PHEROMONES AND OVARIAN GROWTH IN THE AFRICAN CATFISH *CLARIAS GARIEPINUS*

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
Dr H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 21 december 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

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the Department of Fish Culture and Fisheries
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Stellingen

1. De geslachtsorganen van de mannelijke Afrikaanse meerval scheiden feromonen - waarschijnlijk steroïdglucuroniden - uit die de ovariumgroei van soortgenoten bevorderen.

Dit proefschrift; Resink, J.W., 1988, Proefschrift, Rijksuniversiteit Utrecht.

2. Voor een normale gonade-ontwikkeling van mannelijke Afrikaanse meerval is olfactorisch contact met sexe-genoten noodzakelijk: dit geldt niet voor de andere sexe.

Dit proefschrift

3. Mannelijke Afrikaanse meervallen produceren een feromoon dat de vrouwelijke gonade-ontwikkeling beïnvloedt en een feromoon dat de mannelijke gonade-ontwikkeling beïnvloedt; de uitscheiding van beide typen feromonen wordt verschillend beïnvloed door het andere geslacht.

Dit proefschrift

4. Kennis van het vitellogenine gehalte in het bloed van vissen geeft niet altijd betrouwbare informatie over het dooiervormingsproces in het ovarium.

Dit proefschrift

5. Wegnemen van de geslachtsklieren bij mannelijke vissen biedt geen garantie voor de eliminatie van circulerende androgenen.

Dit proefschrift

6. Zonder kennis van de in de natuur voorkomende concentraties, lijkt het afstotend effect van hoge concentraties attractief zaadblaasvocht zoals dit voor de Afrikaanse meerval werd gevonden, een *contradictio in terminis*.

Resink, J.W., Schoonen, W.G.E.J., Albers, P.C.H., Filé, D.M., Notenboom, C.D., Van Den Hurk, R. and Van Oordt, P.G.W.J., 1989.
Aquaculture, 83:137-152.

7. Het verschijnsel dat bij de kweek van aal groeiremming optreedt wanneer dieren verblijfswater van soortgenoten krijgen aangeboden, berust wellicht op de aanwezigheid van feromonen in dat verblijfswater.

8. Het feit dat het visteeltkundig onderzoek zich bevindt op het grensvlak van praktijk en wetenschap, maakt de koersbepaling ervan binnen het wetenschappelijk onderzoek moeilijk.

9. Bij de integratie van de visteelt met de veehouderij in de tropen, wordt te weinig aandacht besteed aan het gevaar voor de consument van zoönosen.

10. De mogelijkheden van het introduceren van de aquacultuur in het kader van de ontwikkelingssamenwerking worden vaak overschat; dit ligt veeleer aan onvoldoende kennis van de sociaal-economische randvoorwaarden van die introductie dan aan de aquacultuur zelf.

Stellingen behorend bij het proefschrift ''Pheromones and ovarian growth in the African catfish'' van J.H. van Weerd.
Wageningen, 21 december 1990.

CONTENTS.

Chapter 1.

PHEROMONES AND REPRODUCTION IN TELEOST FISH

Introduction to the thesis.

PHEROMONES.

Pheromones are defined as 'substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process' (Karlson and Lüscher, 1959). As chemical messengers, pheromones should be active in minute amounts and be relatively species-specific, and only limited molecular overlapping between closely-related species is tolerable (Karlson and Lüscher, 1959). The concept of pheromones was extended to include two types of pheromones (Wilson and Bossert, 1963). Releaser pheromones evoke rapid behavioral responses, whereas primer pheromones have a slower, developmental effect.

In teleosts, a variety of pheromonal actions have been identified (Liley, 1982; Liley and Stacey, 1983). They control social behavior: maintaining hierarchical structures in a population, instilling schooling and homing behavior and eliciting a fright reaction upon release of 'alarm substances' from damaged skin tissue. They have also been implicated in controlling the growth and viability of a population through so-called 'crowding factors' . Pheromones also play a role in teleost reproduction (Colombo *et al.*, 1982; Stacey *et al.*, 1987; Stacey, 1989).

SEX PHEROMONES.

Pheromones affecting teleost reproduction are termed sex-pheromones. They have been shown to regulate spawning behavior and endocrine events related to reproduction in a variety of teleost species (reviews by Colombo *et al.*, 1982; Stacey *et al.*, 1987; Stacey, 1989). In the zebrafish *Brachydanio rerio*, releasing pheromones from ovulated females attract male conspecifics (Van Den Hurk and Lambert, 1983). Releasing pheromones of male origin attract females in the goby *Gobius jozo* (Colombo *et al.*, 1980) and African catfish *Clarias gariepinus* (Resink, 1988). Primer pheromones of female origin increase levels of gonadotropic hormone (GTH) and milt volume (sperm and seminal fluid) in the goldfish *Carassius auratus* (Sorensen and Stacey, 1989). Primer pheromones of male origin may stimulate ovulation in *B. rerio* females (Van Den Hurk *et al.*, 1987). In *C. gariepinus*, ovulated females may induce ovulation (Resink, 1988). This indicates the existence of 'bisexual' primer pheromones which affect both sexes and serve to synchronize gamete maturation between the sexes. This is particularly obvious in spawning of the Pacific herring *Clupea harengus pallasi*, where an unidentified pheromonal component in the milt stimulates spawning in both genders (Stacey and Hourston, 1982).

Being involved in reproduction, a gonadal origin of sex pheromones seems obvious. Female sex pheromones are produced in the ovary, and male sex pheromones in the testes, *e.g.* in *B. rerio* (Van Den Hurk and Lambert, 1983; Van Den Hurk *et al.*, 1987). Males of several teleost species possess specialized structures where sex pheromones are produced. The blenny, *Blennius pavo*, has anal appendices where sex pheromones are produced (Laumen *et al.*, 1974). *G. jozo* has a mesorchial gland, with an abundance of Leydig cells, where sex attractants are produced (Colombo *et al.*, 1980). Catfish species, including *C. gariepinus*, possess a seminal vesicle, in the lobes of which a fluid is produced, containing proteins, polysaccharides and phospholipids (Resink, 1988). Apart from possible functions related to sperm storage and facilitating egg fertilization, the content of the seminal vesicle is responsible for attracting ovulated females (Resink, 1988). However, the skin of *C. gariepinus* may also produce pheromones (Ali *et al.*, 1987). Urine may be the vehicle for gonadally produced pheromones as in the plaice *Pleuronectes platessa* (Canario and Scott, 1989).

THE CHEMICAL NATURE OF SEX PHEROMONES AND MODE OF ACTION.

All teleost sex pheromones identified to date are reproductive hormones or derivatives (Sorensen and Stacey, 1989; Stacey, 1989), whereas non-sex pheromones are of a different nature (proteinaceous) (Colombo *et al.*, 1982). Apart from prostaglandins which, when released from the reproductive tract of *C. auratus* females in response to the presence of ovulated eggs, act as a releaser pheromone eliciting courtship behavior in males (Sorensen *et al.*, 1988), all teleost sex pheromones have been found to be sex steroids or derivatives (Sorensen and Stacey, 1989; Stacey, 1989). The process of glucuronidation, previously thought to consist exclusively of catabolizing redundant steroids (Kime, 1987), plays an important role. Through glucuronidation, the generally apolar steroids are rendered water-soluble, a prerequisite for dispersion in the aquatic environment. The synthesis or presence of steroid glucuronides has been demonstrated in gonads of a variety of teleosts of both sexes, including *C. gariepinus* (Schoonen, 1987). Key enzymes for their synthesis have also been demonstrated in the skin of *C. gariepinus*, and the synthesis there depends on circulating precursor steroids (Ali *et al.*, 1987). Steroid glucuronides have been found to be responsible for several of the primer and releaser effects referred to above. They have also been demonstrated in holding water, evoking a response in conspecifics in *C. auratus* (Van Der Kraak *et al.*, 1989) and in *B. rerio* (Van Den Hurk *et al.*, 1987).

Pheromonal steroid glucuronides act upon the olfactory system, as has been demonstrated by electrophysiological studies (Sorensen *et al.*, 1987; Resink, 1988). In *C. gariepinus*, steroids glucuronidated at the 3α-position are the most potent odorant, evoking a reaction in concentrations as low as 10^{-11} M (Resink, 1988). The existence of an olfactory-brain-pituitary axis has been demonstrated in *C. auratus* (Demski and Dulka, 1984) and *C. gariepinus* (Resink, 1988). In the latter species, the olfactory tract projects into the preoptic area of the hypothalamus, where gonadotropic hormone releasing hormone (GnRH) immunoreactive cells and fibers are located. Some of these fibers have endings between the GTH cells in the pituitary (Resink, 1988).

SPECIES SPECIFICITY AND EVOLUTIONARY ASPECTS OF SEX PHEROMONES.

For pheromones to be species-specific, the basically limited number of sex steroids in teleosts must be differentially used in the various species, especially those with overlapping biotopes. Several explanations have been offered in this respect (Colombo *et al.*, 1982; Stacey, 1989). First, although the number of sex steroids is limited, a relatively minor chemical change in the derived steroid glucuronide may suffice to ensure discrimination between species. Utilization of different blends of a limited number of glucuronides may serve the same purpose. Finally, the mode of release of the pheromone may ensure that the likelihood of heterospecific encounters is minimized.

The use of pheromones to signal one's reproductive status presumably has originated as a side-effect of fortuitous secretion of reproductive hormones or derivatives to the outside world (Liley, 1982). Initially, this was of benefit to the recipient only. In a later evolutionary phase, the benefit became mutual, in that the donor also benefits from the system, *e.g.* by being more successful in encountering a prospective mate (Stacey, 1989). In the latter case, specialized structures for pheromone synthesis or modes of release would be advantageous for both sides.

REPRODUCTIVE PHASES INFLUENCED BY SEX PHEROMONES.

All the sex pheromones referred to above act in the later phases of the reproductive cycle (Colombo *et al.*, 1982; Liley, 1982; Lam, 1983; Liley and Stacey, 1983): priming actions influencing oocyte maturation and ovulation in females, and spermiation in males; releasing actions involving attraction of prospective mates and triggering spawning behavior and release of gametes. Little is known about pheromonal stimulation in early phases of the reproductive cycle (Lam, 1983). Some reports suggest an involvement of primer pheromones in ovarian development in the angelfish *Pterophyllum scalare* (Chien, 1973) and tilapia *Sarotherodon mossambicus* (Silverman, 1978a,b).

THE PRESENT RESEARCH PROJECT.

The experiments forming the basis of this thesis were conducted in the framework of a joint research effort by the Research Group for Comparative Endocrinology of the University of Utrecht and the Department of Fish Culture and Fisheries of the Agricultural University of Wageningen. Its long-term aim was to study the synthesis of steroid glucuronides in the gonads of the African catfish *C. gariepinus*, and to investigate the role of water-soluble steroids in the regulation of oocyte development, ovulation and oviposition. The project was conducted in two phases.

During the first phase attention was focused on fundamental aspects of pheromonal interactions in reproduction of *C. gariepinus*. The results of these studies have been reported in two PhD theses (Schoonen, 1987, Resink, 1988). The main findings were that steroid glucuronides are synthesized in the male seminal vesicles, and also in the female ovary, especially during maturation and ovulation. The testes exhibit little capacity for synthesis of steroid glucuronides. The water fraction of seminal vesicle fluid contains attractants which take effect on post-ovulatory females. The main steroidal components of seminal vesicle fluid are glucuronides of 5ß-reduced C_{19}-steroids. Electrophysiological trials have shown that sex-attracting steroid glucuronides are potent odorants, evoking strong responses after administration to the olfactory epithelium. This finding favors the view that *C. gariepinus* also has an olfactory-brain-pituitary axis, via which sex pheromones exert their influence on gonadal processes. Although stimulation of GTH release and ovulation in females was observed, it could not be demonstrated consistently, and it was suggested that the female response depends on the genetic background of the fish and seasonal differences in the sensitivity of GTH cells in the pituitary to GTH release regulating factors. Moreover, the fact that a response in females was only found when males were in the vicinity suggests the importance of other cues or of an interaction between males and females.

During the second phase of the project, the main topic of research was to investigate the importance of steroid conjugates for ovarian development of *C. gariepinus*, the rationale being that findings might be applied to other fish species which rarely, if ever, enter the stage of ovarian growth in captivity, *e.g.* eels (*Anguilla spp.*). The points of departure for investigations in this second phase were observations that in *C. gariepinus* the presence of males enhances ovarian growth in pubertal females raised under hatchery conditions (Henken *et al.*, 1987) and in wild-caught adult females kept in ponds (Richter *et al.*, 1987).

The ovarian growth stimulating mechanism of male *C. gariepinus* referred to above needs to be analyzed to determine whether chemical cues do indeed play a role. The importance of possible other stimuli, such as those of a tactile, visual, auditory and electric nature, should be determined. Such stimuli have been shown to be important in gonadal development of, for example, *S. mossambicus* (Silverman, 1978a,b) and *P. scalare* (Chien, 1973). Once a chemical signalling system has been identified, its mode of perception should be verified, since chemical substances can be perceived by the senses of taste (gustation; Lamb and Caprio, 1987), or smell (olfaction; Pfeiffer, 1982), which have a different range of action, and are differentially innervated (Hara, 1975). Of the two sensory mechanisms, olfaction can be easily eliminated by removal of the olfactory epithelium (Van Den Hurk and Lambert, 1983) or by disconnecting the olfactory epithelium and the integration centers in the brain (severing of the olfactory nerve; Resink, 1988). Involvement of the sense of smell in perception of the chemical signal indicates a potentially longer range of action than when gustation is the main mode of perception.

A terminology is needed for better understanding of the components involved. In this thesis, we have adopted the terms stimulus donors (the individual presumably releasing the signal), the medium of transport (holding water), and stimulus recipients (the individual receiving the signal and possibly responding with increased gonadal development).

Several aspects are of interest in this context. It might be that donor fishes interact in one way or the other by modulating the intensity of the signal emitted, as has been suggested for *B. rerio* (Chen and Martinich, 1975) and *S. mossambicus* (Silverman, 1978a,b).
In the donor, a gonadal origin of the pheromone or its precursor(s) seems likely, but this needs confirmation by extirpation of the presumed pheromone or pheromone-precursor source.
In the recipient, the endocrine events triggered in response to perception of the stimulus and culminating in enhanced ovarian development may constitute a 'monitoring system' for the presence of pheromonal components in the medium of transport. These endocrine events encompass (Wiegand, 1982; Wallace *et al.*, 1987; Bromage and Cumaranatunga, 1988) the release of GTH, which stimulates ovarian follicles to produce the female sex hormone estradiol-17ß (E_2). E_2 induces hepatocytes to synthesize the female-specific plasma protein, vitellogenin (VTG). VTG is actively sequestered from the maternal blood stream by developing oocytes in the ovary and, after processing, is deposited as yolk material. Through accumulation of yolk, the oocytes increase enormously in size and cause the ovary as a whole to grow. The whole process is known as vitellogenesis.
In the medium of transport (holding water), active components should be identifiable (Van Den Hurk *et al.*, 1987; Van Der Kraak *et al.*, 1989; Stacey, 1989). Based upon existing knowledge about the identity of teleost sex pheromones (see above), holding water could be analyzed for the presence of steroidal compounds. Lastly, when exposed to purified (synthetic) pheromone, recipients should have a

response similar to that found after exposure to holding water, including the endocrine events which culminate in enhanced ovarian development.

OUTLINE OF THE THESIS.

The investigations which form the basis of this thesis cover topics related to the nature and the mode of perception of male sex-stimuli, and the ovarian response to those stimuli. Furthermore, the interaction between males and females with respect to the emission of pheromones is investigated. Finally, the presence of presumed pheromones in holding water is studied, and an attempt is made to elucidate the possible center of their emission. Below, the investigations, as described in subsequent chapters, are summarized:

Chapter 2.	This chapter describes a study with juvenile (pubertal) *C. gariepinus*, in which the nature of the stimuli from donor males enhancing ovarian development (vitellogenesis) in recipient females is studied. The stimuli from donors investigated were those of a tactile, visual, and chemical nature. The response in female recipients was measured in terms of ovarian growth (gonadosomatic index, GSI).
Chapter 3.	In this chapter, an experiment with adult *C. gariepinus* is described, in which the mode of perception (olfactory, gustatory) is investigated by depriving recipients of the sense of smell (anosmia). The response was measured in female recipients which had entered the phase of ovarian recrudescence after hormonally-induced oocyte maturation, ovulation and stripping. The response was measured in terms of GSI and changes in ovarian histology.
Chapter 4.	This chapter describes the dynamics of the response to male sex-stimuli in both pubertal and adult recipient *C. gariepinus*, in terms of the sequence of the endocrine and histological events which accompany vitellogenesis.
Chapter 5.	This chapter covers aspects of interaction between pubertal male and female donor *C. gariepinus* on the emission of pheromonal signals affecting gonadal development (measured in terms of GSI) in pubertal male and female recipients.
Chapter 6.	In this chapter, results are reported of analysis of holding water (the medium of transport of pheromones) which stimulates ovarian

development of pubertal recipient *C. gariepinus* (measured in terms of GSI). Holding water was analyzed for the presence of steroid glucuronides, known to be of gonadal origin and to exert pheromonal influence on the later stages of the female teleost reproductive cycle.

Chapter 7. This chapter describes an attempt to establish the origin of the male donor olfactory stimulus by comparing the response in pubertal female recipient *C. gariepinus* to holding water from intact and gonad-extirpated adult male donor fish. Effectiveness of gonad extirpation was assessed by measurement of plasma androgen levels and gonad remnants; the response in female recipients was measured in terms of GSI.

