., 1971) or by insertion of a foreign object (Spilman and Duby, 1972).

The finding that $PGF_{2\alpha}$ is considerably more effective than $PGE_{2\alpha}$ in stimulating spawning behaviour may be due to differential activity at a single site of action. Alternatively, $PGF_{2\alpha}$ and $PGE_{2\alpha}$ may act at different sites, the lower potency of PGE_2 resulting from insufficient concentration of this prostaglandin in its target tissue following intraperitoneal injection.

The effects of PG injection on hypophysectomized female goldfish which have been treated with salmon gonadotropin or steroids parallel the effect of egg injection on fish receiving similar treatment. Salmon gonadotropin replacement therapy restores the spawning response to injection of either eggs or PG, while steroid treatments are without effect. If egg injection induces spawning behaviour by stimulating PG synthesis and release, then the failure of egg injection to evoke a response in hypophysectomized fish may simply reflect the inability of hypophysectomized fish to respond to PG.

Alternatively, pituitary removal may disrupt many steps in a chain of events leading from the distension of the oviduct, by ovulation or egg injection, to the onset of spawning behaviour.

F. The Regulation of Spawning Behaviour

In the foregoing discussion, more than one mechanism has been suggested to explain the function of each of the endogenous components which influence spawning behaviour. In the present section, the control of spawning behaviour is described by a model (Fig.1) based on what is believed to be the most likely set of mechanisms involved. Implicit in the construction of the proposed model is the assumption that there are fundamental similarities in the endocrine control of sexual behaviour in female goldfish and mammals; without this constraint, the regulation of spawning behaviour could be described by other models consistent with the data presented in this study.

As indicated in Figure 1, gonadotropin stimulates oocyte development and synthesis of steroids which both maintain and sensitize the oviduct and prime hypothalamic (and/or preoptic) areas controlling spawning behaviour. Following ovulation, signals generated by stimulation of the oviduct induce changes in the steroid-primed hypothalamus; this mechanism may involve prostaglandin (PG) at the level of the oviduct and the hypothalamus. Spawning occurs when specific external cues (sexually active male; substrate for egg deposition) are present.

The model attributes the lack of effect of steroids in hypophysectomized fish to the failure of exogenous steroids to duplicate the action of endogenous steroid production and predicts that, in the absence of gonadotropin,

Figure 1. Model of the regulation of spawning behaviour in female goldfish

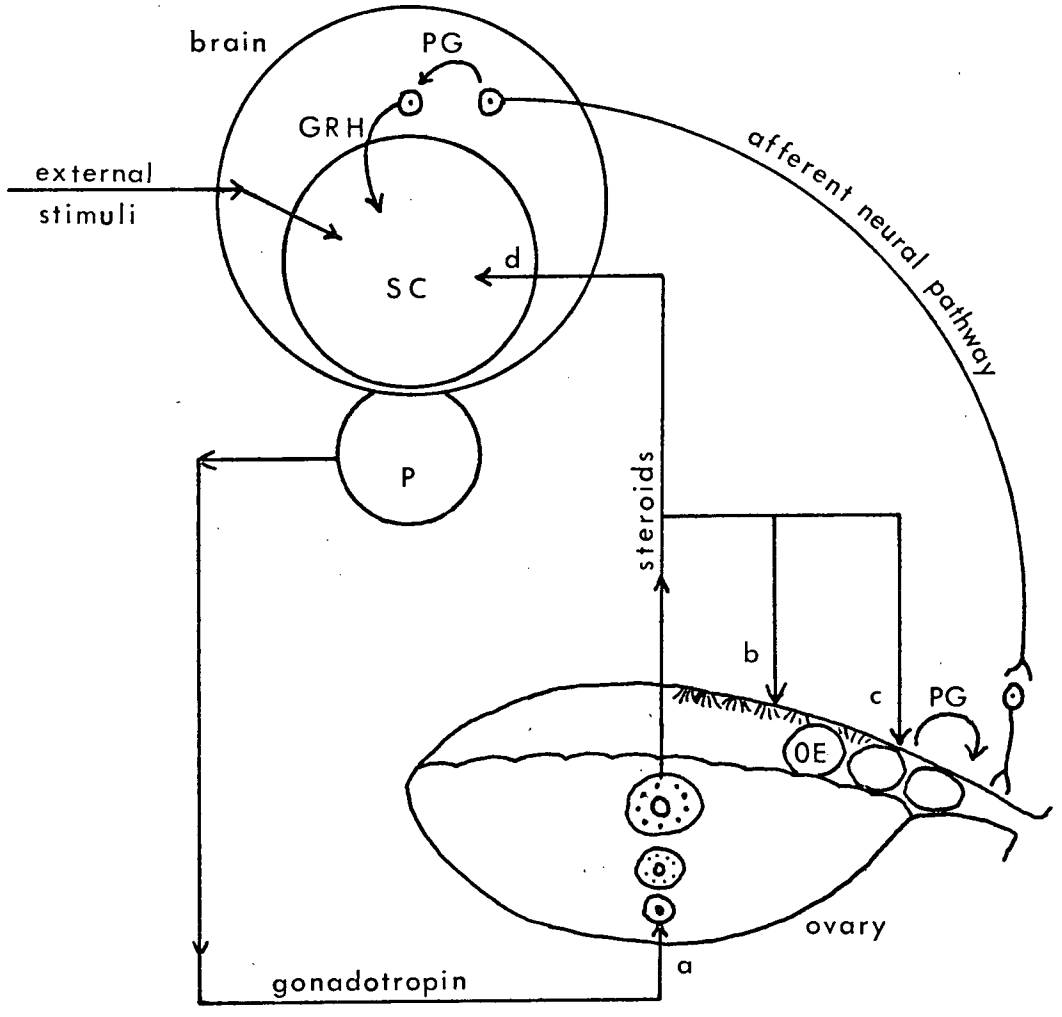
(a) gonadotropin stimulation of oocyte growth and steroidogenesis

(b) steroid stimulation of ovisac and oviduct

(c) steroid action on sensitivity of oviduct

(d) steroid priming of spawning centre

GRH - gonadotropin releasing hormone, OE - ovulated egg, P - pituitary, PG - prostaglandin, SC - spawning centre



MODEL OF THE REGULATION OF SPAWNING BEHAVIOUR

IN THE FEMALE GOLDFISH

some exogenous steroid treatment of hypophysectomized fish should be capable of restoring responsiveness to injection of eggs or PG. This model also attributes the behavioural action of gonadotropin solely to an effect on steroidogenesis. If it could be shown that the ability of PG to induce spawning behaviour persists after ovariectomy, then PG treatment of gonadectomized fish could provide valuable information regarding the roles of gonadotropin and steroids on spawning behaviour.

The model proposes that PG acts both on the oviduct and the hypothalamus to stimulate spawning behaviour. Inhibition of PG-induced spawning by surgical removal of the oviduct would provide evidence that PG acts at this peripheral site. Both induction of spawning by intraventricular injection of PG and inhibition of egg injection-induced spawning by intraventricular injection of indomethacin would provide support for a central action of PG on spawning behaviour.

As indicated in the proposed model, gonadotropin releasing hormone (GRH) is suggested to play a role in spawning behaviour; stimulation of the oviduct following ovulation generates afferent input to the hypothalamus, inducing the release of GRH which then acts on the central neural centre(s) controlling spawning behaviour. The speculation that GRH is involved in spawning behaviour is based mainly on the results of mammalian studies. In the rat, vaginal stimulation induces changes in neuronal activity in brain centres controlling sex behaviour and LH-RH release (Blake and Sawyer, 1972), changes in hypothalamic LH-RH content (Takahashi et al., 1975), and persistent lordosis to manual stimulation (Rodriguez-Sierra et al., 1975); there is much evidence that mechanisms by which genital stimuli induce gonadotropin (and presumably LH-RH) release are widespread

in mammals (reviews by Jochle, 1973, 1975). Furthermore, LH-RH has been shown to induce lordosis in female rats (Pfaff, 1973; Moss and Foreman, 1976) and the effect of PGE_2 on lordosis has been suggested (Hall et al., 1975) to be due to release of LH-RH (see Eskay et al., 1975, and Ojeda et al., 1975, for effects of PG on LH-RH release). These findings, which indicate that genital stimuli may enhance sexual behaviour in female mammals by releasing LH-RH, raise the possibility that ovulated eggs may induce spawning behaviour by a similar mechanism.

There is no published information on the effect of GRH on spawning behaviour of teleosts. However, the findings that plasma gonadotropin titres are greatly elevated in ovulated females of several salmonid species and in ovulating and partially spawned female sockeye salmon (Crim et al., 1975) suggest not only that GRH release may increase during ovulation but also that high rates of release may persist in ovulated fish, perhaps due to the stimulus provided by ovulated eggs. In goldfish, levels of plasma gonadotropin are high on the day of ovulation and return to pre-ovulatory values the following day (Breton et al., 1972). If the presence of ovulated eggs stimulates GRH and gonadotropin release in goldfish, the relatively transient increase in plasma gonadotropin observed by Breton et al. (1972) may have resulted from a decrease in stimulus quality of the ovulated eggs (my observations show that in most cases where ovulated females are not placed with males until the day after ovulation, at least slight egg binding occurs and few or no spawning acts are performed).