Chapter 8. This chapter summarizes the results and discusses the findings in the broader context of teleost reproduction.

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Chapter 2.

AN ANALYSIS OF SEX STIMULI ENHANCING OVARIAN GROWTH IN PUBERTAL AFRICAN CATFISH *CLARIAS GARIEPINUS*.

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An Analysis of Sex Stimuli Enhancing Ovarian Growth in Pubertal African Catfish, *Clarias gariepinus*

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ABSTRACT

Van Weerd, J.H., Sukkel, M. and Richter, C.J.J., 1988. An analysis of sex stimuli enhancing ovarian growth in pubertal African catfish, *Clarias gariepinus*. *Aquaculture*, 75: 181-191.

Ovarian growth in pubertal African catfish was followed during a 100-day period. Different combinations of male sensory stimuli resulted in three distinct levels of ovarian development. A basic level (ovarian weight 5.3-7.5 g; GSI 1.9-2.7%) was reached in controls (no contact with males) and in females having visual and auditory contact with males. An intermediate level (ovarian weight 11 g; GSI 4%) was reached if olfactory contact with males was possible. A maximum level (ovarian weight nearly 20 g; GSI 7.3%) resulted from unlimited contact between the two sexes. The question is discussed whether the difference between maximum and intermediate levels of ovarian growth should be ascribed to a better water quality due to experimental conditions rather than to the presence of male tactile stimuli. The possible existence of male primer pheromones responsible for the observed stimulation of ovarian growth in pubertal African catfish by enhancing vitellogenesis is also discussed.

INTRODUCTION

In teleosts, pheromones have a stimulatory effect on reproduction (e.g. Colombo et al., 1980; Van Den Hurk and Lambert, 1983; Kobayashi et al., 1986; Stacey and Sorensen, 1987). Most of the effects pertain to advanced phases of the reproductive cycle, such as spermiation and ovulation, and sexual behaviour around spawning (Colombo et al., 1982; Liley, 1982; Pfeiffer, 1982; Liley and Stacey, 1983; Stacey, 1983).

Steroid glucuronides of gonadal origin have been identified as pheromones in the goby *Gobius jozo* (Colombo et al., 1980), in the zebrafish *Brachydanio rerio* (Van Den Hurk and Lambert, 1983; Lambert et al., 1986) and in the African catfish *Clarias gariepinus* (Lambert et al., 1986; Resink et al., 1988). In the African catfish, both testes and seminal vesicles are able to produce

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polar steroids and steroid glucuronides (Resink et al., 1987a,b; Schoonen et al., 1987).

Little is known about pheromonal stimulation of early phases of the reproductive cycle (Lam, 1983). In *C. gariepinus*, the presence of males seems to enhance ovarian growth in females. This was observed in pubertal females raised under hatchery conditions (Henken et al., 1987) and in wild-caught adult females kept in ponds (Richter et al., 1987). These observations suggested the existence of a pheromone affecting vitellogenesis in *C. gariepinus* (Huisman and Richter, 1987). These male–female contacts stimulating ovarian growth may, however, include components of visual, auditory, tactile and electric origin as well.

In the present paper, results of a study concerning male stimuli enhancing ovarian growth in pubertal African catfish are described.

MATERIALS AND METHODS

Maintenance of fish

The experiment was carried out with juvenile *C. gariepinus* purchased from a commercial fishfarm (Aquafish, Venhorst, The Netherlands). Upon arrival in our laboratory, they were acclimated for 1 week in a flow-through basin.

At the start of the experiment, when the fish were 137 days old, they were sexed on the basis of external morphological features and divided over the various experimental groups, taking care that the weight range of fish in each group differed as little as possible. During the 100-days experimental period, average weight of the fish increased from 110 to 300 g. Fish were fed Trouvit trout pellets (Trouw, Putten, The Netherlands) at a rate decreasing from 1.3 to 1.1% of their body weight per day, defined as the optimum rate for African catfish in the weight range used (Hogendoorn et al., 1983). All feed was administered by hand at one time.

The experiment was conducted in a two-layer aquarium system at a water temperature of 24–25 °C. Each aquarium (140 l) was divided into two equal compartments (70 l) by means of a partitioning wall. Equal numbers of fish were kept in each compartment. The fresh tap water inflow allowed for complete water exchange in each compartment every 30–45 min. Inflowing fresh water was aerated in small reservoirs placed on top of the aquaria, to avoid gas bubble disease caused by water supersaturated with gases (Boon et al., 1987).

Experimental design

The set-up of the experiment was such, that females were exposed to various combinations of male stimuli (Fig. 1):

(1) Unlimited contact with males. Four groups, containing equal numbers of

male and female fish, were kept in four different compartments. Every compartment received fresh tap water. Females in these groups were thus exposed to all conceivable male stimuli, of tactile (T; possibly including olfactory and/or gustatory components), visual (V), olfactory (O), auditory (A) and electric (E) origin. At the same time, these four groups served as stimulus donors to the other, all-female, recipient groups. Each of the donor groups was linked to a recipient group in such a fashion, that only part of the array of possible stimuli could be perceived by the latter (see below).

(2) Visual (V) and auditory (A) contact with males. A recipient group was enabled to observe donor fish in the adjacent compartment of the same aquarium through a transparent glass partition. Fish were supplied with fresh tap water.

(3) Visual (V), olfactory (O) and auditory (A) contact with males. As in (2), but with a partition consisting of two interspaced glass walls with alternating perforations, allowing for a flow-through of holding water from donor to recipient groups. Electric signals (E) may have been perceived as well.

(4) Olfactory (O) and auditory (A) contact with males. As in (3), but with an opaque partitioning, thus eliminating visual contact. Electric signals (E) may have been perceived as well.

(5) Olfactory (O) contact with males. A recipient group in a compartment in the bottom layer of the aquarium system received holding water supplied by gravity through a siphon from a donor group maintained in a compartment in the top layer of the system. Only olfactory signals could have been perceived by the recipient females.

(6) No contact with males (control; no stimulus). A recipient all-female group received fresh tap water.

Each group contained 40 fish ($n=20$ in Fig. 1). The whole experiment was carried out in duplicate.

To ensure unhampered visual contact, glass partitions were cleaned regularly. Aquaria without glass partitions were cleaned as well, to subject all groups to an equal amount of stress.

Sampling of fish

At the beginning of the experiment (day 0), a separate, initial sample of 20 males and 20 females was taken and fish were weighed individually to the nearest 0.01 g and sacrificed. Two weeks later a final check for sex and total numbers was made. At the end of the experiment all fish were sacrificed. Feeding had been suspended 2 days earlier to avoid bias in weight determinations due to feed remains in the digestive tract. Because of the large numbers of fish to be dissected, the experiment was terminated in a period of 4 consecutive days (days 100–103). A maintenance ration (Hogendoorn, 1983) was given to the groups which were to be sacrificed later in this period so as to avoid additional

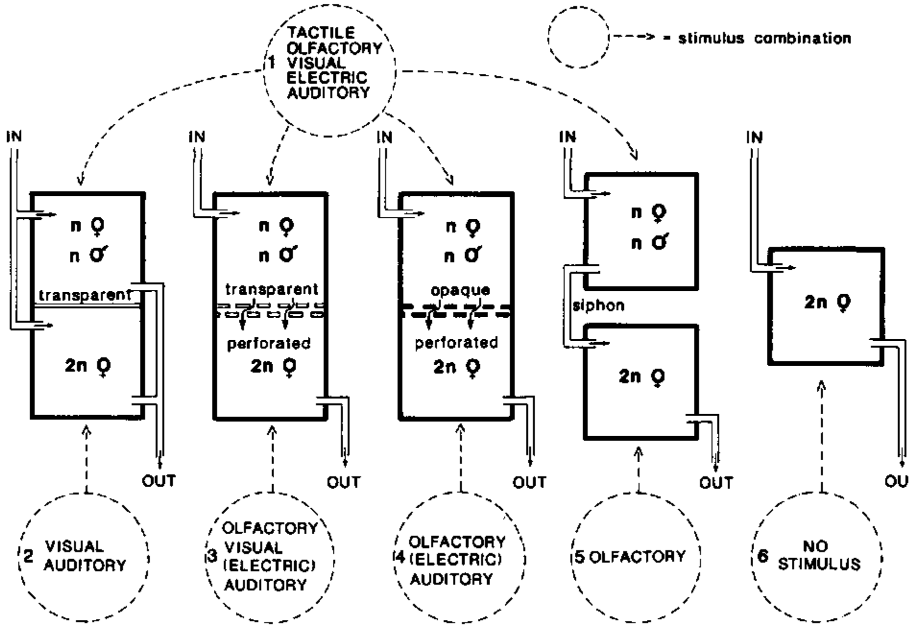

Fig. 1. Schematic representation of the aquarium system, in which groups ($n=20$) of female *C. gariepinus* were exposed to various combinations of male sensory stimuli.

growth. When fish were sacrificed, they were killed in an overdose of 0.2 g/l of TMS (tricaine methanesulphonate; Crescent).

Parameters

Individual values of body weight, gonad weight, gonadosomatic index (GSI in %, calculated for males and females as weight of ovaries or testis/body weight – weight of ovaries or testes $\times$ 100) and seminal vesicle somatic index (SVSI in %, calculated for males as weight of seminal vesicles/body weight – weight of seminal vesicles $\times$ 100), were used as parameters in this study.

Statistical analysis

For each group, data were tested for normality using W values (Shapiro and Wilk, 1965) or Kolmogorov-Smirnov's D values (Sokal and Rohlf, 1969) and for homogeneity of variance using Bartlett's test (Sokal and Rohlf, 1969). Data were normalized by arcsin $\sqrt{x}$ transformation and subsequently, differences between groups were tested with Student's t-test (Sokal and Rohlf, 1969). If normality could not be achieved by transformation, differences were tested with the non-parametric test of Kruskal-Wallis (Sokal and Rohlf, 1969). Calculation of *W* or *D* values was performed using the SAS Univariate procedure (SAS Institute Inc., 1985). All other tests were performed using the BMDP 3D and 9D programmes (Dixon et al., 1983).

If results from groups representing replicates of one treatment are not statistically different, means of these replicates and their standard errors (SEM) are given.

RESULTS

Females

Body weight. In the 100-day experimental period, female body weight increased from 112 g to a mean value ranging from 291 to 303 g for all groups. All values at days 100–103 differed significantly ($P < 0.001$) from the initial values at day 0. Average final body weight between the various groups at days 100–103 did not differ significantly. Apparently, the various conditions resulted in comparable somatic growth.

Ovary weight. Fig. 2. shows mean values of ovary weight, per treatment, presented in order of increasing magnitude. One replicate of the control (no con-

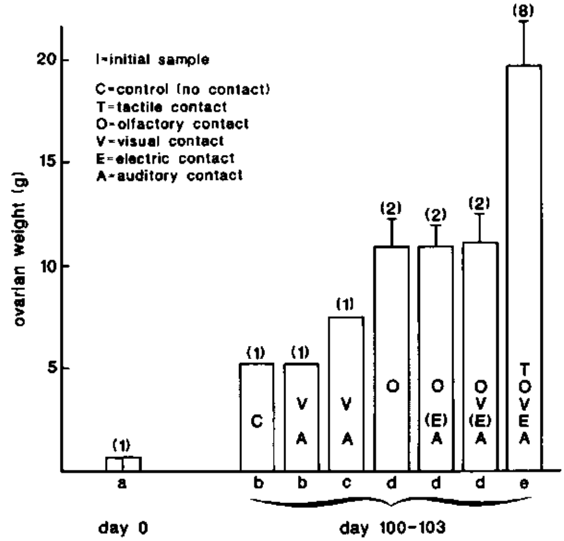

Fig. 2. Initial and final ovarian weight of female *C. gariepinus* exposed to various combinations of male sensory stimuli. Values are means of replicate treatments + SEM. In brackets, number of replicates. Values with the same subscript do not differ significantly. All differences at days 100–103 significant with $P < 0.001$, except b–c ($P < 0.05$) and c–d ($P < 0.01$).

tact) appeared to contain a male (phenotypically female) upon dissection and was excluded from the results.

Ovarian weight appeared to be significantly affected by the various treatments. All values at days 100–103 (fish age 237–241 days) differed significantly ($P<0.001$) from the initial value of 0.85 g at day 0 (fish age 137 days). At days 100–103, three significantly different ($P<0.001$) levels of ovary weight had been reached. A basic level of between 5.3 and 7.5 g was reached in the control treatment (C, no contact) and the treatments in which visual and auditory contact with the mixed-sex group had been possible (VA). Replicates of the latter treatment (VA) differed significantly ($P<0.01$) and are presented separately. An intermediate level of about 11 g was reached in treatments where olfactory, but not tactile stimuli were involved (O, O(E)A, OV(E)A). A maximum level of nearly 20 g was reached in the unlimited contact treatment, in which females received all possible male stimuli (TOVEA).

Gonadosomatic index. GSI values yield the same picture as values of ovary weight. Values at days 100–103 differ significantly ($P<0.001$) from the initial value of 0.8% at day 0. At the end of the experiment, a basic GSI level of between 1.9 and 2.7%, an intermediate level of about 4% and a maximum level of 7.3% can be distinguished. The three levels differed significantly ($P<0.001$). Again, replicates of the VA treatment differed significantly ($P<0.05$).

Males

In the unlimited contact groups, males grew significantly ($P<0.05$) faster than females, attaining a mean weight of 322 g, whereas females reached a mean weight of 303 g. Of the eight groups involved (four similar treatments in duplicate), one group deviated significantly ($P<0.05$) in all parameters except body weight. Mean testes and seminal vesicle weights in the seven comparable groups were 3.5 and 0.9 g, respectively, resulting in an average GSI of 1.09% and an average SVSI of 0.29%. In the eighth group, these figures were 2.5 and 0.7 g for weight of testes and seminal vesicle, respectively, resulting in a GSI of 0.84% and an SVSI of 0.24%. Interaction with the other sex was not influenced, since females in this unlimited contact group did not differ from females in the seven other groups in any of the parameters investigated (see above).

DISCUSSION

In the present study an attempt was made to dissociate the male stimulatory effect on ovarian growth into its component elements. Cues of olfactory, tactile, electric, auditory and visual origin were considered. Three different levels of ovarian weight (Fig. 2) and GSI are reached. Control groups and groups lacking the olfactory stimulus reach a basic level of ovarian development (ovary weight 5.3–7.5 g; GSI 1.9–2.7%). If olfactory cues are offered, an intermediate level is achieved (ovary weight 11 g; GSI 4%). If females are in actual contact with males, a maximum level of nearly triple the basic level is reached (ovary weight 20 g; GSI 7.3%).

Results indicate that neither auditory nor visual contact plays a role in stimulating vitellogenesis in pubertal African catfish, but male factors which are of importance in stimulating ovarian growth were shown to be of olfactory and tactile origin, and an additive effect of the two factors seems to occur (Fig. 2).

However, it could be argued that inhibiting effects on ovarian growth due to environmental conditions (Billard et al., 1981; Gerking, 1982) may have mimicked the effect of tactile stimuli in treatments 3, 4 and 5 of Fig. 1, since females in these groups received water with metabolic wastes from other fish. It is, therefore, possible that the greater ovarian growth observed in females which were fully stimulated by males (treatment 1 in Fig. 1) should be ascribed to a better water quality rather than to the presence of male tactile stimuli. This is supported by the fact that, although tactile stimuli are important in certain phases of reproduction of teleosts and play a key role in spawning, e.g. in *Brachydanio rerio* (Lambert et al., 1986) and in *Carassius auratus* (Partridge et al., 1976), presumably by acting as the final trigger for oviposition and ejaculation, no specific physical contact between the two sexes was observed. In a further series of experiments, the importance of olfaction in the situation where unlimited contact between the sexes is possible, will be investigated by rendering female fish anosmic. At any rate, chemical communication appears to have played an important role in the interaction between sexes. In this respect it is noteworthy that steroid glucuronides of gonadal origin, which have a pheromonal function in the goby (Colombo et al., 1980), the zebrafish (Van Den Hurk and Lambert, 1983; Lambert et al., 1986), and the African catfish (Resink et al., 1988), were recently found to be synthesized in the skin of male African catfish as well (Ali et al., 1987). If released by pubertal male catfish as well, such compounds could then have been perceived by gustation and/or olfaction.

Catfishes, including the related *Clarias batrachus*, possess taste buds, notably on the barbels (Storch and Welsch, 1970; Reutter, 1982), but gustation in general is considered to be of minor importance in fish reproduction (Pfeiffer, 1982). Nevertheless, gustation cannot be excluded in our experiment as a possible contributing factor to the observed effects.

As mentioned earlier, direct contact with males in the mixed-sex group may have included electric signals. Since their transfer through the double-wall partitionings with alternating perforations is doubtful, an effect of possible electric signals was not fully eliminated in the experimental set-up (Fig. 1). Although the American catfish *Ictalurus nebulosus* has a functional electroreceptive system (Knudsen, 1978) and electroreception is employed for other purposes, e.g. accurate location of nearby prey by the Japanese catfish *Parasilurus asotus* (Asano and Hanyu, 1986), a function in social interaction with gametogenesis has, to our knowledge, not been described for teleosts.

Pheromones both as releasers and as primers have been implicated in various phases of teleost reproduction. Their function was reviewed by e.g. Colombo et al. (1982), Liley (1982), Liley and Stacey (1983) and Stacey (1983). Recently, releaser pheromones of male origin involved in attraction of ovulated females were demonstrated in the African catfish (Resink et al., 1987c, 1988). To our knowledge, primer pheromones enhancing gametogenesis have not been the subject of research. Experimental results hinting at the existence of such compounds have been reported for the angelfish *Pterophyllum scalare* (Chien, 1973) and the tilapia *Oreochromis (Sarotherodon) mossambicus* (Silverman, 1978a,b).

Results from the present experiment point to a "vitellogenic" pheromone, as surmised earlier by Huisman and Richter (1987) and Richter et al. (1987). The underlying mechanism which caused the observed increased ovarian growth (increased number and/or size of vitellogenic oocytes) is being investigated at present. The compounds involved might well be similar to the polar steroids and their conjugates, produced in the testes of feral adult African catfish, in the period when recrudescence of gonads takes place. These are the testicular 5β-pregnane-3α, 17α-diol-20-one glucuronide and the highly polar 5β-pregnane-3α, 17α, 20α-triol (Resink et al., 1987b). In the same period, a strong production capacity for testosterone glucuronide is exhibited by the seminal vesicle (Resink et al., 1987b), but since the conjugate itself was not identified in this organ of intact pond-raised male fish at that time (Schoonen et al., 1988), its involvement is unlikely. Although testes and seminal vesicles of males in our experiment have not yet been examined histologically, they seem to have developed normally. However, these pubertal males might produce quite a different spectrum of steroid glucuronides, as compared to adults, and whether these are actually acting as "vitellogenic" pheromones remains to be investigated. It is even possible that female olfactory substances, evoked by the presence of males in mixed-sex groups, are involved in stimulating vitellogenesis in recipient all-female groups. It seems interesting to, in future, identify the source of such "vitellogenic" pheromones and to isolate the active substances or, conversely, to extract these from holding water for biochemical characterization.

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Chapter 3.

PHEROMONAL STIMULATION OF OVARIAN RECRUDESCENCE IN HATCHERY- RAISED ADULT AFRICAN CATFISH *CLARIAS GARIEPINUS.*

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Pheromonal stimulation of ovarian recrudescence in hatchery-raised adult African catfish, *Clarias gariepinus*

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ABSTRACT

Van Weerd, J.H., Sukkel, M., Bin Awang Kechik, I., Bongers, A.B.J. and Richter, C.J.J., 1990. Pheromonal stimulation of ovarian recrudescence in hatchery-raised adult African catfish, *Clarias gariepinus*. *Aquaculture*, 90: 369–387.

Adult female African catfish, *Clarias gariepinus*, were manipulated to enter the phase of ovarian recrudescence by induced ovulation and stripping. These females were exposed to various conspecific stimuli for 33 days. At the end of the experimental period, gonadosomatic indexes (GSIs) were higher in females which had received holding water from a mixed-sex population than in comparable controls. The effect was abolished when recipient females had been rendered anosmic, indicating the presence of chemical substances perceptible through olfaction. Fecundity estimates and histological analysis suggest that the increase in GSI due to pheromonal stimulation is caused by both an increased recruitment of oocytes into the stage of exogenous vitellogenesis and by an enhanced deposition of yolk material in the oocytes. It is concluded that male *C. gariepinus* release pheromones with a stimulatory effect on vitellogenesis. Neither anosmia itself nor metabolites in holding water affected ovarian recrudescence or body weight increase. The biological significance of such pheromones is discussed in the context of observations on reproduction of *C. gariepinus* made in the natural environment.

INTRODUCTION

Ovarian growth in the African catfish, *Clarias gariepinus*, is influenced by the presence of male conspecifics. This phenomenon was observed in pubertal hatchery-reared fish (Henken et al., 1987) as well as in adult fish originating from natural waters and kept in ponds (Richter et al., 1987b). Recently, olfactory and tactile stimuli were found to be responsible for the observed effects in pubertal female African catfish. Olfactory and tactile stimuli of male

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origin, when offered singly or in combination, appeared to enhance ovarian growth, whereas other possible male cues (visual, electric and auditory) were shown to be irrelevant (Van Weerd et al., 1988). In the latter study, however, anosmic control females were not included.

The present study with adult *C. gariepinus* was designed:

(a) To determine whether pheromonal stimulation of ovarian recrudescence in adult *C. gariepinus* occurs.

(b) To elucidate the sensory mechanism through which ovarian recrudescence is affected by conspecific stimuli. Anosmic females were included to gain better insight into the way in which chemical stimuli are perceived.

(c) To investigate whether enhanced ovarian growth is effected through an increased recruitment of oocytes into the stage of exogenous vitellogenesis or through an overall stimulation of vitellogenesis.

MATERIALS AND METHODS

Origin and maintenance of fish

The experiment was carried out with adult *Clarias gariepinus* (age 9 months) with no previous spawning history, purchased from a commercial fishfarm (Aquafish, Venhorst, The Netherlands). Upon arrival in our laboratory, they were acclimated for 1 month in a flow-through basin, and were fed Trouvit trout pellets (Trouw, Putten, The Netherlands) at 1% of their fresh body weight per day (Hogendoorn et al., 1983).

Induction of anosmia and assessment of its effect on ovarian recrudescence

Two weeks before the start of the experiment, all fish were sexed on the basis of external morphological features and a number of female fish was made anosmic by thermocauterization of the olfactory epithelium (Tavolga, 1956; Van Den Hurk et al., 1987). The operation was carried out during anaesthesia (TMS; tricaine methanesulphonate, Sandoz). No sham-operation was performed because inflicting fully comparable damage to the olfactory system without affecting the sensory modality itself was considered impossible. The resulting three groups (males, intact females, anosmic females) were kept separately in flow-through basins for a period of 2 weeks until the experiment started.

Effectiveness of the cauterization was assessed 2 weeks after the operation (start of the experiment; day 0) and at the end of the experiment (day 33), by macroscopically checking for the presence of olfactory epithelium. In addition, samples of anosmic females were taken at random at the end of the experiment and processed for microscopic evaluation of the presence of olfactory epithelium. Possible adverse effects of cauterization on reproductive performance were also assessed at day 0 by comparing egg quality and quantity (see measurements) when fish were induced to spawn. Moreover, body weight and ovary weight of intact and anosmic females in comparable treatments were studied at day 33.

Artificially induced spawning and selection of experimental fish

Maturation and ovulation of oocytes was induced by giving a single intraperitoneal injection of pimozide (PIM) combined with luteinizing hormone-releasing hormone analogue (LHRHa) (Des-Gly^{10}, D-Trp^{6}, Pro-ethylamide^{9}) at a dose of respectively 5 and 0.05 mg/ml per kg body weight (De Leeuw et al., 1985; Richter et al., 1987a). Injected fish were housed individually and were stripped after 12.5 h.

Female fish which had been successfully stripped were divided over the various treatment groups at day 0 (see stimuli investigated, below). Male fish were selected on the basis of their body weight, taking care that this approximated that of the females.

Experimental design

Stimuli investigated. Eight stimulus combinations which could affect ovarian recrudescence were investigated. A flow-diagram of stimuli, with the corresponding treatments of intact and anosmic fish, is shown in Fig. 1.

1 – Unlimited contact between males and females. Equal numbers of male and intact female fish were kept in two compartments receiving fresh water. Females in these groups were thus exposed to both relevant male stimuli, tactile (T) and olfactory (O). Holding water from these groups was mixed and redistributed over two other, all-female groups (treatments 3 and 5, see below). The holding water contains olfactory stimuli (O) and metabolites (M).

2 – Tactile contact of females with males. Equal numbers of male and anosmic female fish were kept in two compartments receiving fresh water. Females in these groups were thus exposed to only one male stimulus, tactile (T). Holding water from these groups was mixed (see also below) and partly (50%) redistributed to a group containing intact females. The holding water supplying this group (treatment 4, see below) possibly contains a different male stimulus due to an unnatural interaction between males and anosmic females (treatment 2).

3 – Olfactory contact of females with males and intact females. A group of intact females received holding water from the unlimited contact groups (treatment 1) thus being exposed to olfactory stimuli (O) and to metabolites of the holding water (M).

4 – Olfactory contact of females with males and anosmic females. A group of intact females received holding water from treatment 2, thus being exposed to a possibly different male olfactory stimulus ([O]) and metabolites present in the holding water (M).

5 – Anosmic females were exposed to olfactory stimuli (O) and metabolites

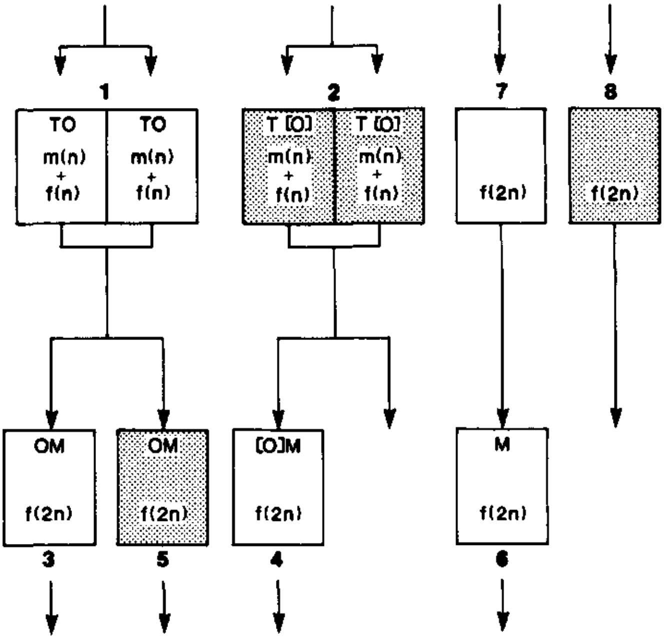

Fig. 1. Flow diagram of stimuli with a possible effect on ovarian recrudescence in *C. gariepinus*. Arrows indicate in- and outflow of water. m = males, f = females, (n) = number of fish.
Bold numbers refer to treatments mentioned in the text. Open blocks designate compartments containing intact females; shaded blocks designate compartments containing anosmic females, with (2) or without (5 and 8) intact males.
M = metabolites in holding water from donor compartments (1, 2 and 7) entering the recipient compartments (3, 5, 4 and 6).
Male stimuli: T = tactile; O = olfactory, [ ] = stimulus from males maintained with anosmic females.

(M) present in the holding water of the unlimited contact group (treatment 1). These females were thus not able to perceive olfactory stimuli.
6 – Intact females received holding water from a similar group of intact females (treatment 7, see below), thus being exposed to metabolites present in the holding water (M), without male olfactory stimuli.
7 – Intact females received fresh water, thus not being exposed to any stimulus. Holding water from this group, containing metabolites (M), was led to a similar group of females (treatment 6, see above).
8 – Anosmic females were supplied with fresh water. No stimulus was offered nor could be perceived. This group was included to assess the effect of cauterization itself on ovarian development.

The whole experiment was carried out in duplicate, resulting in a total of 20 treatment groups.

Set-up of aquaria. The experiment was conducted in a two-layer aquarium system, similar to the one described earlier (Van Weerd et al., 1988). 140-l aquaria were divided into two equal compartments of 70 l. Each of the resulting 20 compartments contained 14–18 fish (n=7–9 in Fig. 1). Water temperature was kept at 24–25 °C. Holding water from donor compartments was siphoned by gravity to recipient compartments. The fresh tap water and holding water inflow allowed for complete exchange in each compartment every 30–45 min. Inflowing fresh water was aerated in small reservoirs placed on top of the aquaria, to avoid gas bubble disease caused by water supersaturated with gases (Boon et al., 1987).

Maintenance and feeding of experimental fish. The experiment was carried out in spring under semi-natural Dutch light periodicity circumstances, i.e. 12 h light and 12 h darkness (Richter et al., 1987b). During the 33-day experimental period, fish were fed Trouvit trout pellets (Trouw, Putten, The Netherlands) at a rate decreasing from 1.02 to 0.97% of their body weight per day (Hogendoorn et al., 1983). All feed was administered by hand at one time.

Metabolites in the holding water. Biomass in all aquaria was such that the quality of holding water supplying treatments 3, 4, 5 and 6 never exceeded limits set for *C. gariepinus* by Viveen et al. (1985). i.e., oxygen above 3 ppm, nitrate below 0.25 ppm, nitrate below 250 ppm, ammonia below 0.05 ppm.

Measurements

Initial samples (day 0). Of seven randomly chosen females of both intact and anosmic groups, body weight (before stripping) was compared and the pseudo-gonadosomatic index (pGSI in %, calculated as weight of stripped eggs/body weight before stripping × 100; Richter et al., 1985) was calculated. An egg sample from these fish was fertilized to compare egg quality (De Leeuw et al., 1985; Richter et al., 1985).

Another seven stripped females from both intact and anosmic groups were sacrificed, their body weight and gonad weight were recorded, and gonadosomatic index (GSI) in % was calculated as [ovary weight/(body weight − ovary weight) × 100]. Gonads were fixed for histology.

All stripped females of the treatment groups mentioned above were weighed individually to the nearest 0.01 g.

Final sample (day 33). Individual weight and ovary weight of all females were recorded, and the average GSI in each treatment group was calculated. A random subsample of five females from each treatment group was taken, and gonads were fixed for histology and relative fecundity estimates. When fish were sacrificed, they were killed in an overdose of 0.2 g/l TMS.

Histology. A part of the left ovary was fixed in Carnoy's fluid (Romeis, 1968). After dehydration and embedding in paraffin, 5–10-μm sections were made from the mid-portion of the ovary. Sections were stained in haemalum–eosin (Romeis, 1968). The relative proportion of the various oocyte stages was determined by counting all oocytes present in a representative ovary section, and expressing the numbers as percentage of the total. Staging was based upon the morphological criteria given by Richter and Van Den Hurk (1982), i.e., oocytes in previtellogenesis, in endogenous vitellogenesis (synthesis of cortical alveoli) and in exogenous vitellogenesis, the latter category also including postvitellogenic oocytes. This simplification served to avoid errors in discriminating between late exogenous vitellogenesis and postvitellogenesis when oocytes were not sectioned through the nucleus and thus diameter could not provide a reliable criterion. Furthermore, average diameter of oocytes in the stage of exogenous vitellogenesis (including postvitellogenesis) was calculated by taking, from at least 40 oocyte cross-sections with a visible nucleus, the mean of the largest and the smallest diameter.

Relative fecundity. From the right ovary, a quantity of ca. 0.2 g of oocytes was taken from the mid-section, weighed to the nearest 0.1 mg and fixed in 4% formalin (Clay and Clay, 1981). Vitellogenic oocytes larger than 300 μm were counted under a binocular microscope at low magnification. The resulting average individual vitellogenic oocyte weight was used to calculate the total number of exogenous vitellogenic oocytes which had been present in the ovary, thereby considering the contribution to the ovary weight of small oocytes (<300 μm) and non-oocyte ovarian tissue negligible. Relative fecundity was arrived at by dividing the calculated number of oocytes in exogenous vitellogenesis by body weight.

Statistical analysis

Measurements of intact and anosmic fish in the initial sample were tested for normality using W values (Shapiro and Wilk, 1965) or Kolmogorov–Smirnov's D values (Sokal and Rohlf, 1969), and for homogeneity of variance using Bartlett's test (Sokal and Rohlf, 1969). Normality was achieved after arcsin $\sqrt{x}$ transformation and differences between intact and anosmic females were tested with Student's t test (Sokal and Rohlf, 1969).

Measurements in the final sample were tested within treatment groups for normality and homogeneity of variance as described above. If necessary, data were normalized by arcsin $\sqrt{x}$ transformation and, subsequently, differences between groups of each treatment (treatment groups) were tested with Student's t-test (Sokal and Rohlf, 1969). If normality could not be achieved by transformation, differences were tested with the non-parametric test of Kruskal–Wallis (Sokal and Rohlf, 1969). Since results between treatment groups were found to be not statistically different, data were pooled to calculate treatment means, and differences between treatments were tested as described above. Data concerning GSI, histology and fecundity of females from the random subsample of each treatment group (five females) were treated similarly. Calculation of *W* or *D* values was performed using the SAS Univariate procedure (SAS Institute Inc., 1985). All other tests were performed using the BMDP 3D and 9D programs (Dixon et al., 1983).

RESULTS

Cauterization and its effects

Macroscopic inspection did not reveal the presence of olfactory epithelium, although in a few cases, microscopic evaluation showed that some patches could still be present. This has not been taken into account in evaluating the results, because in nearly all cauterized females the nares appeared to be closed off due to regrowth of the skin after the cautery procedure. Fig 2A shows absence (cauterized female) and Fig. 2B presence (intact female) of olfactory epithelium.