The hypothesis that GRH plays a role in spawning behaviour could be tested indirectly by determining changes in plasma gonadotropin levels following injection of ovulated eggs. Behavioural observation following

intraventricular injection of mammalian LH-RH would provide a more direct approach; this technique has been used in examining the effects of neurohypophyseal hormones on the spawning reflex response of Fundulus (Peter, 1976). However, as there is evidence that mammalian LH-RH and teleost GRH may be different (Deery, 1974), failure to induce spawning behaviour with mammalian LH-RH would not eliminate the possibility that endogenous GRH is involved.

G. A Comparative Approach to the Study of Female Sexual Behaviour

In the preceding discussion, which considered the regulation of spawning behaviour in terms of mechanisms proposed to regulate sexual behaviour in female mammals, it has been assumed that similarities in the endocrine control of reproduction in mammals and teleosts may be paralleled by similarities in the endocrine control of reproductive behaviour. Although this approach has been useful in interpreting experimental results, it affords only a limited view of the relationship between mechanisms controlling reproductive behaviour in goldfish and mammals. A more comprehensive view of this relationship may result from considering these behaviours in an evolutionary context.

In the course of vertebrate evolution, reproductive strategies have changed greatly, the presumed ancestral mode of reproduction, external fertilization, giving rise to internal fertilization associated with oviparity, viviparity, etc. Regardless of the strategy employed, however, the formation and fate of the oocytes involves a similar series of stages: pituitary-stimulated development, ovulation, passage of ova or embryos through the coelomic cavity (gymnovarian teleosts) or reproductive tract (cystovarian teleosts, mammals) where they may be held for varying periods,

expulsion of ova or foetuses. What has changed drastically is the stage at which the male intervenes and sexual behaviour occurs. For example, in female goldfish and frogs, where fertilization is external, sexual behaviour and the release of ova are synchronous. In oviparous lizards and birds, and in viviparous guppies and mammals, the advent of internal fertilization has been accompanied by an advancement in the timing of sexual behaviour in relation to oviposition and parturition. Thus, as the role of the female in reproductive behaviour changed from donator of ova (external fertilization) to recipient of sperm (internal fertilization), the occurrence of sexual behaviour became temporally dissociated from the expulsion of sexual products; in contrast, the persistent role of the male as gamete donator necessitated a close temporal association between sexual behaviour and gamete release in all male vertebrates.

It is proposed that in the ancestral, externally fertilizing female vertebrate (i.e. goldfish) a single mechanism, primed by endocrine factors associated with ovarian development and activated by physical stimulation of the genital tract following ovulation, performed the dual function of regulating sexual behaviour and releasing gametes. As the timing of sexual behaviour shifted with the evolution of internal fertilization, the mechanism controlling female sexual behaviour came to be influenced by hormonal factors associated with earlier stages in oocyte development. For example, stimuli from ovulated eggs are required to induce spawning behaviour in female goldfish, whereas estrogen alone is sufficient to stimulate receptivity in female mammals in which sexual behaviour precedes ovulation. However, as stimulation of the genital tract affects sexual behaviour in female mammals, the ability of female mammals to respond to

steroids alone may be due to elaboration of a basic mechanism regulating sexual behaviour in all female vertebrates.

These highly speculative proposals, which greatly simplify complex processes, are offered in the hope of providing a conceptual basis for comparing the regulation of sexual behaviour and related phenomena in female vertebrates.

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APPENDIX

SOME ASPECTS OF THE HISTOLOGY OF REGRESSED OVARIES

In this study, the ovarian histology of experimental fish has been used both in assessing the effects of various injected hormones and in determining the completeness of hypophysectomy and ovarian regression. The process of selecting ovarian characteristics which could be correlated with various endocrine states revealed several aspects of ovarian histology which apparently have not been reported previously. Degeneration of previtellogenic (first growth phase) oocytes was observed many times in intact and hypophysectomized fish; a series of stages of degeneration is described below and some information is provided concerning the effects of steroids and gonadotropin. Cytological features of preatretic early yolk vesicle stage oocytes also are discussed.

As the existence of degenerating previtellogenic oocytes (DPVOs) was not discovered until completion of all experiments, no special techniques were employed in preparing ovaries for histological examination. All ovaries with DPVOs were routinely fixed in Bouin's Fluid and wax embedded (Paraplast). Blocks were cut at 5μ and stained with either haematoxylin and eosin, Mallory trichrome connective tissue stain (Gurr, 1962), or PAS and light green.

As tissues were sampled from various areas of the ovary and as the cutting orientation was not standardized, no quantitative assessment of ovarian histology was undertaken.

DPVOs were found in the ovaries of intact, temperature-regressed females and in hypophysectomized fish, though the incidence was much higher in the latter group.

In the early stages of atresia (Fig.2), DPVOs were very difficult

to distinguish from degenerating early yolk vesicle stage oocytes (DEYVOs). However, as atresia progresses, the follicles of the DEYVOs develop into corpora atretica, passing through stages α to δ as described by Khoo (1975) for more advanced oocytes: i.e. the follicular layer hypertrophies, the oocyte contents are taken up by the granulosa and invading macrophages (Figs. 3 and 4), the hypertrophied follicle first surrounds an empty cavity (Figs. 4 and 5), and finally collapses into an irregular mass of cells. On the other hand, DPVOs do not form corpora atretica, there is very little or no follicular hypertrophy, and although macrophages invade and remove the contents of the oocyte, there is no collapse of the follicle, which eventually surrounds a space approximately the size of the evacuated oocyte (Fig. 6).

It is difficult to be certain that all small degenerating follicles that form corpora atretica also contain yolk vesicles, as the amount of yolk vesicle material may be very small (one vesicle/5μ section). Staining with PAS and light green has proven effective in detecting small amounts of yolk vesicles, but this method is complicated by the fact that the yolk nucleus (an accumulation of mitochrondria) not only is taken up by the hypertrophied follicle cells (as are the yolk vesicles), but, as with the yolk vesicles, is strongly PAS-positive (Fig. 7).

The first recognizable stage of atresia in previtellogenic oocytes involves migration of the nucleus toward the egg membrane, breakdown of the nucleus, disappearance of any difference in staining properties of nucleoplasm and cytoplasm, and a general decrease in the basophilia of the two (Fig. 8). The early stages of the loss of staining may be due simply to the dilution of the cytoplasm with the weakly staining nucleoplasm;

in later stages, the weaker staining is likely due to changes in cytoplasmic constituents as the cytoplasm takes on a more granular appearance and eventually is composed of aggregated debris. Whether other follicular events precede these early changes is not known. However, a common finding in ovaries that contain DEYVOs is that the yolk vesicles in the non-degenerating oocytes no longer maintain a normal cytoplasmic distribution (Figs. 9 and 10), but are very close to, if not touching, the oocyte membrane (Figs. 11 and 12). In all other aspects the oocytes appear normal. As the yolk vesicles in oocytes which have just become atretic are not visible in the cytoplasm but can be found in the hypertrophied follicular layer (Fig.13), it appears that yolk vesicles migrate to the extreme periphery of the cytoplasm prior to the onset of atresia in early yolk vesicle stage oocytes. No other cytoplasmic or nuclear abnormalities are evident at this time.

Migration of the nucleus toward the periphery is seen commonly in DPVOs and DEYVOs and has been reported by Yamazaki (1961) and observed in the present study to occur in oocytes with more advanced yolk vesicle formation (Fig.14). In the early vitellogenic and previtellogenic oocytes, the nucleus moves as a unit toward the egg membrane, at which point some or all of the nucleoli are released. The follicular layer may (Fig.15) or may not (Fig.16) be hypertrophied. In many if not all instances, the nuclear membrane, often retaining a large (10 - 12μ) spherical structure with highly variable staining properties (Figs. 17 and 18), returns to the centre of the oocyte, where it may persist after follicular hypertrophy has begun. It is believed that it is this stage which has been interpreted by Khoo (1974) as evidence of pregnenolone-induced yolk

granule formation.

It is not known whether nuclear migration occurs in DPVOs which have no detectable follicular development. However, in oocytes degenerating at this stage, the nucleoli do not remain at the periphery of the cell but are found in the general cytoplasm after nuclear breakdown. In oocytes which have become atretic at a slightly more advanced stage in which the follicle is partially developed, small numbers of nucleoli can be found within the scattered hypertrophied follicular cells, the rest remaining in the cytoplasm (Fig.19). As degenerating oocytes are found in which hypertrophy of follicular cells and uptake of nucleoli occur and yet in which no yolk vesicles can be detected, it appears that follicular envelopment of the oocyte is completed prior to yolk vesicle formation. It seems that follicular hypertrophy in the partially enveloped previtellogenic oocyte is transient and that no corpus atreticum is formed.