Body weight before stripping, pGSI and egg-quality parameters of intact and anosmic females, upon stripping at day 0, are given in Table 1. In the 14-day post-operative period, body weight, pGSI, fertilization % and hatching %

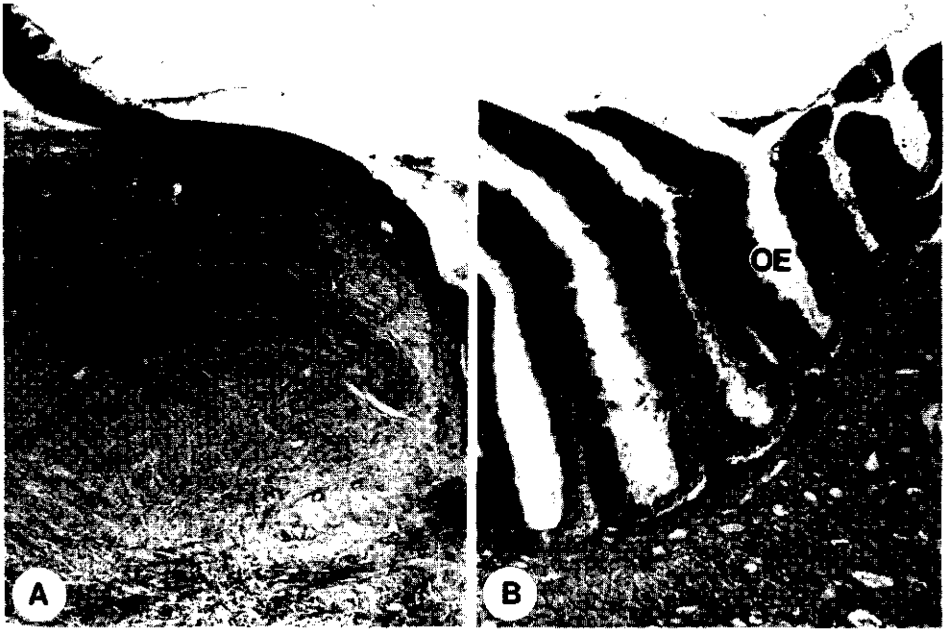

Fig. 2. Absence (A) and presence (B) of olfactory epithelium (OE) in cauterized and control female *C. gariepinus*, respectively (56×).

TABLE 1

Body weight before stripping and egg-quality parameters of female *C. gariepinus*, 14 days after induction of anosmia (mean values ± s.d.). Differences between intact and anosmic females were not significant ($P > 0.05$)

	Body weight (g)	pGSI (%)	Fertilization (%)	Hatching (%)	*n*
Intact	421.76 ± 81.71	14.3 ± 7.8	74.8 ± 20.4	59.4 ± 27.3	7
Anosmic	443.69 ± 65.34	16.3 ± 4.8	70.2 ± 19.6	61.6 ± 19.9	7

n = number of females.

TABLE 2

Initial (day 0) and final (day 33) data of female *C. gariepinus* in various treatments (mean ± s.e.m.). Treatment numerals refer to Fig. 1

Day 0		Day 33			
Treatment	Body weight (g)	Body weight (g)	Ovary weight (g)	GSI (%)	*n*
1	382.97 ± 4.93	464.45 ± 0.76	53.07 ± 3.06	13.10 ± 0.98	2[a]
2	359.52 ± 10.82	467.92 ± 5.08	44.76 ± 3.55	10.67 ± 1.07	4
3	395.18 ± 16.73	480.00 ± 39.47	47.04 ± 6.17	11.04 ± 0.71	2
4	361.97 ± 2.02	430.22 ± 3.53	37.48 ± 0.98	9.88 ± 0.77	2
5	358.01 ± 1.43	455.48 ± 4.48	38.29 ± 0.16	9.32 ± 0.23	2
6	381.24 ± 19.57	453.65 ± 20.26	35.82 ± 1.38	8.68 ± 0.16	2
7	368.84 ± 9.19	440.37 ± 21.80	36.63 ± 2.28	9.28 ± 0.74	2
8	333.04 ± 8.17	418.25 ± 21.72	38.33 ± 1.98	10.41 ± 0.13	2

n = number of treatment groups.

[a] Two treatment groups omitted due to low survival.

were not affected by anosmia. Effects of anosmia at day 33 (after a 47-day post-operative period) are mentioned below.

Factors affecting ovarian recrudescence

Mean initial body weight and mean final body weight, ovary weight and GSI of females from treatments 1–8 are mentioned in Table 2. Initial values of these parameters, determined in the separate sample of seven stripped females at day 0, were 334.16 ± 0.78 (mean body weight ± s.e.m.), 13.84 ± 0.91 (mean ovary weight ± s.e.m.) and 4.35 ± 0.46 (mean GSI ± s.e.m.). All parameters had increased during the 33-day experimental period, whereas mor-

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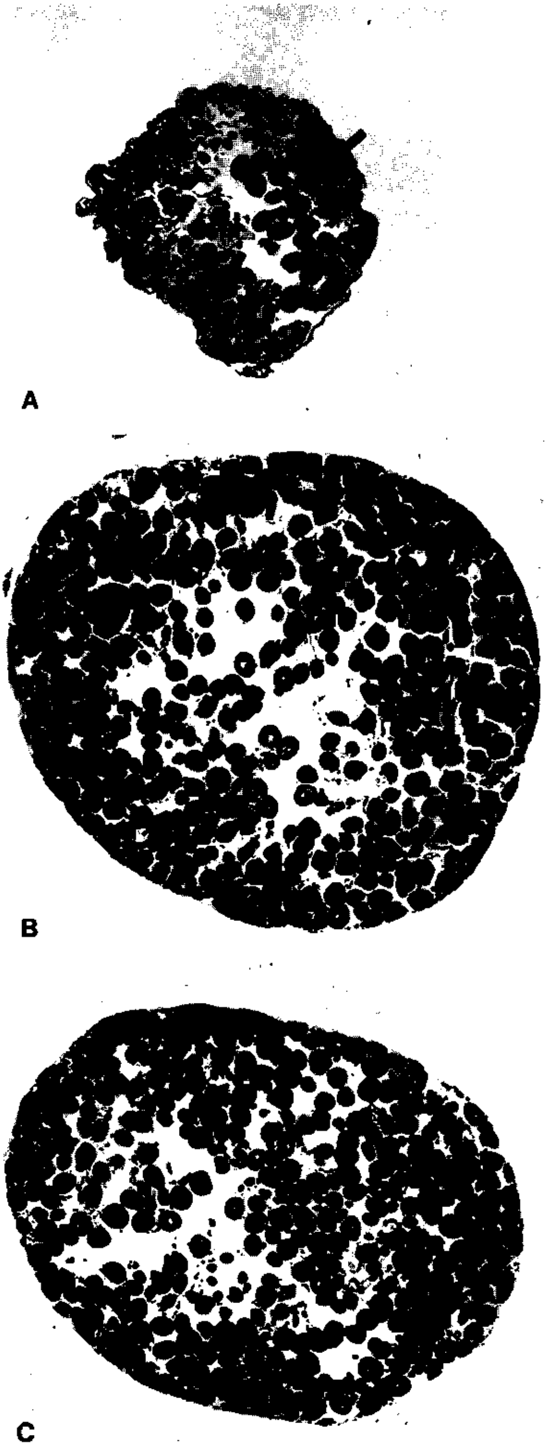
Fig. 3. Histology of ovary of *C. gariepinus* (3.5×).

(A) Ovary upon stripping (day 0). Some ovulated eggs still visible inside the ovary (arrow).

(B) Ovary of female from treatment 1, 33 days after stripping. GSI of 13.2%.

(C) Ovary of female from treatment 2, 33 days after stripping. GSI of 10.1%.


A
B
C

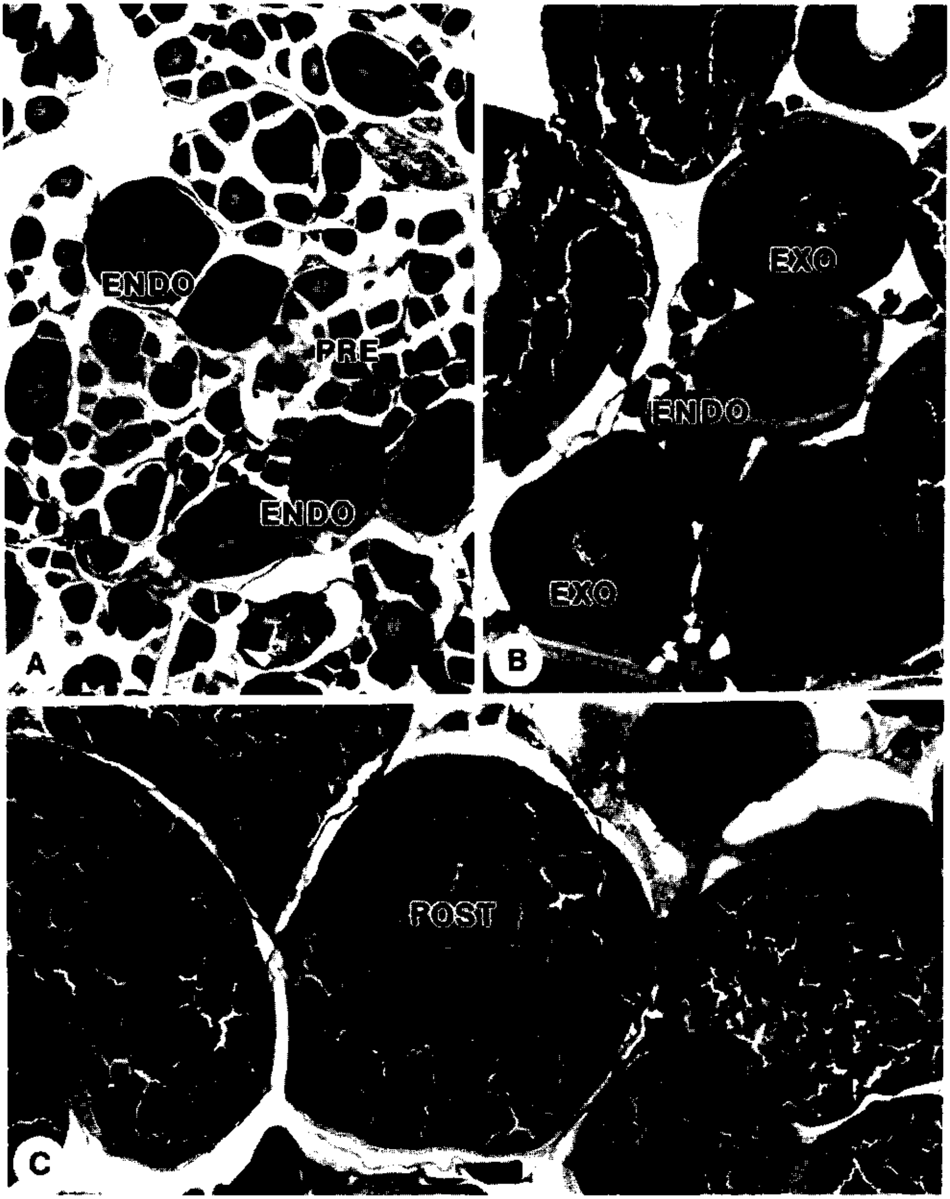


Fig. 4. Histology of oocyte stages of *C. gariepinus* (56×).
(A) Oocytes in previtellogenesis (PRE) and endogenous vitellogenesis (ENDO).
(B) Oocytes in endogenous vitellogenesis (ENDO) and exogenous vitellogenesis (EXO).
(C) Postvitellogenic oocytes (POST).

tality was negligible in all but two treatment groups (treatment 1). The data for these groups were therefore not included in further calculations.

Effects of the factors (i) anosmia, (ii) metabolites present in the holding water, (iii) olfactory stimuli present in holding water from males and intact females, (iv) olfactory stimuli present in holding water from males and anosmic females, and (v) tactile stimuli from males were investigated by making relevant comparisons between treatments.

Body weight. Neither anosmia (comparison of treatments 7 and 8) nor metabolites present in the holding water (comparison of treatments 6 and 7) affected final body weight. Mean final body weights of females in treatments 7 and 8 did not differ significantly (440.4 ± 21.8 and 418.3 ± 21.7 g, respectively), nor did mean final body weights of females in treatments 6 and 7 (453.7 ± 20.3 and 440.4 ± 21.8 g, respectively).

Ovarian development. Anosmia (comparison of treatments 7 and 8) and metabolites present in the holding water (comparison of treatments 6 and 7) did not affect ovarian growth. On the contrary, final GSI values of anosmic fish are slightly, but not significantly, higher than controls (comparison of treatments 7 and 8). Ovarian growth was, however, significantly enhanced when olfactory stimuli from males and intact females were perceived (comparison

TABLE 3

Effect of factors investigated on final GSI of female *C. gariepinus*. Comparisons based on pooled results of treatment groups (mean ± s.e.m.). Treatment numerals refer to Fig. 1

Factor	Treatment	GSI (mean ± s.e.m)	
Anosmia	7	9.28 ± 0.74	NS
	8	10.41 ± 0.13	
Metabolites	6	8.68 ± 0.16	NS
	7	9.28 ± 0.74	
Olfactory stimuli from males + intact females	1	13.10 ± 0.98	S
	2	10.67 ± 1.07	
	3	11.04 ± 0.71	S
	5	9.32 ± 0.23	
Olfactory stimuli from males + anosmic females	3	11.04 ± 0.71	NS
	4	9.88 ± 0.77	
Tactile stimuli from males	2	10.67 ± 1.07	NS
	8	10.41 ± 0.13	

NS = not significantly different.
S = significantly different at $P < 0.05$.

TABLE 4

Effect of olfactory stimuli from males + intact females on final GSI, diameter of vitellogenic oocytes, relative proportion of oocyte stages and relative fecundity of female *C. gariepinus*. Comparisons based on pooled results of subsamples from treatment groups (mean ± s.e.m.). Treatment numerals refer to Fig. 1

Parameter	Comparison of treatments					
	1	2		3	5	
GSI (%)	13.81 ± 1.24	10.36 ± 0.81	S	11.25 ± 0.70	9.63 ± 0.08	NS
Diameter of oocytes in exog. vitellogenesis (μm)	912 ± 15	871 ± 17	S	841 ± 15	855 ± 1	NS
Relative proportion of oocytes in stage (%):						
previtellogenesis	65.9 ± 4.1	72.9 ± 2.3	S	70.8 ± 1.0	75.9 ± 0.0	S
endog. vitellogenesis	1.6 ± 1.0	1.9 ± 0.2	NS	1.9 ± 0.1	1.4 ± 0.5	NS
exog. vitellogenesis	32.4 ± 4.0	25.2 ± 2.2	S	27.3 ± 1.1	22.7 ± 0.4	NS
Relative fecundity (number of oocytes/g body weight)	132.3 ± 15.0	100.7 ± 9.1	S	112.3 ± 7.1	100.9 ± 1.3	NS

NS = not significantly different.
S = significantly different at $P < 0.05$.
endog. = endogenous; exog. = exogenous

of treatments 1 and 2 and treatments 3 and 5). When olfactory stimuli originated from males and anosmic females, ovarian growth was less than in the situation when olfactory stimuli were received from males and intact females, but this difference was not significant (comparison of treatments 3 and 4), thus providing no evidence for a different male olfactory stimulus due to an unnatural interaction between males and anosmic females. In addition, female anosmia did not affect male gonadal development (data not shown). Male tactile stimuli did not bring about a significantly enhanced ovarian development (comparison of treatments 2 and 8).

Fig. 3 shows the histological development of the ovary from day 0 (Fig. 3A) to ovaries at day 33 containing postvitellogenic oocytes, in a female representative of treatment 1 (GSI 13%; unlimited contact with males) (Fig. 3B) and in a female representative of treatment 2 (GSI 10%; tactile contact with males and absence of pheromonal stimulation) (Fig. 3C). The three stages distinguished and counted in the histological analysis are illustrated in Fig. 4: Fig 4A shows oocytes in the stage of previtellogenesis and endogenous vitellogenesis, Fig. 4B shows exogenous vitellogenic oocytes, and Fig. 4C shows postvitellogenic oocytes.

In Table 4, only the comparisons clarifying the effect of olfactory stimuli from males + intact females are presented, since only this effect is significant (Table 3). The effect is expressed in terms of the parameters GSI, diameter of exogenous vitellogenic oocytes, relative proportion of oocyte stages, and relative fecundity. Figures are means from subsamples ($n=5$) from each treatment group.

In Table 4, the effect of olfactory stimuli from males and intact females on GSI is significant only in one comparison (treatments 1 and 2). Differences in mean diameter of oocytes in exogenous vitellogenesis correspond to GSI differences. This suggests that ovarian development is related to oocyte growth through an increased deposition of yolk material. There is a similar good correspondance between differences in the relative proportion of oocytes in previtellogenesis and in exogenous vitellogenesis, and differences in GSI. This indicates that ovarian development is also related to an enhanced recruitment of previtellogenic oocytes. This notion is confirmed by the fact that results on differences in relative fecundity closely follow results concerning GSI differences, indicating a close correlation between the two parameters (Table 4).

DISCUSSION

Induction of anosmia has often been used to confirm involvement of olfaction in intraspecific communication, for example by Tavolga (1956) in the goby, *Bathygobius soporator*, and by Van Den Hurk and Lambert (1983), Golubev (1985) and Van Den Hurk et al. (1987) in the zebrafish, *Brachydanio rerio*, all performed by cauterization of the nasal epithelium. In all these experiments, anosmia served as control for short-term responses to olfactory stimuli, i.e. releasing effects in the goby (sex-discriminatory and courtship behaviour; Tavolga, 1956) and the zebrafish (attraction; Van Den Hurk and Lambert, 1983) and priming effects (ovulation) in the zebrafish (Van Den Hurk et al., 1987). In our experiment, however, we included anosmic fish as controls for the relatively long-term recrudescence response to olfactory stimuli, and results have not been biased by this prolonged period of anosmia. Anosmic females had even slightly higher final GSI values than controls. Stabell and Refstie (1980), comparing groups of anosmic and intact *Salmo salar*, also observed no negative effect of anosmia on ovarian development.

When discussing in our earlier paper (Van Weerd et al., 1988) the respective roles of olfactory and tactile cues in stimulating ovarian development of pubertal African catfish, we argued that metabolites in the holding water from donor fish could have inhibited ovarian growth in recipient fish, since adverse environmental conditions have been implicated in negatively influencing gonad development in teleosts (Billard et al., 1981; Gerking, 1982; Sumpter et al., 1987). In the present experiment however, intact females receiving holding water from another group of intact females (containing metabolites but no male olfactory stimulus) did not differ significantly in any respect, and thus a negative effect of metabolites in the holding water was not observed.

Male tactile cues did not stimulate ovarian development in the present study. In this respect, adult *C. gariepinus* seem to differ from pubertal ones, since there, male tactile stimuli possibly do play a role in ovarian development (Van Weerd et al., 1988).

For pubertal *C. gariepinus*, we postulated the existence of male pheromones stimulating vitellogenesis (Van Weerd et al., 1988), and the present study confirms the involvement of pheromones since the ovarian response to olfactory stimuli in the holding water was abolished by anosmia. Pheromones play an important role in reproduction of teleosts (Colombo et al., 1982; Liley, 1982; Liley and Stacey, 1983; Stacey, 1983; Lambert et al., 1986; Stacey et al., 1986, 1987). Recently, they have been demonstrated in the African catfish as well (Resink et al., 1987b, 1989a,b; Van Oordt, 1987). All observations described, however, relate to advanced phases of the teleost reproductive cycle: oocyte maturation and ovulation in females (Van den Hurk et al., 1987; Resink et al., 1989b) and spermiation in males (Stacey and Sorensen, 1986). To our knowledge, pheromonal stimulation of gametogenesis has not been demonstrated in teleost species other than *C. gariepinus* (Van Weerd et al., 1988).

All teleost reproductive pheromones identified are reproductive hormones or their derivatives (Sorensen and Stacey, 1989; Stacey, 1989). In holding water from a mixed-sex population of adult *C. gariepinus* which stimulated ovarian development of pubertal female conspecifics, we recently identified several steroids and steroid glucuronides (Van Weerd et al., in press) of both male and female origin. These polar derivatives of reproductive hormones might have been involved in the present experiment as well, since Resink et al. (1987a) found that two of these compounds, 5β-pregnane-3α,17α-diol-20-one-glucuronide and 5β-pregnane-3α,17α,20α-triol, can be produced by male *C. gariepinus* during the natural period of gonadal recrudescence of females, and they suggested that these compounds may thus have a function in stimulation of vitellogenesis of *C. gariepinus.*

The role of females in mixed sex groups in the present and other studies (Van Weerd et al., 1988 and in press) regarding the effect of holding water is still unclear. The present results do not indicate that unlimited mutual contact between the sexes is a prerequisite for holding water to be effective in stimulating female ovarian development. The difference between the effect of holding water from males with intact or with anosmic females is not significant.

Stimulation of ovarian growth can be effected through two distinct mechanisms. On the one hand, the rate of oocyte growth may be stimulated, resulting in larger oocytes responsible for the increased ovarian growth. On the other hand, the total number of oocytes partaking in vitellogenesis may increase, through a stimulated recruitment of previtellogenic oocytes into the phase of vitellogenesis. Results of the present study suggest that both mechanisms are involved in pheromonally stimulated ovarian recrudescence of *C. gariepinus.*

Histological analysis shows that the diameter of oocytes in exogenous vitellogenesis is affected in females which had been receiving olfactory cues. This means that the rate of oocyte growth has been stimulated, and there is increased deposition of yolk material. However, histological analysis also shows that the relative proportion of previtellogenic oocytes had decreased in favour of that of oocytes in exogenous vitellogenesis when females respond to olfactory cues with increased ovarian development. This means an increased recruitment of oocytes into vitellogenesis; the fact that endogenous vitellogenic oocytes are present in very low proportions indicates that this stage is very short-lived. Relative fecundity is higher in pheromonally stimulated females, and this also suggests an increased recruitment of oocytes into exogenous vitellogenesis. Both mechanisms have been described in the literature as being responsible for ovarian growth in other teleost species. For *Oncorhynchus mykiss*, Bourlier and Billard (1984) reported that increased GSIs due to photoperiodic manipulation were caused by a larger oocyte diameter. In contrast, in *Heteropneustes fossilis*, GSI and relative fecundity showed a parallel increase when hypophysectomized females were treated with gonadotropins (Sundararaj and Anand, 1972; Sundararaj et al., 1972). As in *C. gariepinus* (this study), ovarian development is apparently caused by the two mechanisms in *Gobius niger*, where gonadotropins not only stimulated oogenesis, but particularly vitellogenesis (oocyte growth), when administered to hypophysectomized females (LeMenn and Burzawa-Gerard, 1985).

In the present experiment, differences in ovarian development of adult female *C. gariepinus* during recrudescence are clear after slightly more than 4 weeks. In pubertal fish, vitellogenesis takes 3 months for completion (Van Weerd et al., 1988). It would therefore seem attractive to use hatchery-reared adult females, artificially induced to spawn, to develop a bio-assay for the olfactory compounds involved. However, experimentation with adult *C. gariepinus* in numbers sufficient for adequate statistical analysis proved more cumbersome than when using pubertal fish, and results are less distinct. For example, the difference between minimum and maximum ovary weight attained in the present experiment is 17.25 g or some 33% of the maximum. In pubertal fish, this difference is some 73% (cf. fig. 2 of Van Weerd et al., 1988). This warrants continuation of similar experiments with pubertal fish. The longer period of response to pheromones in pubertal catfish may be overcome by monitoring other parameters of vitellogenesis, e.g., plasma levels of gonadotropin, oestradiol, and vitellogenin (Whitehead et al., 1983).

The biological significance of pheromones stimulating ovarian growth may be understood when placed in the context of observations made in nature. In the Hula Nature Reserve in Israel, where throughout the breeding period (May–August) spawning of *C. gariepinus* is observed, spent females with obvious atresia of oocytes cannot be found before August (Van Den Hurk et al., 1984/1985). Viveen et al. (1985) mention that after spawning, a second spawning can take place within a few weeks, induced by rainfall or by inflow of water from an upstream source, resulting in several spawnings per year. This would entail a rapid recrudescence after each spawning. In this respect it is noteworthy that in our laboratory, adult female catfish raised in a mixed-sex population can be successfully stripped of viable eggs at intervals as short as 1 week (Hogendoorn and Vismans, 1980). Since *C. gariepinus* generally are together in dense populations during the breeding period, it would be possible for vitellogenic pheromones to exert their action especially during this period.

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Chapter 4.

MALE-INDUCED SHIFTS IN PATTERN OF VITELLOGENESIS DURING PUBERTY AND RECRUDESCENCE OF FEMALE AFRICAN CATFISH *CLARIAS GARIEPINUS*.

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SUMMARY.

Vitellogenesis was monitored in *Clarias gariepinus* females during puberty and after artificially induced ovarian recrudescence. In two experiments (with pubertal and adult females, respectively), the effect of male stimulation of vitellogenesis was assessed by weekly sampling of females kept with males or exposed to male+female holding water. The parameters examined were the gonado somatic index (GSI), the proportion and diameter of exogenous vitellogenic oocytes, and plasma levels of estradiol (E_2) and vitellogenin (VTG). In pubertal females the pattern and sequence of appearance of these parameters are in general agreement with data from other teleosts: a peak in E_2 values precedes a peak in VTG, resulting in an increase of GSI and in a concomitant change in yolk deposition in the oocytes. Stimulation causes both a shift in timing and an increase in levels of plasma E_2 and VTG, resulting in faster ovarian growth. In adult females, stimulation of vitellogenesis, although resulting in distinct differences in GSI and ovarian development, is not accompanied by a shift in timing of plasma E_2 and VTG. E_2-values tend to be higher in stimulated adult females. These differences between first and subsequent cycles are thought to be due to the extremely short recrudescence period of *C. gariepinus* in captivity.

INTRODUCTION.

Sex pheromones of male origin, alone or in combination with male tactile stimuli, stimulate vitellogenesis in female African catfish, *Clarias gariepinus*. This has been demonstrated in pubertal females, entering their first cycle of ovarian development (Ch. 2), and in adult females entering a subsequent cycle (recrudescence) after induced maturation and ovulation of eggs (Ch. 3). In these two studies, the parameters to measure stimulation of ovarian development were the gonado somatic index (GSI), in the latter study supplemented with histological measurements. The studies provided 'end-point' measurements, giving no insight into how and when differences between treatment groups and controls were established.

Vitellogenesis in teleosts encompasses a cascade of endocrinological events, culminating in ovarian development (recent reviews by *e.g.* Wiegand, 1982; Wallace *et al.*, 1987; Bromage and Cumaranatunga, 1988). Gonadotropic hormone (GTH) stimulates ovarian production of estradiol-17ß (E_2), which in turn stimulates the production of a female-specific plasma protein of hepatic origin, vitellogenin (VTG). Circulating VTG is selectively sequestered from the blood stream by growing oocytes and deposited after processing as exogenous yolk material in the oocyte.

Hence, the progress of vitellogenesis can be monitored by measurement of these parameters and ample information is available from the salmonid species *Oncorhynchus mykiss* (review by *e.g.* Bromage and Cumaratunga, 1988), *Salmo trutta*, (Norberg *et al.*, 1989; Billard, 1987), *Salvelinus fontinalis* (Tam *et al.*, 1986), *Salvelinus leucomaenis* (Kagawa *et al.*, 1981), *Salmo salar* (Dickhoff *et al.*, 1989) and from a variety of other species, including catfishes (*e.g. Clarias batrachus*, Singh and Singh, 1987; *Heteropneustes fossilis*, Lamba *et al.*, 1983; *Ictalurus nebulosus*, Burke *et al.*, 1984; *Ictalurus punctatus*, MacKenzie *et al.*, 1989). Such studies show that plasma levels of E_2 and/or VTG are indicative of the initiation or progression of vitellogenesis at times when GSI is not (yet) a valuable parameter. Changes in ovarian histology, on the other hand, seem useful only to confirm GSI-based indications.

The present study was conducted to gain insight in the endocrine and histological aspects of vitellogenesis and ovarian growth in the African catfish, *C. gariepinus*. We furthermore compared the dynamics of these parameters in females stimulated by the presence of males or by exposure to 'male + female' holding water with the dynamics in control females. An attempt was made to assess whether some of these parameters would enable us to detect enhancement of vitellogenesis earlier than on the basis of an increased GSI. The available RIA for *C. gariepinus* GTH (Goos *et al.*, 1986) probably measures GTH II, predominant at the time of ovulation and spermiation (review by Kawauchi *et al.*, 1989). This assay was thus not suitable for monitoring vitellogenesis.

The study was conducted with pubertal *C. gariepinus*, in their first reproductive cycle and with adult fish, of which females entered a subsequent reproductive cycle after induced maturation, ovulation, and stripping of ovulated eggs.

MATERIALS AND METHODS.

General.

Two experiments were carried out. In experiment 1, pubertal fish were used. In experiment 2, adult fish were used, of which females entered their second reproductive cycle.

Pubertal and adult male and female *C. gariepinus* were purchased from commercial hatcheries (Fleuren Fishfarms, Someren, The Netherlands and Aquafish, Venhorst, The Netherlands, respectively), and were kept in 'store' in flow-through tanks until use in the experiments. Pubertal fish in experiment 1 were 4 months old. Males were prepubertal, with immature testes, and seminal vesicles macroscopically non-detectable. Females were previtellogenic, with small reddish ovaries lacking vitellogenic oocytes. Adult fish in experiment 2 were 7 months old. Fish of both sexes were mature and in prespawning condition.

During the experiment, fish were fed Trouvit trout pellets (Trouw, Putten, The Netherlands) at a ration of $8.4 g/kg^{0.8}/day$ (Hogendoorn *et al.*, 1983). Water flow was 1.5-2 l/min and water temperature was maintained at 25±1 °C.

The experiments were carried out in early spring, under semi-natural Dutch light periodicity circumstances, *i.e.* 12 hrs light and 12 hrs darkness (Richter *et al.*, 1987b).

Experimental set-up.

Three treatments were realized in both experiments. The first consisted of females held with equal numbers of males. Females in this group were thus exposed to the two male cues shown earlier to be of importance, of tactile and olfactory nature (Ch. 2; Ch. 3). These males and females will be designated as donor groups. Their holding water was supplied to groups of recipient females, the latter being exposed to olfactory cues only. Control groups received fresh water without conspecific stimuli.

In experiment 1 (pubertal fish), 70-l aquaria were used. Four units consisting each of 2 donor, 1 recipient and 1 control group were stocked at the beginning of the experiment. Each of the 2 donor aquaria of a unit contained 30 males and 30 females. They received fresh water. Holding water of the 2 aquaria was mixed, and half of it was led to the recipient aquarium, which contained 60 females. The control aquarium also contained 60 females. In this way, equal initial densities and water supply rates were realized in all aquaria. An initial sample of 10 fish from both sexes was taken upon stocking the aquaria. Thereafter, the 4 units were sampled weekly in an alternating fashion. Each unit was thus sampled only once per 4 weeks, to minimize stress. At sampling, the number of fish in each aquarium was reduced by 10, by removing ad random 5 males and 5 females from each of the two donor aquaria and 10 females from recipient and control aquaria. Since the experiment lasted 13 weeks, none of the aquaria was depleted completely and all fish sampled had received equal 'social stimulation'.

In experiment 2 (adult fish), 140-l aquaria were used to house the donor groups. Similar aquaria, but divided into two 70-l compartments accommodated the recipient and control groups. A total of 8 units of donor, recipient and control aquaria were stocked. A unit was stocked after fish had been sexed, and females had been induced to ovulate and stripped of ovulated eggs. Ovulation was induced with a single intraperitoneal injection of pimozide and an LHRH-analogue (buserilin-acetate), at a dosage of 5 and 0.05 mg/kg, respectively (De Leeuw *et al.*, 1985; Richter *et al.*, 1987a). The donor aquarium of each unit contained 15 males and 15 females, and received fresh water. Holding water was led to the recipient compartment, which contained 15 females. The control compartment contained 15 females and received fresh water. This way, equal densities and water supply rates were realized in the donor aquarium and the recipient and control compartments of a unit. An initial sample of 15 males and 15 stripped females was taken. After an interval ranging from 1 to 8 weeks, all fish of a unit were sacrificed, resulting in 8 sampling points with intervals increasing from 1 to 8 weeks between induced ovulation and stripping, and sacrifice.

At sampling, a blood sample was taken. Subsequently, fish were killed by an overdose of TMS (Tricainemethanesulfonate; Sandoz).
At random, 5 females from each treatment were designated to constitute a subsample for histological studies and plasma parameters.

Parameters.

Gonad indices.

Of all fish sampled, female body weight (measured to the nearest 0.1 g) and ovary weight (0.01 g) were recorded to calculate GSI (%). Male body weight (0.1 g), weight of testes (0.01 g) and weight of seminal vesicles (0.01 g) were recorded to calculate GSI (%) and SVSI (seminal vesicles somatic index; %). Indices were calculated as 100*{weight of organs/(body weight - weight of organs)}.

Histology.

From the 5 females of the subsample, ovaries were fixed in Ca-formalin, embedded in paraffin and sectioned (5-10 μm). Sections were stained with Haemalum-Eosin (Romeis, 1968). The relative proportion of the various oocyte stages was determined by counting in a representative ovary section all oocytes present, and expressing the numbers as percentage of the total. Staging was based upon the morphological criteria given by Richter and Van Den Hurk (1982), *i.e.* oocytes in previtellogenesis, in endogenous vitellogenesis (synthesis of cortical alveoli) and in exogenous vitellogenesis, including postvitellogenesis. Oocytes of the latter category are mature, *i.e.* sensitive to maturation inducing hormones. This simplification served to avoid errors in discriminating between late exogenous vitellogenesis and postvitellogenesis when oocytes were not sectioned through the nucleus and diameter could thus not provide a reliable criterion. Furthermore, average diameter of oocyte in the stage of exogenous vitellogensis (including postvitellogenesis) was calculated by taking from at least 40 oocyte cross-sections with a visible nucleus the mean of the largest and the smallest diameter.

Plasma parameters.

Blood samples (1-3 ml) were collected before sacrifice of females and mixed with 20-60 μl of Na-citrate (4%) and centrifuged. Plasma was stored at -20 °C until further use. Plasma of the 5 females of the subsample was analyzed for estradiol and vitellogenin.
Estradiol (E_2). E_2 was determined by radioimmunoassay (RIA), as decribed in Schulz (1984), with the following modifications. Plasma aliquots of 200-400 μl (exp 1) and 800 μl (exp 2) were mixed with an equal volume of RIA-buffer, heated

for 1 hr at 80 °C, and centrifuged to precipitate denatured plasma proteins. Samples were then extracted three times with ether, and the combined ether phase was evaporated. The dry residue was reconstituted in RIA-buffer, to a volume equivalent to or lower than the original plasma volume extracted. Extraction efficiency was checked by adding recovery tracer to a selected number of samples. Of each sample, volumes of 100 and 20 µl (exp 1) and of 100, 20 and 5 µl (exp 2) were measured, to test for parallelism. Sensitivity of the assay was 7.8 pg/tube. Further details are described by Schulz (1984). Values of individual fish below the detection threshold of 7.8 pg/tube were entered as zero-values in subsequent calculations. As a consequence, group averages presented below may underrate reality.
Vitellogenin (VTG). VTG was determined by polyacrylamide gel electrophoresis (PAGE), as described in Van Bohemen (1981), with a modification in the quantification procedure. Polyacrylamide slab gels (11%) were prepared, to which sodium-dodecyl-sulphate (SDS) treated plasma samples were applied. The location of the resulting VTG-monomer on the gel had been verified earlier (unpublished data, Van Weerd *et al.*) by comparing plasma from males with that from E_2-injected males (250 ng/g body weight/day during 7 days; Van Bohemen, 1981). Calibration with a mixture of standard proteins had revealed that the molecular weight of the VTG-monomer was 152,000 D (unpublished data, Van Weerd *et al.*). VTG concentrations in blood plasma were measured by applying to a gel a dilution series of bovine serum albumin (BSA, fraction V; Sigma) and a maximum of 15 plasma samples. Plasma samples were diluted with buffer 1:10 or 1:20. Densities of VTG-bands were compared with those of the BSA standard curve, by means of a laser densitometer (LKB, 2202 Ultroscan) and concentrations are expressed as µg BSA-equivalents/ml plasma. All determinations were carried out in duplicate, and the variation never exceeded 10%. The sensitivity of the densitometer was 0.070 µg of VTG (in BSA-equivalents)/lane. Outcomes of individual fish below the detection threshold of 0.070 µg/lane were entered as zero-values in subsequent calculations. As a consequence, group averages presented below may underrate reality.