In the early atretic stages of oocytes with developed follicles, nucleoli recently deposited at the periphery are usually clumped in a small area; the degenerating nucleus often is seen close to these nucleolar concentrations (Figs. 8 and 15). Eventually the nucleoli are distributed fairly evenly within the hypertrophied follicular cells (Fig.3); it is not known how this is accomplished. Unlike the situation in degenerating oocytes without developed follicles, nucleoli are not found free in the cytoplasm.

In DPVOs and DEYVOs, the stage of nuclear migration is also characterized by invasion of macrophages which initially tend to accumulate in and around the degenerating nucleus. In some instances,

the nucleoli at this stage are found in clusters (Fig.20). Two readily distinguishable types of macrophage are found both in DPVOs and in DEYVOs. The most common, which will be referred to as M-1 type macrophages, are small, usually spherical but occasionally ellipsoidal cells with clear cytoplasm and small central nuclei (Figs. 6, 20, and 21). The second type (M-2) is a larger, roughly spherical cell with granular cytoplasm and a large, eccentric, bilobed nucleus (Figs.6 and 22). As cells similar to both M-1 and M-2 macrophages are usually very numerous in the channels between the ovarian lamellae and often are found adjacent degenerating oocytes (Figs. 23 and 24), it appears that the free macrophages of DPVOs and DEYVOs are of extrafollicular origin. Although this was not quantified, it appeared that the less common M-2 macrophage occurred most frequently in the less developed atretic oocytes.

Macrophages are present in DPVOs from the time of nuclear degeneration until all oocyte constituents have been removed. When this has occurred, the non-hypertrophied follicular envelope may remain intact around the cavity (Fig.25); however, many of these envelopes apparently disintegrate. Thus, in many long-term hypophysectomized , and in several intact, regressed fish, it appeared that extensive atresia of previtellogenic oocytes had created large open spaces in the ovary (Figs. 26 and 27). This was not simply the result of atresia of large yolky oocytes and shrinkage of the resulting corpora atretica, as in that process the entire ovary decreases in volume while maintaining close association of normal and atretic oocytes (Fig.28). In addition, corpora atretica are persistent structures which are evident for at least several months after their formation.

After cytoplasmic debris has been removed, DEYVOs resemble the γ - stage atretic follicles described by Khoo (1975), except that they are smaller (usually less than 50μ in cross section). Eventually these small structures collapse to form persistent δ- stage corpora atretica.

No experiments were conducted to investiage the endocrine basis of degeneration of early yolk vesicle or previtellogenic oocytes. However, some incidental observations are worth noting.

In none of the experiments reported in this study was there any indication that treatment with exogenous steroids induced the formation of yolk vesicles. There was, however, some indication that injection of estradiol or 5α - dihydrotestosterone delayed onset of atresia in early yolk vesicle oocytes following hypophysectomy (Experiment 5). For example, whereas 5 of 8 fish receiving estradiol and 4 of 8 fish receiving 5α - dihydrotestosterone had small numbers of normal yolk vesicles in their oocytes, there were no normal yolk vesicles in any of 6 saline-treated fish. That this indicated inhibition of atresia rather than induction of yolk vesicle formation is suggested by the fact that whereas all 6 saline-injected females had many DEYVOs, these were found in low numbers in only 4 of the estradiol and 4 of the dihydrotestosterone groups. In addition, yolk vesicles associated with the egg membrane were abundant in 6 of the fish receiving estradiol and in 5 of the fish receiving dihydrotestosterone, whereas these structures were seen in only 3 of the fish receiving saline, and then only in low numbers. This is interpreted to mean that in fish treated with saline, the proposed transition of normal early yolk vesicle oocytes to oocytes with membrane vesicles and finally to DEYVOs has proceeded more rapidly than it

has in fish receiving estradiol or dihydrotestosterone.

In all three experiments involving hypophysectomy and injection of pituitary material, there was good evidence that both homogenized goldfish pituitaries and partially purified salmon gonadotropin (SG-G100) inhibited atresia following hypophysectomy. In the two experiments in which replacement therapy began within one month of hypophysectomy, none of 11 fish receiving homogenized pituitaries (Experiment 6) and only 1 of 10 fish receiving SG-G100 (Experiment 7) had DPVOs in their ovaries. In contrast, following estradiol treatment, 7 of 8 fish in Experiment 6 and 8 of 10 fish in Experiment 7 had DPVOs. SG-G100 appeared to be less effective in inhibiting atresia in long-term hypophysectomized goldfish, as the ovaries of 4 of 15 females treated with SG-G100 in Experiment 11 contained DPVOs; however, this was still a lower incidence of atresia than that found in fish treated with estrogen (8 of 12 fish) or saline (13 or 16 fish). As females receiving SG-G100 also had more yolk vesicles associated with the egg membrane, it is possible that some of the atresia involved oocytes in which yolk vesicles were induced by the gonadotropin.

Most studies of oocyte atresia in teleost ovaries have dealt with the degeneration of second growth stage (vitellogenic) oocytes (references cited by Khoo, 1975) which are generally accepted to be dependent on the pituitary (review by Dodd, 1972). Previtellogenic oocytes have been suggested to be independent of pituitary influence (Dodd, 1972) and atresia of these oocytes has received scant attention in the literature. Beach (1959) described corpora atretica in goldfish which he believed were derived from previtellogenic oocytes; however, as these structures had a hypertrophied granulosa layer, they may have developed from early yolk

vesicle stage oocytes. MacKay (1973) reported 'small corpora atretica apparently derived from degeneration of previtellogenic oocytes' in ovaries of firetail gudgeons (Hypseleotris gallii) receiving methallibure treatment. As these structures, found only in females given long-term (2 month) methallibure treatment, appeared to have no granulosa layer, it is likely they were DPVOs.

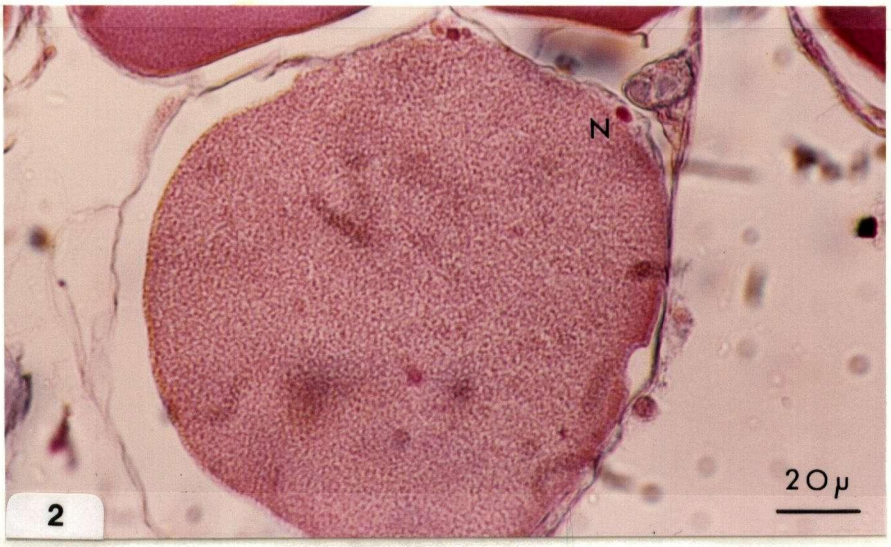
The results of the present study indicate that the correlation between the post-hypophysectomy degeneration of teleost oocytes and the presence of yolk deposition is not as simple as has been suggested in earlier work. Although these findings are only suggestive, they point to a greater pituitary influence on previtellogenic oocytes than has been recognized previously.

APPENDIX FIGURES

Figure 2: Early stage of degenerating early yolk vesicle (DEYVO) or previtellogenic (DPVO) oocyte. A few red nucleoli (N) are seen at the periphery. Mallory trichrome.

Figure 3: Follicular hypertrophy more advanced than in Figure 2. Mallory trichrome.

Figure 4: Advanced DEYVO from intact regressed fish. Hypertrophied follicle surrounds empty cavity. Note that presumptive nucleoli (N) are still numerous. Mallory trichrome.