Statistics.

Males.

Average body weight, testes and seminal vesicle weight and GSI and SVSI were analyzed by means of the non-parametric Mann-Whitney test. Averages at a given sampling date were compared with those of the previous sampling dates (within-group analysis).

Females.

Within-group analysis of average body weight, ovary weight, GSI, relative proportion of oocyte stages and diameter of exogenous vitellogenic oocytes was performed similar to the procedure for male data, by means of Mann-Whitney test. Within-group analysis of E_2 and VTG data was also performed by means of Mann-Whitney test.

Between-group analysis of the above-mentioned parameters was conducted by comparing average values of donor, recipient and control treatments at a given sampling date by means of Mann-Whitney test.

The difference with respect to body weight, ovary weight and GSI based upon the total sample (10 females in exp 1 and 10-15 females in exp 2) and based upon the subsample (5 females in both experiments) was tested by comparison of linear regression curves, calculated with individual data.

Of exp 2, data from the initial sample with respect to ovary weight and GSI were omitted, because at that time, the just-stripped ovary was for the major part filled with fluid, and its weight did not reflect the presence of follicles only.

P-values ≤ 0.05 are considered significant.

All statistical analyses were performed using the BMDP statistical software (Dixon *et al.*, 1988).

RESULTS.

General.

Sampling.

In females, there was no significant difference between data based upon the total sample (10 females in exp 1 and 10-15 females in exp 2) and the subsample (5 females in both experiments), with regard to average bodyweight, ovary weight and GSI. Average values of these parameters mentioned below therefore relate to the total sample, whereas data regarding proportion and growth of exogenous vitellogenic oocytes, and plasma parameters are based upon the subsample.

Ovary samples of donor females in the first week of experiment 2 were lost.

Mortality and somatic growth.

Mortality was negligible in the 2 experiments.

Body weight increased in both experiments. In exp 1, from 39.5 g at week 0 to 147.1 g at week 13 in males and from 47.2 g at week 0 to 152.0 g (donor), 182.9 g (recipient) and 177.0 g (control) at week 13 in females. In this experiment, average female bodyweight was not significantly different between the 3 treatments. In exp 2, body weight increased from 312.9 g at week 0 to 531.5 g at week 8 in males and from 331.2 g at week 0 to 493.7 g (donor), 392.5 g (recipient) and 398.6 g (control) at week 8 in females. In this experiment, average female body weight was significantly higher in fish from the donor treatment, than in those from the other 2 treatments. Because of these variations in average body weight, results with respect to gonad weight will be presented and discussed in terms of GSI (male and female) and SVSI (male) only.

Experiment 1.

Male gonad indices.

GSI increased from 0.02% at week 0 to 0.87% at week 13. The most dramatic increase ocurred from week 11 onwards.
SVSI could not be determined until week 6, because the seminal vesicles were still absent. It rose gradually from 0.06% at week 6 to 0.43% at week 13, with the sharpest increase from week 12 onwards. At the end of the experiment, males were generally still immature, without sperm-filled cysts at the testes' edges.

Females.

GSI and ovarian development (fig. 1).

GSI (fig 1A), the proportion (fig 1B) and diameter of exogenous vitellogenic oocytes (fig 1C) increased in all 3 treatments. A large variation is observed in all 3 parameters. This is caused by 1 or 2 (out of 5) 'advanced' animals (one individual from the donor group at week 6 had 20% exogenous vitellogenic oocytes). Females from donor groups, receiving the strongest male stimulus, displayed significantly higher GSI values than the other 2 groups from week 6 onwards (fig 1A). These values did not differ between recipient and control groups. Both the proportion of exogenous vitellogenic oocytes (fig 1B) and their diameter (fig 1C) did not differ significantly between the 3 groups, allthough an overall tendency for donor females to have a larger oocyte diameter was observed. Exogenous vitellogenic oocytes (fig 1B) appeared earlier in donor and recipient groups (week 6) than in the control group (week 7).

Plasma parameters (fig. 2).

Plasma E_2 levels (fig 2A) were low (*ca.* 0.3 ng/ml) for the first 4-5 weeks in all treatments. From week 5 onward, changes occurred. E_2 levels tended to be highest in donor females and lowest in control females. A bi-modal profile was seen, in which an initial elevation (between weeks 6 and 8) preceded a second, steeper increase in E_2-levels (from week 8-10 onwards). The second increase started earlier and reached higher values in donor females than in recipient and control females.

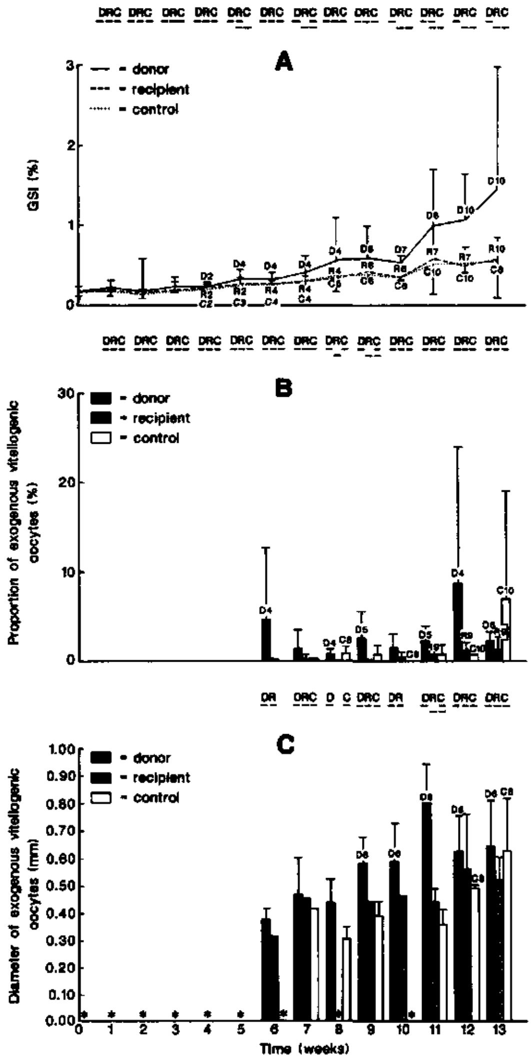

Fig 1. Experiment 1. Development of GSI (A), proportion of exogenous vitellogenic oocytes (B) and diameter of exogenous vitellogenic oocytes (C), of pubertal female *C. gariepinus*, from donor, recipient and control treatments ($\overline{X}$+SD or $\overline{X}$-SD).

Results of between group analysis (Mann-Whitney test, see text) are mentioned above the figure. Per sampling date, significant differences ($P \leq 0.05$) between donor, recipient and control treatments, respectively, are indicated by horizontal lines with different levels.

Results of within-group analysis (Mann-Whitney test, see text) are mentioned in the figure. Per sampling date of treatment D (donor), R (recipient) and C (control), significant difference ($P \leq 0.05$) with the nearest previous sampling date is indicated, specified by week number. No indication means no significant difference with values of any of the previous sampling dates.

* = absent

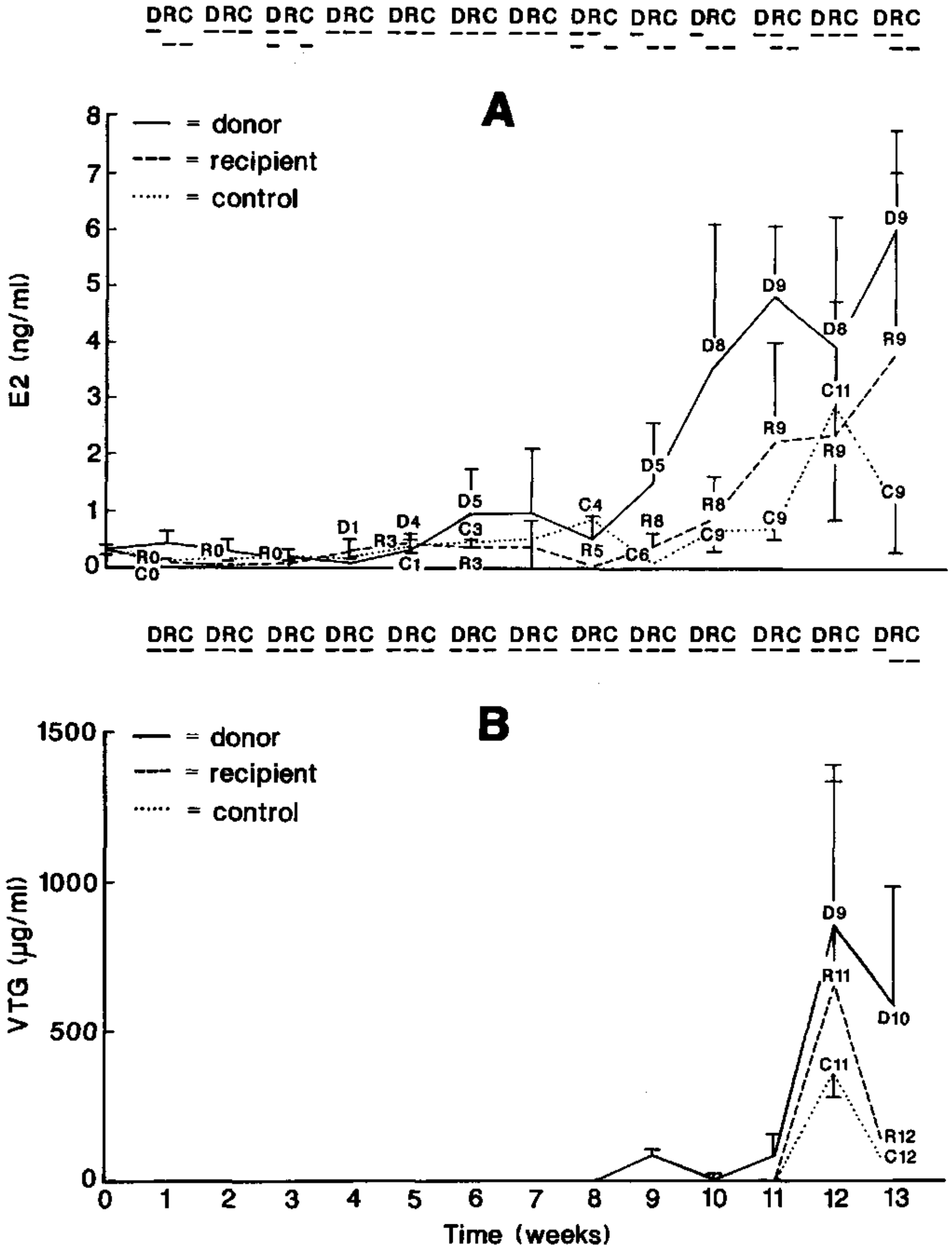

Fig 2. Experiment 1. Plasma levels of estradiol (E2) (A) and vitellogenin (VTG) (B), of pubertal female *C. gariepinus*, from donor, recipient and control treatments ($\overline{X}$+SD or $\overline{X}$-SD).

Results of between group analysis are mentioned above the figure; results of within group analysis in the figure. See legend to fig 1, for explanation of symbols.

Plasma VTG levels (fig 2B) were below the detection threshold in all groups until week 9, when it could be detected in females from the donor treatment. VTG levels tended to be highest in donor females and lowest in control females.

Experiment 2

Males gonad indices.

GSI increased from 0.48% at week 0 to 1.12% at week 8. SVSI did not increase during the experiment, but fluctuated around 0.37%.

Females.

GSI and ovarian development (fig. 3).

GSI (fig 3A), proportion of exogenous vitellogenic oocytes (fig. 3B) and diameter of exogenous vitellogenic oocytes (fig. 3C) increased in all 3 treatments. The initial GSI, after stripping, had decreased to minimum values at week 1. From week 1 onwards, GSI of donor and recipient females is significantly higher than in females of the control group. Recipient females have higher GSIs than control females. The proportion of exogenous vitellogenic oocytes (fig. 3B) tended to be highest in donor females and lowest in control females, with recipients taking an intermediate position. A plateau is reached in all 3 groups at week 3-4. The average diameter of exogenous vitellogenic oocytes (fig 3C) reached a maximum from week 4 and onwards. This parameter did not differ significantly between groups, although donor females tended to have highest diameters.

Plasma parameters (fig. 4).

Plasma E_2-values (fig. 4A) dropped in all 3 treatments from an initial high to minimum values at week 1. Thereafter E_2 values increased, with a second 'dip' 3-5 weeks later. Differences between donors, recipients and controls were generally not significant, although the former two groups tended to display highest levels. Plasma VTG levels (fig 4B) present an irregular picture, with large variations. In all 3 treatments, initial VTG levels dropped from week 0 to week 2, reached a peak at week 3 and dropped once more to a minimum at week 4 (in donors and controls) or week 5 (in recipients). VTG levels then rose again and displayed an irregular pattern. Differences between treatments were not significant, in general.

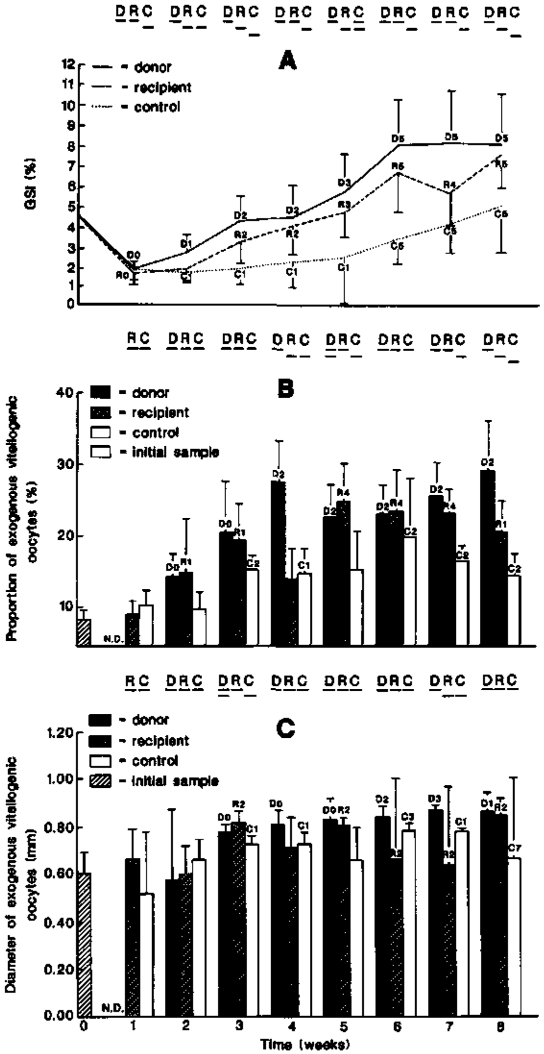

Fig 3. Experiment 2. Development of GSI (A), proportion of exogenous vitellogenic oocytes (B) and diameter of exogenous vitellogenic oocytes (C), of adult female *C. gariepinus*, from donor, recipient and control treatments ($\overline{X}$+SD or $\overline{X}$-SD).

Results of between group analysis are mentioned above the figure; results of within group analysis in the figure. See legend to fig 1, for explanation of symbols.

N.D. = not determined, data lost.