N
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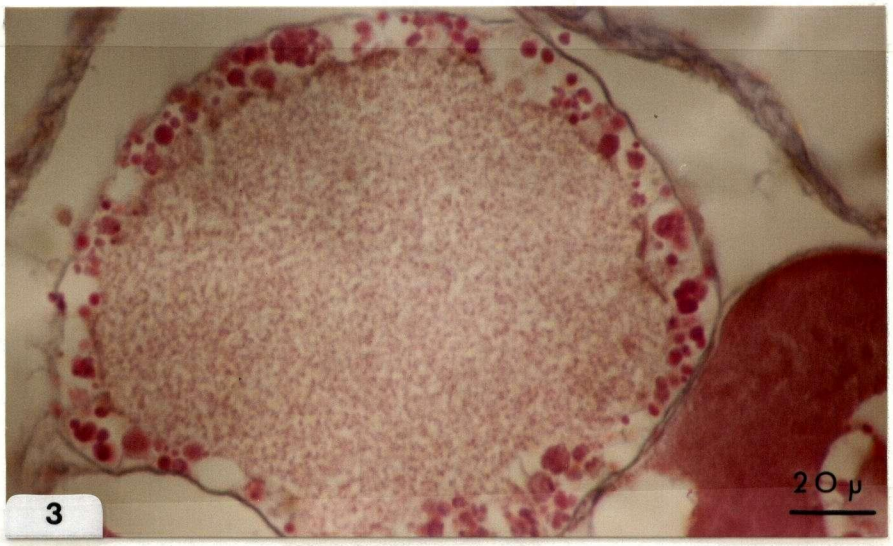
2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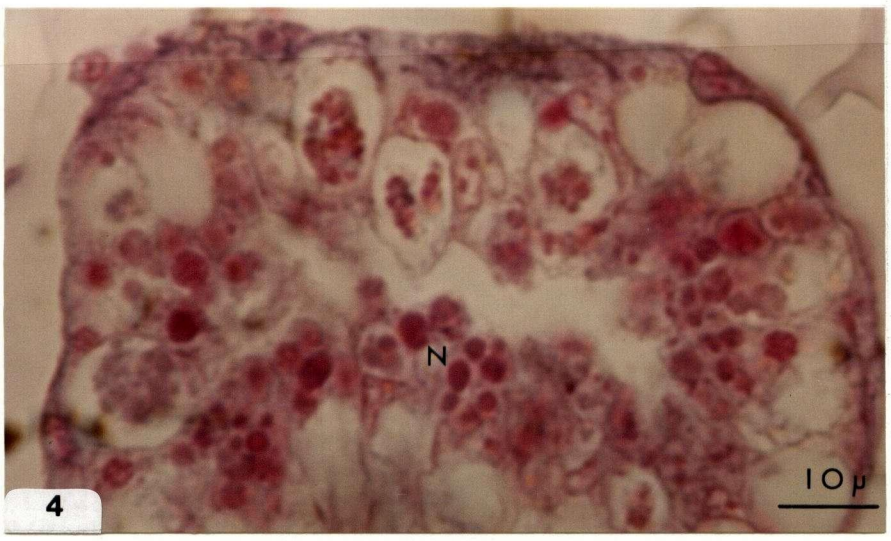
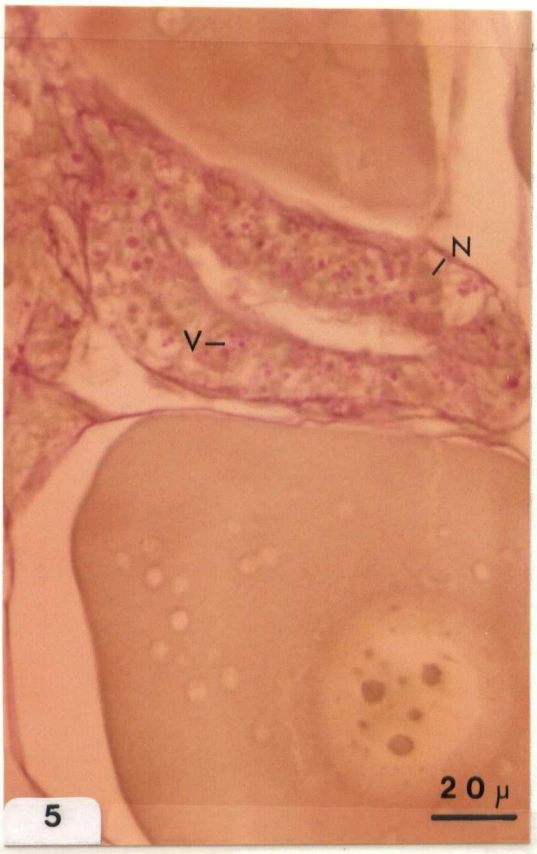

N
10 μ
4

Figure 5: Advanced DEYVO from female goldfish hypophysectomized for 2 months. PAS-positive yolk vesicles (V) and green nucleoli (N) are still obvious. PAS-light green.

Figure 6: Advanced DPVO from fish hypophysectomized for 2 months. Most oocyte contents have been removed and several M-1 (1) and M-2 (2) macrophages remain. Note absence of follicular hypertrophy. Mallory trichrome.

Figure 7: Yolk nucleus in oocyte of fish hypophysectomized for 4 months. PAS-light green.

Figure 8: Nuclear migration stage in oocyte from fish hypophysectomized for 4 months. Note breakdown of nuclear structure and loss of cytoplasmic basophilia. Mallory trichrome.


N
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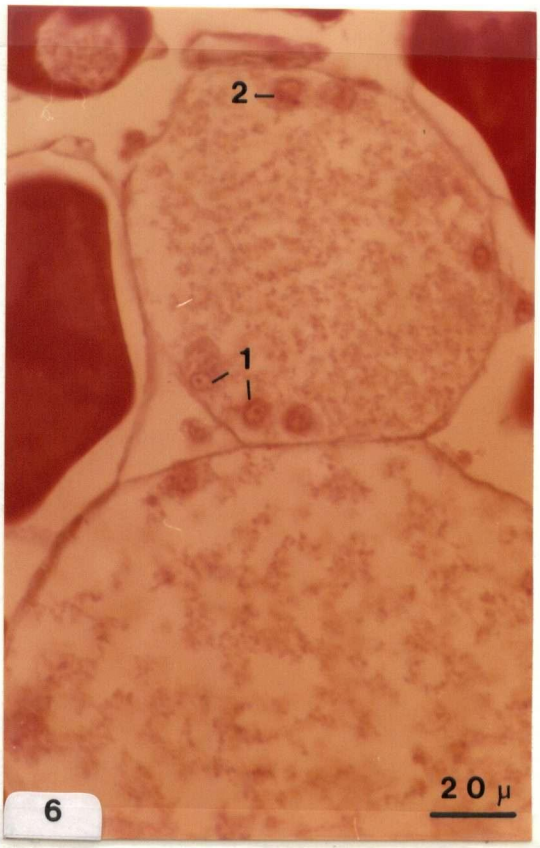
20 µ
5


2
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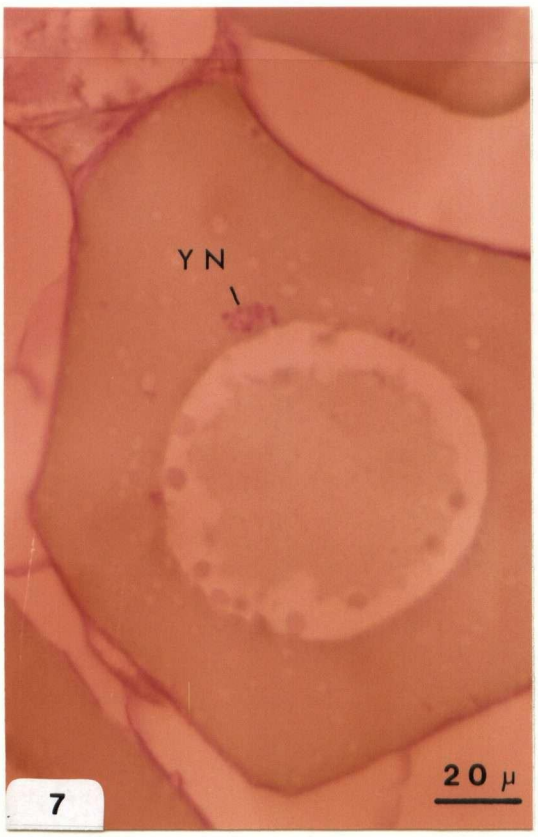
Y N
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7

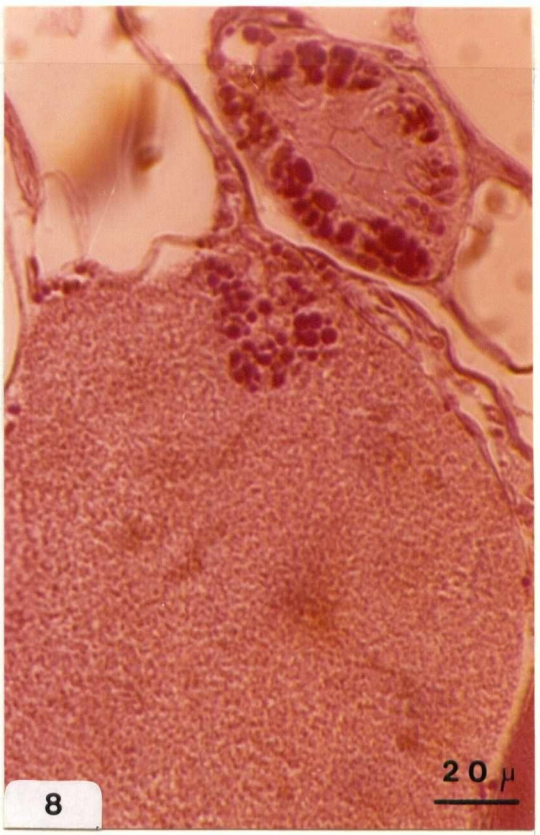
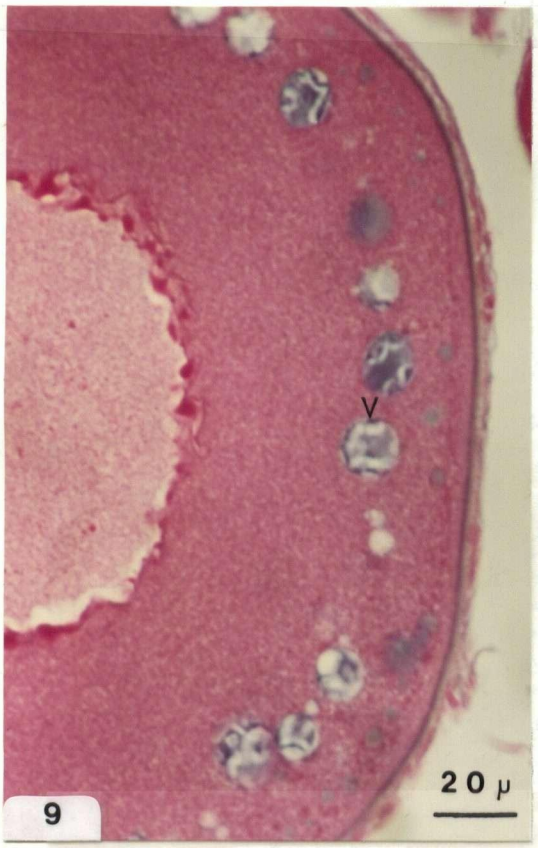

20 µ
8

Figure 9: Normal configuration of yolk vesicles (V). Note vesicles tend to form a ring and do not contact the oocyte membrane. Mallory trichrome.

Figure 10: As in Figure 9 but stained with PAS-light green.

Figure 11: Yolk vesicles (V) associated with membrane of presumptive pre-atretic oocyte. From fish hypophysectomized for 2 months. Mallory trichrome.

Figure 12: As in Figure 11 but stained with PAS-light green.


V
20 µ
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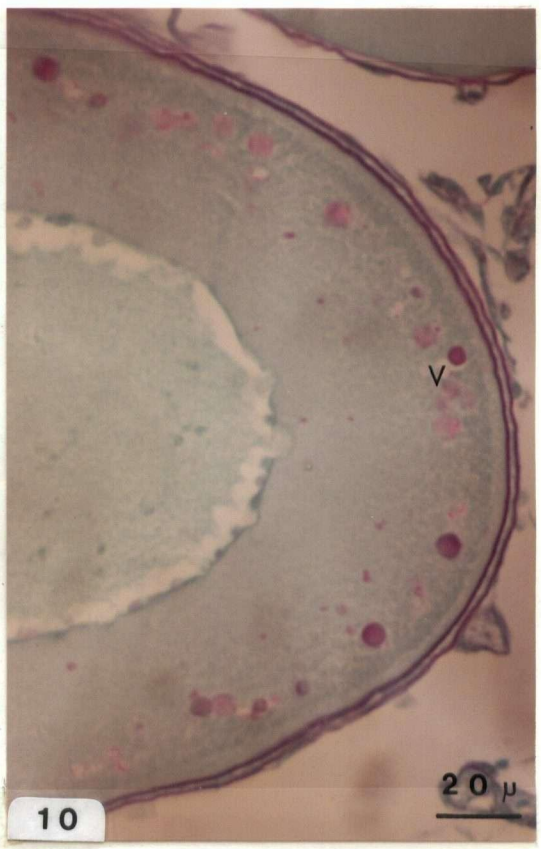
V
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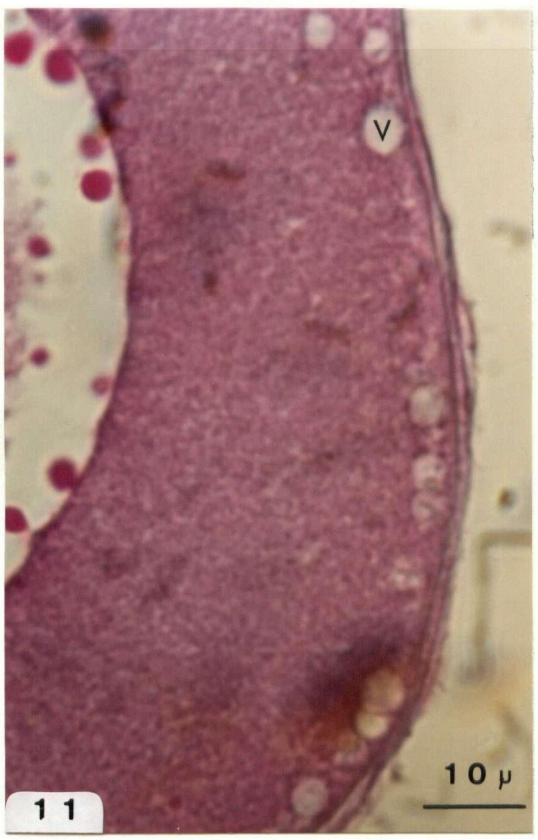

V
10 µ
11

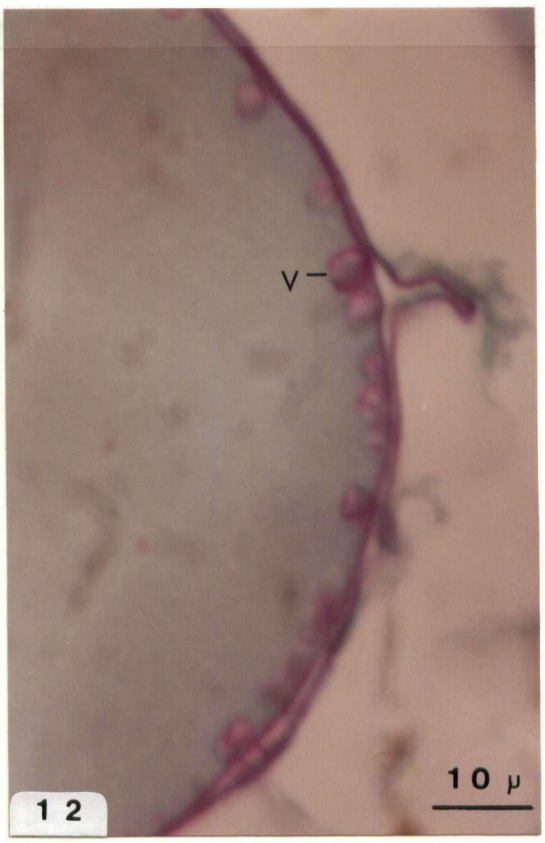
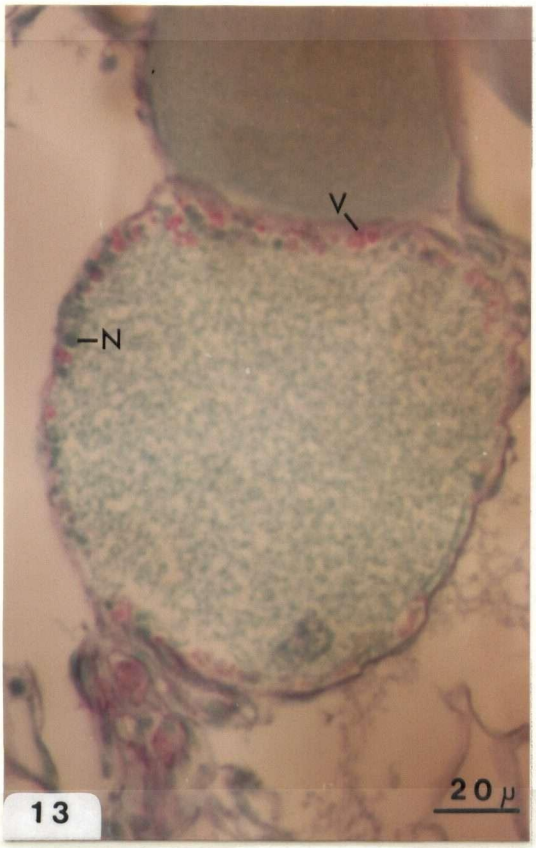

V
10 µ
12

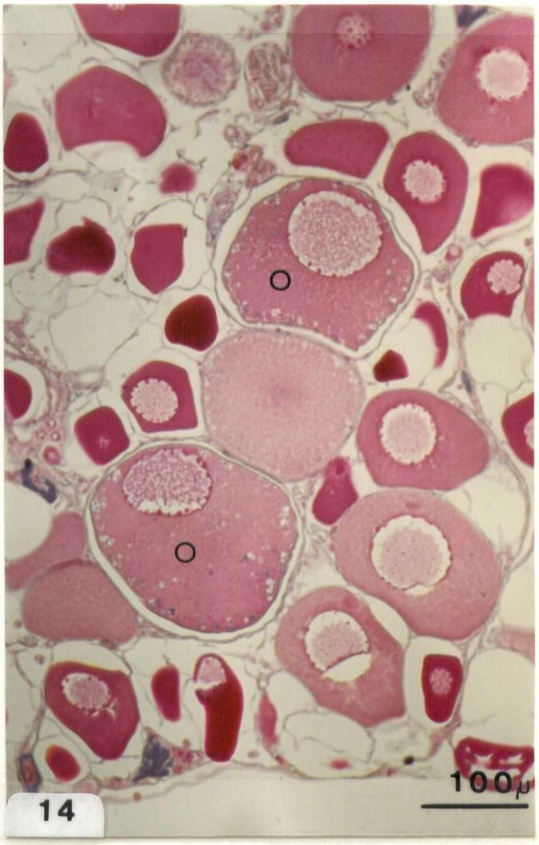
Figure 13: Yolk vesicles (V) and presumptive nucleoli (N) in hypertrophied follicular layer from fish hypophysectomized for 2 months. PAS-light green.