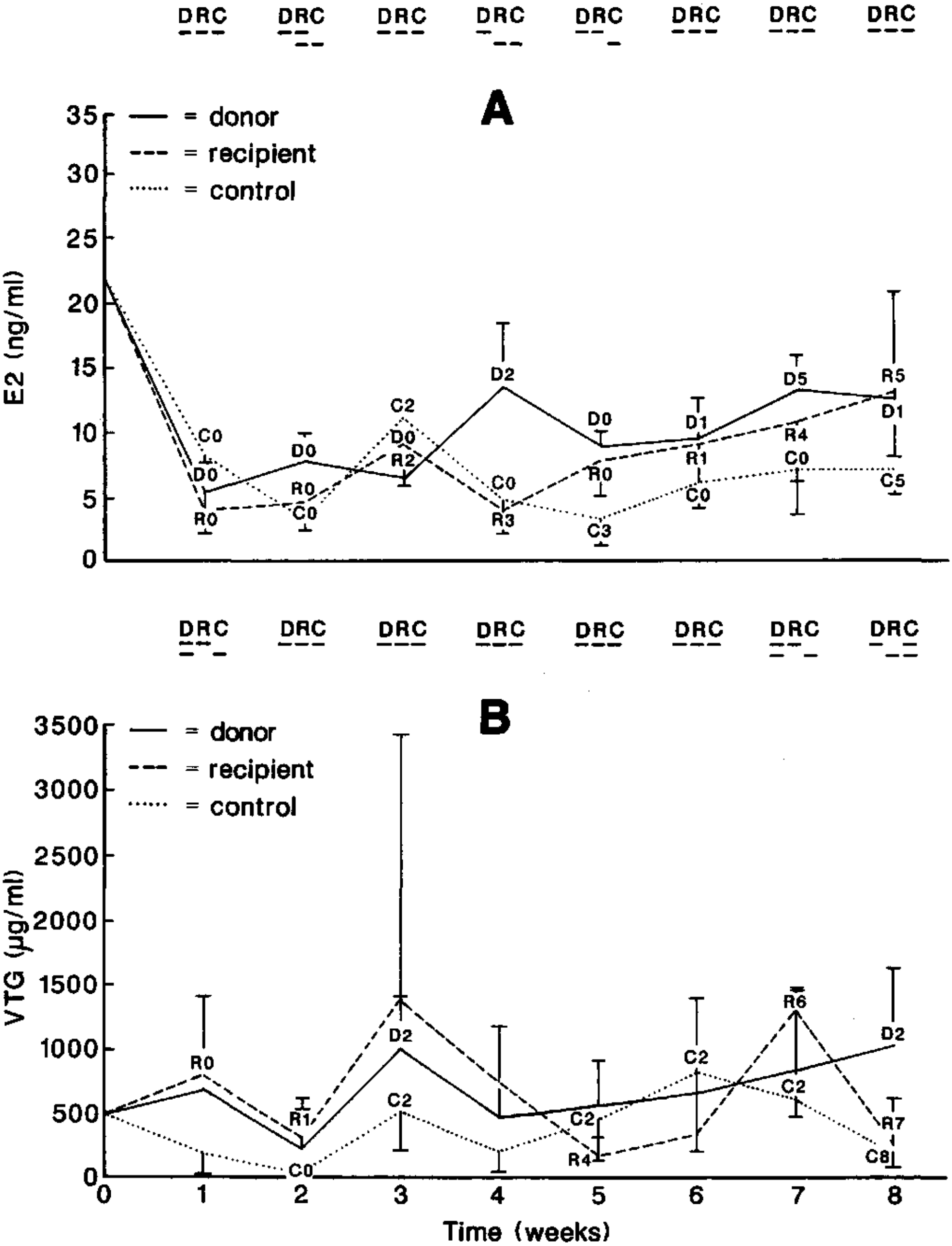

Fig 4. Experiment 2. Plasma levels of estradiol (E2) (A) and vitellogenin (VTG) (B), of adult female *C. gariepinus*, from donor, recipient and control treatments ($\overline{X}$+SD or $\overline{X}$-SD).

Results of between group analysis are mentioned above the figure; results of within group analysis in the figure. See legend to fig 1, for explanation of symbols.

DISCUSSION.

Development of males.

Gonadal development of pubertal males and adult males is in agreement with data from previous experiments (Ch. 2; Richter *et al.* ,1982). Pubertal males of the present study were still immature at the end of the experiment. The appearance of their seminal vesicles coincided with an increase of GSI and a first peak in E_2 of donor females (see also below). Since the seminal vesicles of adult *C. gariepinus* are a major source of pheromonal steroid glucuronides in *C. gariepinus* (Resink *et al.*, 1989b,c; Schoonen and Lambert, 1986, 1987; Schoonen *et al.*, 1987, 1988), it may be that the seminal vesicle of relatively immature males is already capable of producing steroid glucuronides, which could play a role in stimulation of vitellogenesis (Resink *et al.*, 1987a; Ch. 6).

Development of females.

In both pubertal and adult females, gonadal development in terms of GSI, proportion and diameter of exogenous vitellogenic oocytes was slower than in previous experiments (Ch. 2; Ch. 3). In all treatment groups, the final proportion and mean diameter of exogenous vitellogenic oocytes of pubertal females is much lower than in adult females. This indicates that pubertal females at an age of 7 months had reached the state of active vitellogenesis. Their gonadal development does not proceed synchronously and this explains the large variation in the ovarian parameters. In adult females, the proportion of vitellogenic oocytes reached a plateau and the mean diameter of these oocytes was over 0.8 mm. At this stage, they are sensitive to maturation inducing hormones (Richter and Van Den Hurk, 1982). Increases in GSI occurring after week 4 should be attributed to a continuous recruitment of oocytes into vitellogenesis and a simultaneous gradual slight increase in diameter of mature oocytes.

Quantitative aspects of E_2 and VTG.

Maximum E_2 values at the end of the experiment were *ca.* 6 ng/ml in pubertal donor females. They are in the same order of magnitude as values E_2 levels reported by Sumpter *et al.* (1984) for juvenile *O. mykiss* (4-6 ng/ml). Maximum E_2 levels of *ca.* 22 ng/ml were encountered in adult females upon stripping at week 0, whereas subsequent maxima were *ca.* 13 ng/ml. These levels were consistently higher than even the final levels of pubertal females. The initial values of adults at week 0 represent the stage of postovulation. In this stage, Van Dam *et al.* (1989) found E_2 levels in *C. gariepinus* of just below 30 ng/ml. In the related catfish *C. batrachus*, E_2 levels rise from less than 1 ng/ml in the season of previtellogenesis (preparatory phase) to a maximum of 35 ng/ml just before the spawning season (Singh and Singh, 1987). In other catfish species, peak values range from *ca.* 6 ng/ml in *I. nebulosus* (Burke *et al.*, 1984) and *ca.* 9 ng/ml in H. fossilis (Lamba *et al.*, 1983) to *ca.* 43 ng/ml in *I. punctatus* (MacKenzie *et al.*, 1989). Thus it seems, that adult *C. gariepinus* has E_2 levels comparable to those of other catfish species.

It is difficult to compare the VTG levels of the present experiment with data from other workers, because of differences in techniques employed. We measured the VTG monomer, whereas VTG measurement by other workers usually concerns the intact (dimeric) form (*e.g.* Norberg and Haux, 1988; Sumpter, 1985; Tyler and Sumpter, 1990). Van Bohemen (1981), who employed essentially the same method as in the present experiment, found VTG levels in adult *O. mykiss* of 0.1 mg/ml in May to 12.9 mg/ml in November, two months before spawning. Pubertal females of the present study exhibited maximum VTG concentrations of 860 µg/ml, and adult females maximum values of 1350 µg/ml.

The pattern of vitellogenesis during puberty and recrudescence.

In *O. mykiss*, a rise in plasma E_2, generally precedes a rise in plasma VTG (Whitehead *et al.*, 1978, 1983; Van Bohemen, 1981; Scott and Sumpter, 1983a,b; Elliot *et al.*, 1984; Bromage and Cumaranatunga, 1988) and GSI (Lambert *et al.*, 1978). Elevated VTG-levels either precede or accompany an increase in GSI (Van Bohemen, 1981; Riazi and Fremont, 1988; Sumpter *et al.*, 1984). Increases in GSI are accompanied by increases in both the diameter of oocytes in exogenous vitellogenesis and an increase in proportion of these oocytes (Riazi and Fremont, 1988; Sumpter *et al.*, 1984). In catfish species, a rise in E_2 either precedes (in *I. punctatus*; MacKenzie *et al.*, 1989) or coincides with (in *H. fossilis*; Lamba *et al.*, 1983) a rise in VTG. The GSI is always the last parameter to rise, in *C. batrachus* (Singh and Singh, 1987) and in *H. fossilis* (Lamba *et al.*, 1983). In *I. punctatus* and *H. fossilis*, respectively, the GSI rise also occurs simultaneously with increases in proportion (Anand and Sundararaj, 1974) or diameter and proportion of exogenous vitellogenic or postvitellogenic oocytes (MacKenzie *et al.*, 1989).

In pubertal female *C. gariepinus* of the present study, E_2 levels rise before VTG starts to appear. Sampling was done with weekly intervals and the E_2 pattern was bimodal. An initial, relatively modest, peak is followed by a second more steady increase towards maximum values at the end of the experimental period. Major changes in GSI occur simultaneously with further elevations of E_2 (second peak) and VTG and an increasing GSI is accompanied by an increase in both the relative proportion and the mean diameter of exogenous vitellogenic oocytes. A bi-modal pattern suggests a priming action of the first increase, in which hepatocytes are sensitized to synthesize VTG in response to the following rise in E_2. Such a priming action would not be unlike the role suggested for estrone (E_1) in *O. mykiss* by Van Bohemen (1981). However, since cross-reactivity with E_1 of the antibody used in the E_2-RIA in the present study is 3.3 % (Schulz, 1984), and circulating E_2 levels are twice as high as E_1 levels in *C. gariepinus* (Van Dam *et al.*, 1989), a contribution of E_1 itself to the pattern in the present study can be ruled out.

During recrudescence, a subsequent reproductive cycle is initiated and completed, with a sequence of events essentially similar to the one described above. However, in the present study the sequence of events in adult *C. gariepinus* appears to be irregular. E_2 levels, after dropping from postovulatory values at week 0, show a much less clear pattern than in pubertal females, and levels remain between 5 and 12 ng/ml. VTG levels fluctuate between 100 and 1350 µg/ml and their is no clear relation with yolk deposition in the oocytes. The picture may have been confounded by the fact, that during the first week(s) of the experiment in particular, plasma VTG at least partly still mirrors the previous reproductive state (postvitellogenesis) of the animal, perhaps because of a low clearance rate from the plasma. This 'overshoot' effect was also present with respect to VTG in spent *S. trutta* (Norberg *et al.*, 1989). Another reason for the irregularity of the pattern in adult *C. gariepinus* as compared to that of pubertal females may be the already very compressed cycle of ovarian recrudescence. Adult *C. gariepinus* can be stripped of viable eggs after hormonally-induced ovulation with intervals as short as 2 weeks (Hogendoorn and Vismans, 1980). Furthermore, Elliot *et al.* (1984) and Bromage *et al.* (1982a) found lower VTG levels, but larger egg size in *O. mykiss* with an extended reproductive cycle, pointing to the possibility of an increased uptake at a constant production rate determining circulating VTG levels. Similarly, Ruby *et al.* (1987) found a higher VTG level, when its uptake had been hampered in *S. salar* by exposure to sublethal cyanide. It may thus be, that differences between treatments in the present experiment with respect to VTG levels, particularly in adult females, are not only the consequence of preceding changes in circulating E_2 levels, but also of differential uptake in the ovaries.

Burke *et al.* (1984) sampled adult *I. nebulosus* on a weekly basis in the period of most dramatic changes. The authors found a bi-phasic E_2-peak, of which the first phase preceded a rise in GSI, and a second phase followed the rise in GSI. De Mones *et al.* (1989), found a transient rise in E_2 in postovulatory *O. mykiss*, and suggested priming of the liver for VTG production. Interestingly, Thomas *et al.* (1987) found E_2 in *Cynoscion nebulosus* during subsequent ovarian cycles to reach peak values when fully-grown oocytes were present and to decline afterwards to a constant and lower level until the end of the reproductive season. During the latter period, however, continuous recruitment of vitellogenic oocytes occurs in this species, and the authors conclude that after the initial E_2-peak, VTG production can be maintained with lower E_2 levels, once exogenous vitellogenesis is underway. Pankhurst and Conroy (1987) found a bi-phasic peak not in E_2 but in testosterone (T) in *Parapercis colias* and suggest that a bimodal increase in sex steroids prior to spawning is a feature of teleost species with rapid recrudescence. Also *C. gariepinus*, at least in captivity, is capable of reaching the stage of postvitellogenesis after a very short period of recrudescence, as mentioned above. However, a bi-phasic pattern in E_2 in adult females of the present study is much less clear than in pubertal females.

Male-induced shifts in pattern of vitellogenesis during puberty and recrudescence of females.

We showed earlier that the effect of males on vitellogenesis of pubertal and adult females has a tactile and an olfactory component (Ch. 2; Ch. 3). The olfactory component was shown to be a pheromonal one (Ch. 3). Also in the present study pheromonal stimulation of vitellogenesis ocurred, especially in adult females. The effect of sex-pheromones in teleosts involves the brain-pituitary axis (Dulka *et al.*, 1987). This pathway was recently demonstrated in *C. gariepinus* as well (Resink *et al.*, 1989a,d). In pubertal females, ovarian development in terms of GSI and histology was faster in the donor groups than in the recipient and control groups, the latter two being essentially similar. With respect to E_2 and VTG levels, however, differences, albeit often not significant, were also noted between recipients and controls, both in terms of concentration and timing. Exposure to holding water of pubertal females caused the plasma E_2 pattern to occur earlier and to reach higher values and had a similar effect on the VTG profiles. Apparently these changes were able to effect an enhancement of ovarian development (GSI and histology) only in the case of maximum stimulation (olfactory and tactile cues, donor group). It is possible that the effects of the 'intermediate degree of stimulation' (exposure to holding water) would have been noticeable at the ovarian level in due course, as in Ch. 2. The present results of the various degrees of male stimulation of vitellogenesis confirm results obtained earlier (Ch. 2), but not in terms of ovarian development. Apparently, in early vitellogenic *C. gariepinus*, GSI measurements and to a lesser extent, histological criteria are insufficient to monitor changes induced by male conspecifics. This is recognized in other teleosts as well, and the limitations to use GSI and histology to describe vitellogenesis during puberty have been discussed by *e.g.* Billard *et al.* (1978).

In adult females, on the other hand, ovarian development in terms of GSI and histology was faster in the donor and the recipient treatment than in the control treatment, the former two being essentially similar. The only noticeable difference with respect to plasma parameters is the fact that control females have the lowest overall E_2 plasma levels, in particular during the last 4-5 weeks of the experiment. In several teleost species, the female reproductive cycle can be altered by means of day-length manipulation, through involvement of the brain-pituitary-axis (Bromage and Cumaranatunga, 1988). This is evident from changes in peak values or timing of GTH (*e.g.* Bromage *et al.*, 1982a,b; Scott and Sumpter, 1983b; Bourlier and Billard, 1984). In most studies concerning day-length manipulation to shorten the ovarian cycle in *O. mykiss* (*e.g.* Bromage *et al.*, 1982b; Duston and Bromage, 1986, 1987; Bromage and Cumaranatunga, 1988; Cyr *et al.*, 1988), peak-values of E_2 and VTG are not dramatically different between females with short or long cycles, but the sequence of events leading to ovarian growth is compressed. Similar findings were reported for *Dicentrarchus labrax* (Zanyu *et al.*, 1989). However, changes in height of E_2 and/or VTG levels have been reported (Whitehead and Bromage, 1980; Bromage *et al.*, 1982a). In some cases, VTG-values tend to be lower in extended cycles (Elliot *et al.*, 1984; Bromage *et al.*, 1982a), accompanied by a larger egg size at ovulation (Elliot *et al.*, 1984), but this may be an indirect result, stemming from an increased uptake of VTG in vitellogenic oocytes, rather than a direct response to alterations in level or timing of E_2 (see above). In contrast, Carillo *et al.* (1989) found a lower proportion of exogenous vitellogenic oocytes in *D. labrax* from shortened cycles and Bourlier and Billard (1984) found oocyte diameter and final GSI before ovulation to be higher in *O. mykiss* from shortened cycles. It appears from the present study, that stimulation of vitellogenesis in pubertal female *C. gariepinus* largely conforms with light regimen stimulated females from most of the studies cited above: not only the height of plasma levels, but also their timing is affected. In adult females of the present study, however, stimulation seems to have affected the maxima of the various parameters involved, rather than their timing. This is perhaps due to the 'compressed' nature of the recrudescence period in period in *C. gariepinus*, as discussed above.

Finally, although the present study gave us more insight in the process of vitellogenesis during puberty and ovarian recrudescence of *C. gariepinus*, and in the mechanism through which stimulation by males results in differences in ovarian development, it did not provide us with a practical tool to detect enhanced vitellogenesis earlier than on the basis of GSI. However, GSI increases are reflected in increasing E_2 levels (pubertal and adult females) and VTG levels (pubertal females), and these, especially if based on a larger sample size, may be used to assess stimulation of vitellogenesis, without the necessity to sacrifice the fish.

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Chapter 5.

STIMULATION OF GONADAL DEVELOPMENT BY SEXUAL INTERACTION OF PUBERTAL AFRICAN CATFISH *CLARIAS GARIEPINUS.*

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SUMMARY.

Stimulation and inhibition of gonadal development by intersexual contact was studied in pubertal African catfish, *Clarias gariepinus*. The effect of a possible interaction was studied by evaluation after a 98-days experimental period, of gonadal development in combinations of intact and anosmic males and females. In addition, separate groups of males and females, respectively, were exposed to holding water from these combinations. A tentative model of stimulation of gonadal development by intersexual contact in pubertal fish was developed. Males stimulate ovarian development of females by both olfactory and tactile cues. In addition, males seem to enhance gonadal development of other males through olfactory stimulation via holding water. In contrast, females tended to inhibit male gonadal development, especially through tactile cues. It seems that although males are hampered by female tactile stimuli in their gonadal development and ability to stimulate male gonadal development, their ability to stimulate female gonadal development is not affected.

INTRODUCTION.

In African catfish, *Clarias gariepinus*, males stimulate ovarian development of vitellogenic females. This was shown to be the case in pubertal fish (Ch. 2; Ch. 4) and in adult fish, of which females had entered recrudescence (Ch. 3; Ch. 4). Holding water from a mixed-sex population of adult males and females also enhances ovarian development in pubertal female *C. gariepinus*, both previtellogenic and vitellogenic (Ch. 6). These studies indicated that male stimulation of female gonadal development is of olfactory and tactile nature. Visual and other components are irrelevant (Ch. 2; Ch. 3).