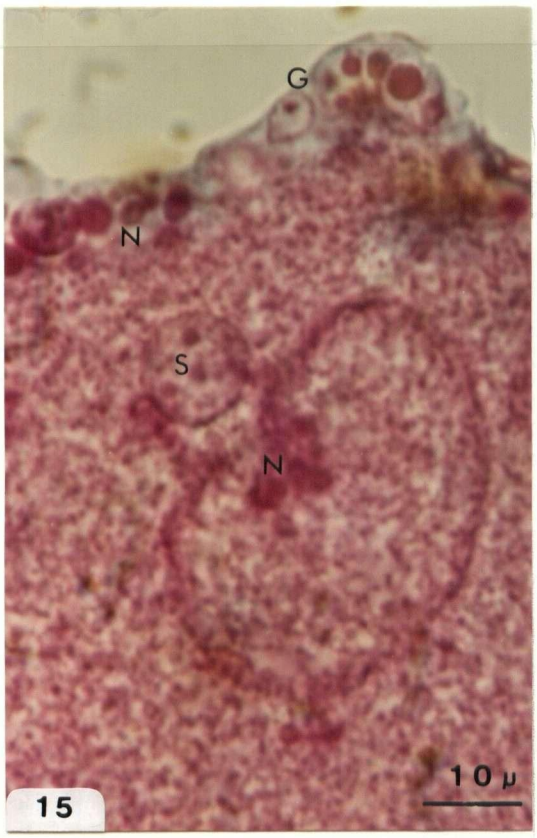
Figure 14: Presumptive pre-nuclear breakdown stage oocytes (O) in ovary of fish hypophysectomized for 2 months. Note abnormal yolk vesicle distribution. Mallory trichrome.

Figure 15: Degenerating nucleus at early stage of atresia. Spherical body (S) appears outside nuclear membrane and is very lightly stained. Presumptive nucleoli (N) are seen both within the nuclear membrane and within the presumptive granulosa cells (G) in the slightly hypertrophied follicle. From intact regressed female. Mallory trichrome.

Figure 16: Peripheral nucleus with spherical body (S) and several nucleoli (N). Follicle is not hypertrophied. Mallory trichrome.


V
N
20 µ
13


O
O
100 µ
14


G
N
S
N
10 µ
15

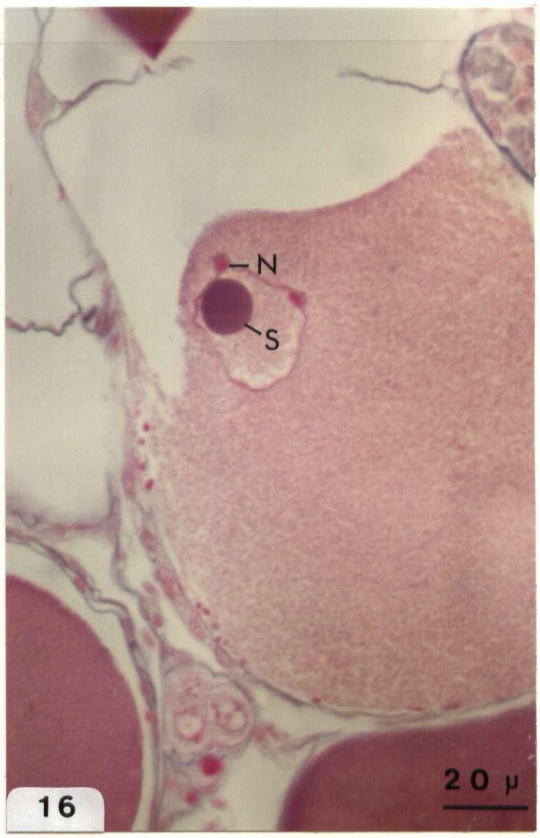
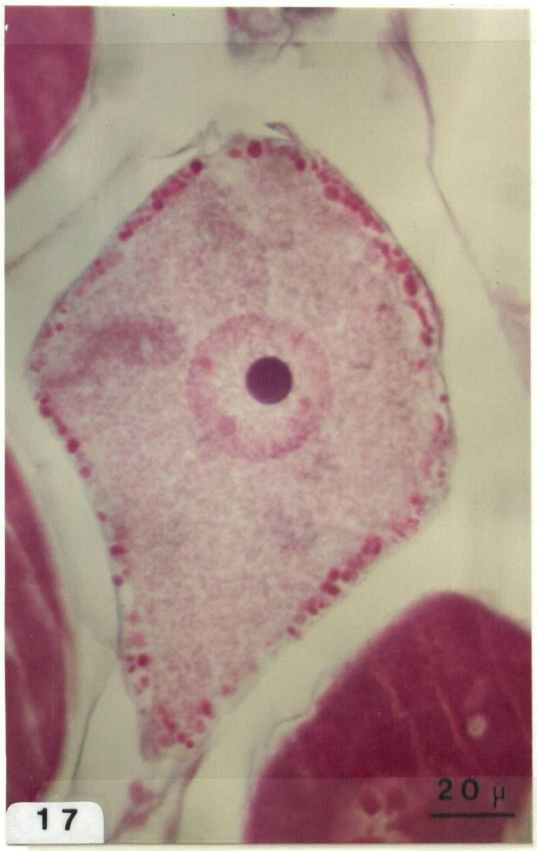

N
S
20 µ
16

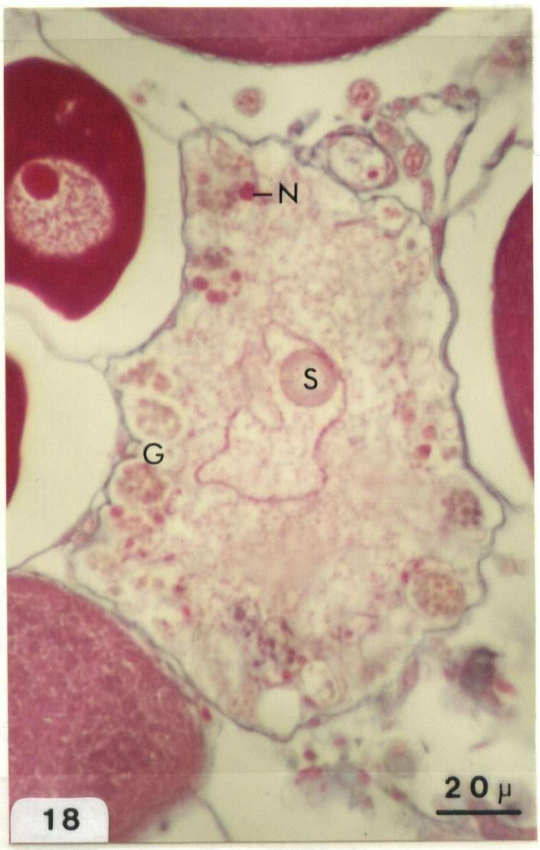
Figure 17: Presumptive DEYVO from fish hypophysectomized for 2 months. Nucleus is unusual as it stains differently than the cytoplasm and appears to retain some nucleoli. Mallory trichrome.

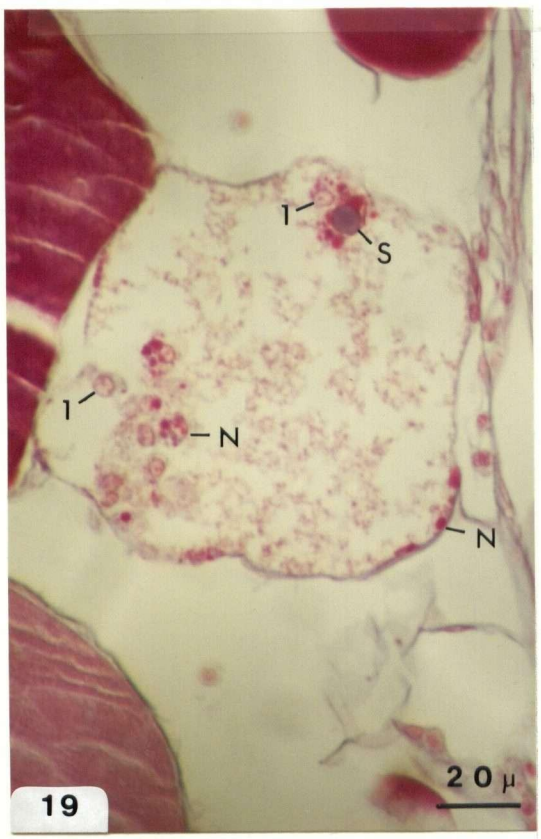
Figure 18: Advanced DEYVO from intact, regressed fish. Degenerated nucleus with spherical body (S) is present. Hypertrophied granulosa cells (G) and presumptive nucleoli (N) can also be seen. Mallory trichrome.

Figure 19: Advanced DPVO from intact, regressed fish. Presumptive nucleoli (N) are seen both in the slightly hypertrophied follicle and in the cytoplasm. Spherical body (S) and M-1 macrophages (1) also are present. Mallory trichrome.

Figure 20: Clumped nucleoli (N) from degenerating nucleus of atretic oocyte of fish hypophysectomized for 4 months.M-1 macrophages (1) can be seen. Mallory trichrome.


20 µ
17


N
S
G
20 µ
18


l
S
l
N
N
20 µ
19

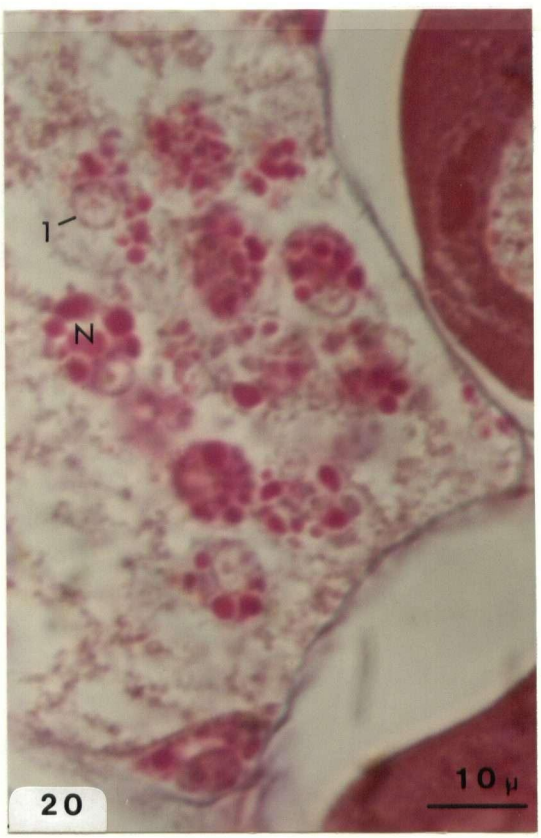
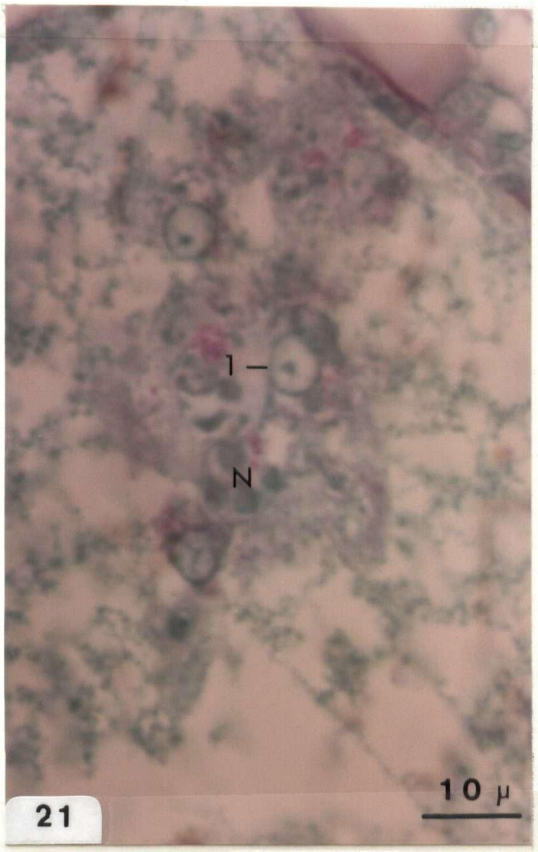

l
N
10 µ
20

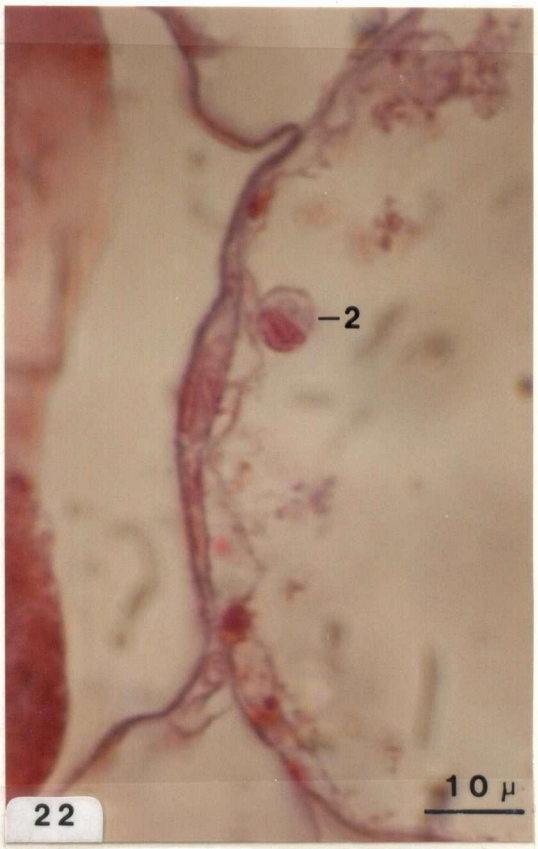
Figure 21: M-1 macrophage (1) and presumptive nucleoli (N) in nuclear debris of oocyte of fish hypophysectomized for 4 months. PAS-light green.

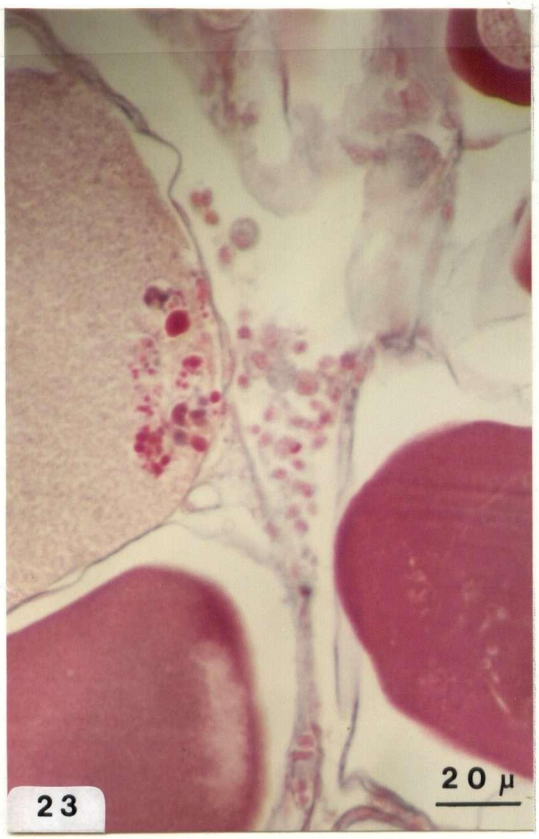
Figure 22: M-2 macrophage (2) in atretic oocyte of intact, regressed fish. Mallory trichrome.

Figure 23: Macrophage aggregation adjacent to early atretic oocyte. Mallory trichrome.

Figure 24: Macrophages entering early atretic oocyte in ovary of intact regressed fish. Mallory trichrome.


1
N
10 μ
21


2
10 μ
22


20 μ
23

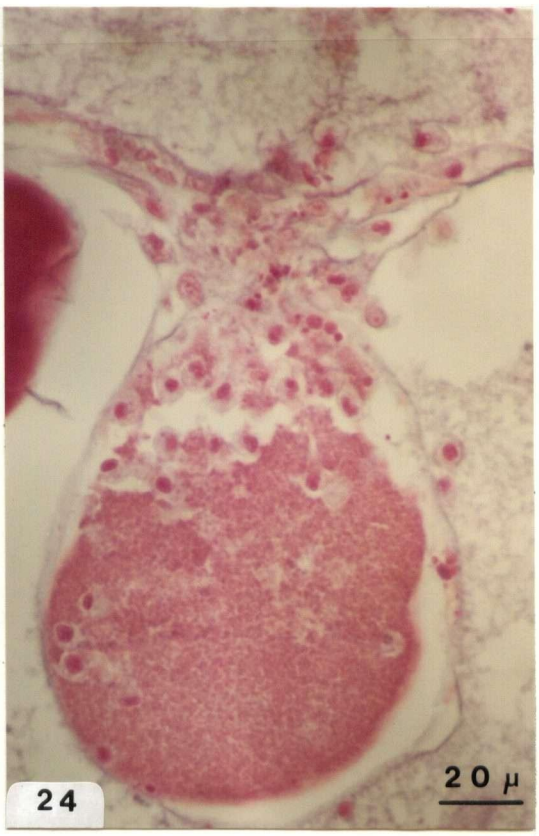
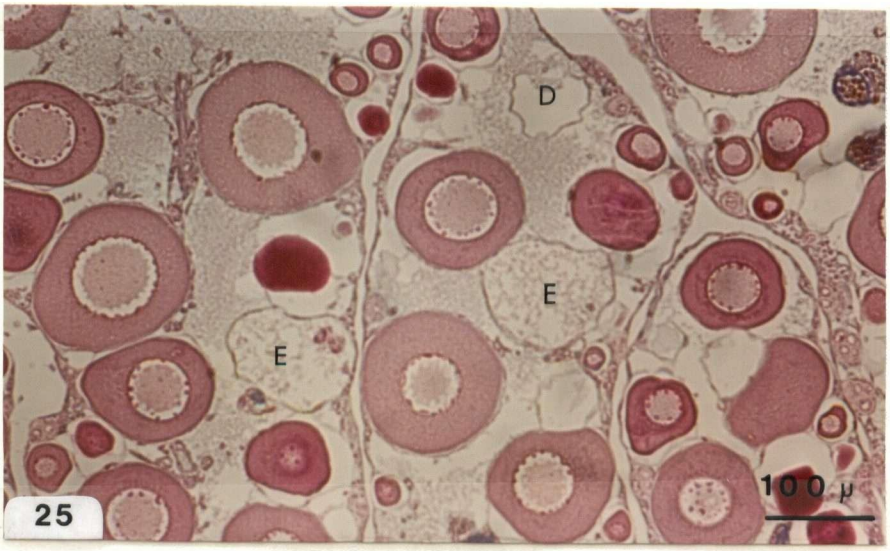

20 μ
24

Figure 25: Ovary of female goldfish hypophysectomized for 4 months. Two nearly evacuated DPVOs (E) can be seen. Older presumptive DPVOs (D) are apparently disintegrating. Mallory trichrome.

Figure 26: Ovary of fish hypophysectomized for 4 months showing open spaces apparently caused by extensive atresia of previtellogenic oocytes. Mallory trichrome.

Figure 27: As in Figure 26 but at a lower magnification. Mallory trichrome.



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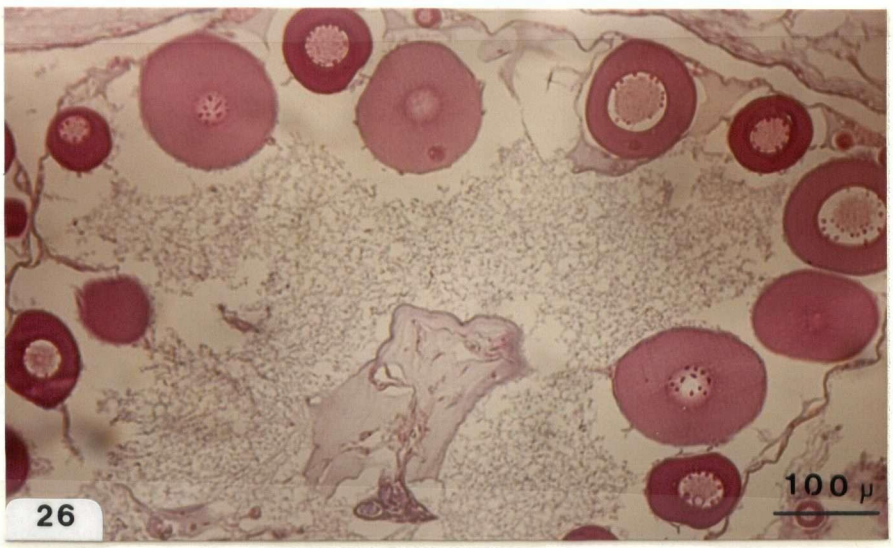

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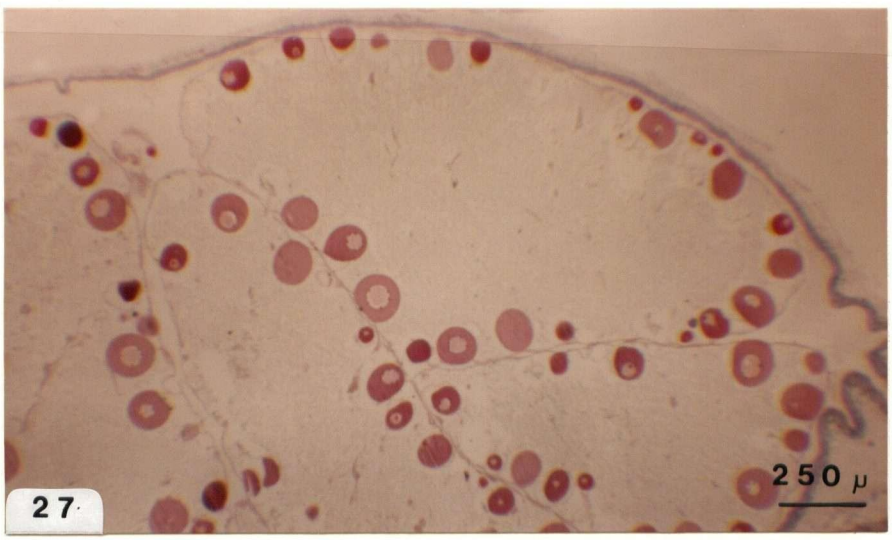
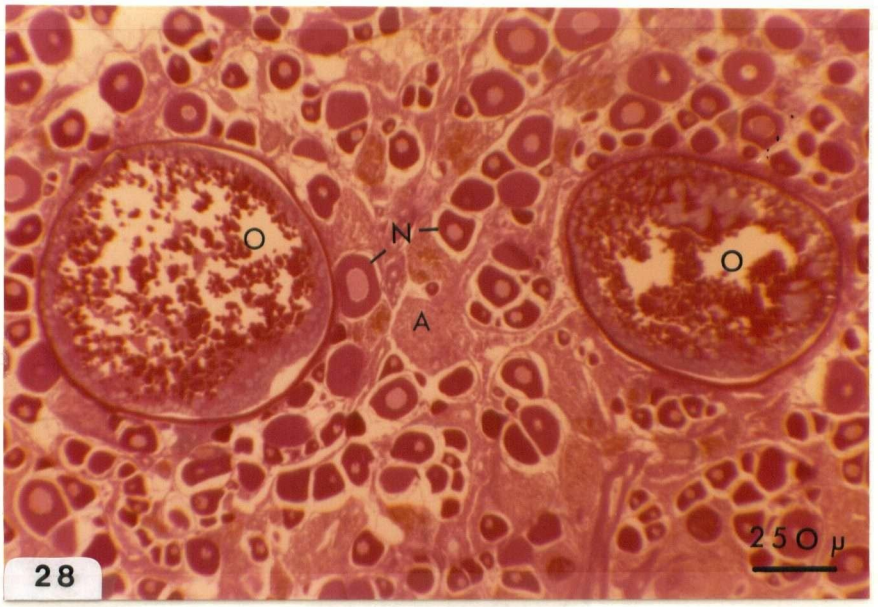


27

Figure 28: Ovary of female goldfish hypophysectomized for 2 months showing close association of normal (N) and atretic (A) oocytes. The two large oocytes (O) are ovulated oocytes (from another female) which were injected through the ovipore. Mallory trichrome.

Figure 29: Macrophage aggregation adjacent to injected ovulated oocyte. Detail from Figure 28. Mallory trichrome.



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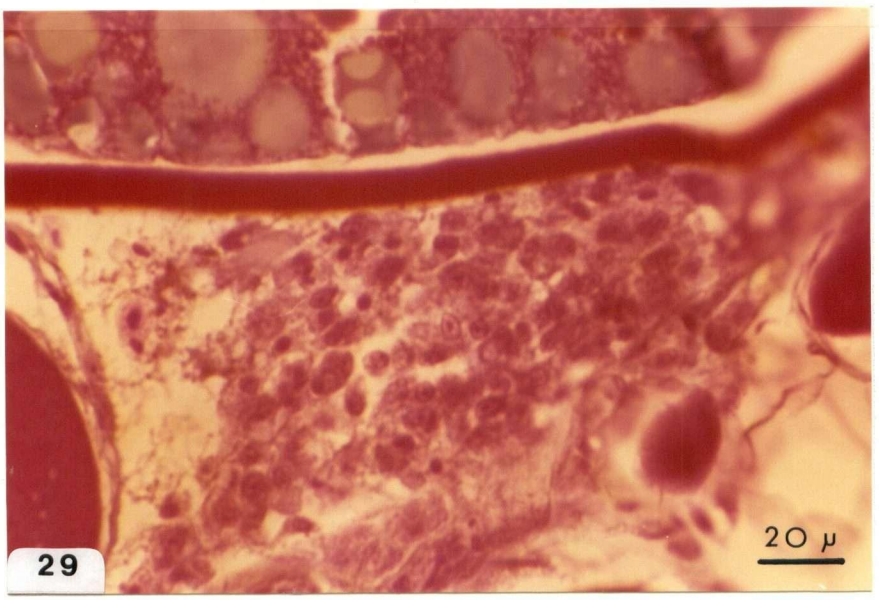

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