Male *C. gariepinus* possess, in addition to the testes, seminal vesicles, which have been meticulously described (Resink, 1988). Situated caudally to the testes, the seminal vesicles consist of 36-44 oblong lobes. Both testes and seminal vesicles are connected to the sperm duct, which ends in the urogenital papilla. Three main functions of the seminal vesicles have been suggested: sperm storage (Nawar, 1960), aiding in fertilization of eggs (Sundararaj and Nayyar, 1969) and production of pheromones. This latter aspect has been extensively researched and the presence of sex-attracting substances (Resink *et al.*, 1987), of steroidal nature was demonstrated (Resink *et al.*, 1989 a,b). These findings pertain to the spawning phase of the species, but a role of the seminal vesicles as production site of pheromones involved in stimulation of ovarian growth has been suggested (Ch. 2; Ch. 4).

In some teleosts, not only female, but also male reproductive parameters have been shown to be influenced by conspecific stimuli, in *e.g. Sarotherodon (Tilapia) mossambicus* (Silverman, 1978b,c) and in *Trichogaster trichopterus* (Cheal and Davies, 1974). An interaction between the sexes with respect to emission of sexual

stimuli has been suggested for *S. mossambicus* (Silverman, 1978a) and *Brachydanio rerio* (Chen and Martinich, 1975).
The present study was conducted to investigate the possible emission of female cues influencing male gonadal development in *C. gariepinus*, parallel to the situation in females referred to above. In addition, we investigated to what extent interaction between the sexes would modify the emission of stimuli enhancing gonadal development in the opposite sex. Lastly, by reducing the degree (intensity) of interaction through elimination of the olfactory sense, we attempted to analyze the interaction and the importance of the composing elements for emission of olfactory stimuli.

MATERIALS AND METHODS.

Origin and maintenance of fish.

Pubertal male and female *C. gariepinus* were purchased from a commercial hatchery (Aquafish, Venhorst, The Netherlands). At the start of the experiment, their age was 5 months. Females were previtellogenic, with an average body weight of 117 g. Males had an average body weight of 128 g. Fish were kept in a flow-through basin for 1 week, until sexing and induction of anosmia, and were fed during this and the entire experimental period Trouvit trout pellets (Trouw, Putten, The Netherlands) at a daily ration of 8.4 $g/kg^{0.8}$ (Hogendoorn *et al.*, 1983).

Sexing of fish and induction of anosmia.

Three weeks before the start of the experiment, fish were sexed on the basis of external morphological features and kept separate. One week later (two weeks before the start of the experiment), a number of males and females was made anosmic by thermocauterization of the olfactory epithelium. The resulting 4 groups of fish (anosmic males, anosmic females, intact males, intact females) were kept separate until the start of the experiment. No sham operation was performed but instead, the effect of anaesthesia during the operation and of anosmia was checked at the end of the experiment, by comparison of anosmic with intact fish (Ch. 3). Effectiveness of the cauterization was checked two weeks after the operation and at the end of the experiment, by macroscopically checking for the presence of olfactory epithelium. Regrowth of olfactory epithelium was not confirmed microscopically, because this was shown earlier to parallel macroscopic assesment (Ch. 3).

Table 1. Intersexual contact in pubertal African catfish, *C. gariepinus*.

Treatment*	Recipient fish*	Cue	via	from**
1a	M_I	none	fresh water	-
1a	F_I	none	fresh water	-
1b	M_I	olfactory	holding water	non-interacting F_I(1a)
1b	F_I	olfactory	holding water	non-interacting M_I(1a)
1c	M_I	olfactory	holding water	non-interacting F_I(1a) + M_I(1a)
1c	F_I	olfactory	holding water	non-interacting M_I(1a) + F_I(1a)
2a	M_I	olfactory + tactile	direct contact	interacting F_I(2a)
2a	F_I	olfactory + tactile	direct contact	interacting M_I(2a)
2b	M_I	olfactory	holding water	interacting F_I+M_I(2a)
2b	F_I	olfactory	holding water	interacting M_I+F_I(2a)
3a	M_I	olfactory + tactile	direct contact	interacting F_A(3a)
3a	F_A	tactile	direct contact	interacting M_I(3a)
3b	M_I	olfactory	holding water	interacting F_A+M_I(3a)
3b	F_I	olfactory	holding water	interacting M_I+F_A(3a)
4a	M_A	tactile	direct contact	interacting F_I(4a)
4a	F_I	olfactory + tactile	direct contact	interacting M_A(4a)
4b	M_I	olfactory	holding water	interacting F_I+M_A(4a)
4b	F_I	olfactory	holding water	interacting M_A+F_I(4a)
5a	M_A	none	fresh water	-
5a	F_A	none	fresh water	-

* Numbers and letters refer to treatments (see fig. 1)

** origin of stimulus donor(s) between brackets

M = male, F = female, I = intact, A = anosmic.

Cues were transmitted via water (olfactory) or direct contact (tactile) and came from interacting and non-interacting males and females.

Experimental design.

For both males and females, 10 stimulus combinations were investigated. These are mentioned in table 1 and illustrated by the diagram of fig 1. Males and females receive olfactory and/or tactile cues from non-interacting or interacting, intact and/or anosmic fish, via holding water or in direct contact. As a control, intact and anosmic fish receive fresh water, without any cue. A pilot experiment had shown that there is no significant influence on overall growth performance and gonadal development in either anosmic or intact fish, when both are kept together. The effect of metabolites in holding water on gonadal development has been investigated earlier and was found to be negligable (Ch. 3). Gonadal development was used as the parameter to detect modulations in the emission of stimuli from pubertal conspecifics.

Each treatment consisted of two duplicate groups of 18 fish each, resulting in a total of 34 treatment groups. The duration of the experiment was 98 days.

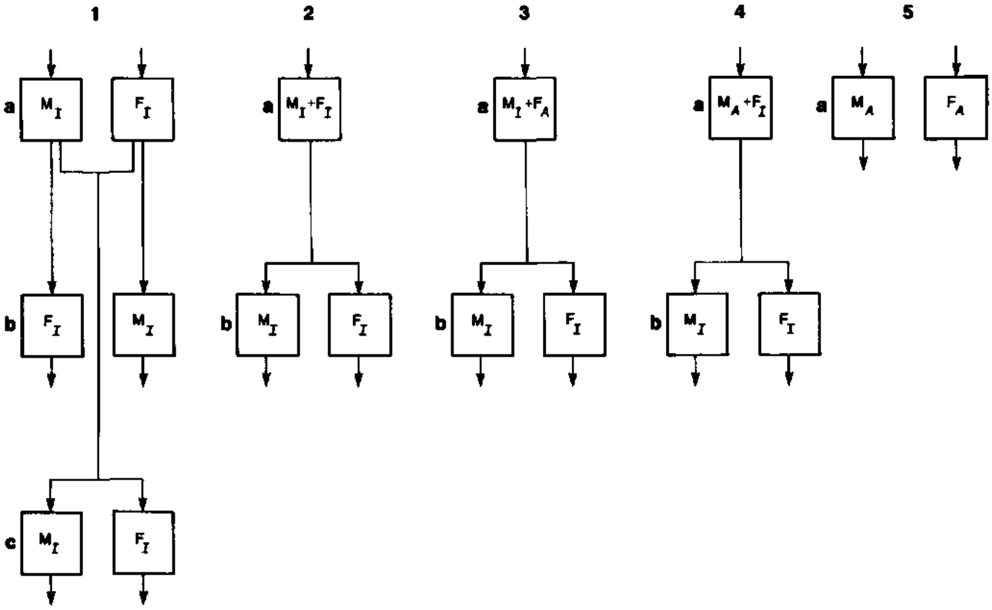

Figure 1. Diagram illustrating experimental design to study intersexual contact in pubertal African catfish (*C. gariepinus*). M=male, F=female, I=intact, A=anosmic.

Maintenance units (1-5) contain aquaria designated 'a' receiving fresh water and 'b' and 'c' receiving holding water. Arrows indicate direction of water flow. A number in combination with a letter refers to a treatment. Transmission of cues is summarized in table 1.

Set-up of aquaria.

The experiment was conducted in a two-layer aquarium system, described earlier (Ch. 2; Ch. 3). Seventeen aquaria of 70 l were divided into two compartments of 35 l. Each of the resulting 34 compartments was stocked with 18 fish. Water temperature was kept at 24-25 °C.
Fig. 1 shows that compartments received either fresh water or holding water. Fresh water inflow was 1.0-1.2 l/min. As one compartment receiving fresh water provided holding water for 2 other compartments (fig. 1), holding water inflow was 0.5-0.6 l/min.

Sampling and measurements.

At the start of the experiment, initial samples (n=18) of anosmic and intact males and females were taken. At the end of the experiment, all fish were sacrificed. Fish were killed by an overdose of TMS (Tricaine methanosulfonate; Sandoz).
Body weight (to the nearest 0.1 g) and gonad weight (to the nearest 0.01 g) were determined and male and female GSI (gonado somatic index; as 100*weight of ovary or testes/(body weight - weight of ovary or testes)) and male SVSI (seminal vesicle somatic index; as 100*weight of seminal vesicles/(body weight - weight of seminal vesicles)) were calculated.

Statistics.

Data concerning body weight, gonad weight and gonad indices (GSI, SVSI) were not distributed normally. Hence, results were compared by the non-parametric Mann-Whitney test. Results of duplicate treatments were compared and combined if not statistically different.
P-values ≤ 0.05 are considered significant.
All statistical analyses were performed using the BMDP statistical software (Dixon *et al.*, 1988).

RESULTS.

General.

Mortality among females ranged from 0 to 16%. Mortality among males was slightly higher, from 0 to 22%. All mortality occurred during the first 3 weeks of the experiment, due to fighting injuries after stocking of the aquaria. This is a common phenomenon in culture of *C. gariepinus*.
Anosmic and intact females and anosmic and intact males, respectively, of the

initial sample did not differ significantly in bodyweight, gonad weight, GSI and SVSI.

At the end of the experiment, twenty fish had regenerated olfactory epithelium, as revealed by macroscopic inspection. In 12 of these 20 cases, however, the skin had grown over and closed the nares. The 8 fish with regenerated olfactory epithelium and open nares were found in different groups. Their parameters were within the first quartile from the median of the same treatment groups, and they have been included in subsequent calculations.

Bodyweight.

Female body weight increased significantly from 120.8 g at the start of the experiment to values ranging from 261.8 g to 377.1 g. Male body weight increased significantly from 120.8 g to values ranging from 323.6 g to 393.5 g.

Because of significant differences in final bodyweight between treatments, gonadal development will be presented below in the form of somatic indices, *i.e.* GSI in both sexes and SVSI in males.

Table 2A. Gonadal development in pubertal males of *C. gariepinus*.

treatment	GSI±SEM	statistics*	SVSI±SEM	statistics*
1 a	0.39/0.67**	abc/d	0.14/0.23**	ade/bc
1 b	0.36±0.01	abc	0.17±0.01	ab
1 c	0.51±0.05	e	0.23±0.02	c
2 a	0.27±0.04	af	0.11/0.19**	de/abc
2 b	0.38±0.00	bc	0.16±0.00	ad
3 a	0.32±0.00	abf	0.15±0.00	ade
3 b	0.38±0.01	abc	0.16±0.00	ad
4 a	0.32±0.04	abcfg	0.15±0.01	ade
4 b	0.41±0.04	c	0.16±0.03	ad
5 a	0.18±0.00	g	0.12±0.00	e

For explanation see fig. 1 and table 1.

*** different letters indicate significant difference**

**** differing duplicates**

Gonadal development.

Males (table 2A).

Both GSI and SVSI had increased significantly, from initial values of 0.11% (GSI) and 0.06% (SVSI) to values ranging from 0.18% (GSI) and 0.11% (SVSI) to 0.67% (GSI) and 0.23% (SVSI). Duplicates of treatment 1a differed significantly with respect to GSI and SVSI and those of treatment 2a with respect to SVSI. These values are presented separately. In all other cases, results of duplicate treatments have been pooled. Significant differences in both GSI and SVSI were found between the various treatments, but less prominent than in females (see below). The highest values were observed in intact males receiving fresh water (treatment 1a, *cf.* table 1) and in intact males receiving holding water from non-interacting males and females (treatment 1c, *cf.* table 1). Lowest values were recorded in non-interacting anosmic males (treatment 5a, *cf.* table 1) and in 1 duplicate of intact males interacting with intact females (treatment 2a, *cf.* table 1)

Table 2B. Gonadal development in pubertal females of *C. gariepinus*.

treatment	GSI±SEM	statistics*
1 a	0.42/0.57**	a/bc
1 b	1.06±0.38	bd
1 c	0.92±0.52	bd
2 a	1.45±0.09	e
2 b	1.01±0.09	bd
3 a	0.77±0.15	ab
3 b	0.83±0.10	b
4 a	1.51±0.19	de
4 b	0.63±0.01	bc
5 a	0.57±0.02	ac

For explanation see fig. 1 and table 1.

*** different letters indicate significant difference**

**** differing duplicates**

Females (table 2B).

In all treatments, GSI had increased significantly, from an initial value of 0.23% to values ranging from 0.42% to 1.51%. Duplicates of treatment 1a differed significantly and are presented separately. In all other cases, results of duplicate treatments have been pooled. There were significant differences in final GSI, between the various treatments. The highest values were found in intact females interacting with males (treatments 2a and 4a, respectively, *cf.* table 1). Lowest values were recorded in non-interacting females (treatment 1a and 5a, *cf.* table 1).

Treatment effects.

By making appropriate comparisons of treatments, we could determine i) the effect of the cauterization procedure on gonadal development, ii) the effect of olfactory and/or tactile stimuli from the opposite sex on gonadal development, iii) the effect of olfactory and/or tactile stimuli on gonadal development under conditions of complete or partial interaction of the sexes and iv) the effect of olfactory stimuli from the same sex on gonadal development.

Males (table 3A).

Cauterization significantly depressed GSI and, to a lesser extent, SVSI (1a-5a). Comparisons of treatments involving male anosmia (4a and 4b) were therefore not considered meaningful and were excluded from interpretation of results. Male gonadal development was not or negatively (a significant depression of GSI was found in 1 duplicate) affected by female tactile+olfactory stimuli (1a-2a). It was not or negatively (a significant depression of GSI was found in one duplicate) affected by female olfactory stimuli (1a-1b). It was negatively affected in terms of GSI and not in terms of SVSI by female tactile stimuli (2a-2b). Mutual contact of sexes modulated olfactory stimuli (1c-2b) and resulted in a depression of male GSI and SVSI. There was no difference in modulation of olfactory stimuli between partial (due to anosmia of females) and complete mutual contact of sexes on gonadal development of males (2b-3b). Male olfactory stimuli significantly enhanced male gonadal development (1b-1c).

Females (table 3B).

Cauterization did not affect GSI (1a-5a). Comparisons of treatments including anosmic females were, therefore, maintained. Female GSI was positively affected by male tactile+olfactory stimuli (1a-2a). It was positively affected by male olfactory stimuli, resulting in increases in GSI in two comparisons (1a-1b; 2a-3a), except in one duplicate (of 1a-1b). Female GSI was also positively affected by male

Table 3A.

Effects of various stimuli on gonadal development of pubertal males of *C. gariepinus*.

Stimuli	comparison of treatments	GSI	stat.*	SVSI	stat.*
cauterization	1a 5a	0.39/0.67 0.18	S/S	0.14/0.23 0.12	NS/S
female tactile+ olfactory stimuli	1a 2a	0.39/0.67 0.27	NS/S	0.14/0.23 0.11/0.19	NS/S/NS/NS
female olfactory stimuli	1a 1b	0.39/0.67 0.36	NS/S	0.14/0.23 0.17	NS/NS
female tactile stimuli	2a 2b	0.27 0.38	S	0.11/0.19 0.16	NS/NS
modulation of male and female olfactory stimuli[a]	1c 2b	0.51 0.38	S	0.23 0.16	S
modulation of male and female olfactory stimuli[b]	2b 3b	0.38 0.38	NS	0.16 0.16	NS
male olfactory stimuli	1b 1c	0.36 0.51	S	0.17 0.23	S

For explanation, see table 1.

a by complete interaction of sexes

b by partial interaction of sexes due to presence of F_A

*** stat. = statistics:**

S = significantly different,

NS = not significantly different.

Table 3B.

Effects of various stimuli on gonadal development of pubertal females of *C. gariepinus*.

Stimulus	comparison of treatments	GSI	statistics
cauterization	1a	0.42/0.57	NS/NS
	5a	0.57	
male tactile + olfactory stimuli	1a	0.42/0.57	S/S
	2a	1.45	
male olfactory stimuli	1a	0.42/0.57	S/NS
	1b	1.06	
	2a	1.45	S
	3a	0.77	
male tactile stimuli	2a	1.45	S
	2b	1.01	
modulation of male and female olfactory stimuli[a]	1c	0.92	NS
	2b	1.01	
modulation of male and female olfactory stimuli[b]	2b	1.01	NS
	3b	0.83	
female olfactory stimuli	1b	1.06	NS
	1c	0.92	

For explanation, see table 1.

[a] by complete interaction of sexes

[b] by partial interaction of sexes due to the presence of F_A

S = significantly different;

NS = not significantly different.

tactile stimuli (2a-2b). Mutual contact of sexes did not modulate olfactory stimuli (1c-2b). There was no difference in modulation of olfactory stimuli between partial (due to anosmia of females) and complete mutual contact of sexes on ovarian development (2b-3b). Female olfactory stimuli did not enhance female gonadal development (1b-1c).

DISCUSSION.

The present study confirms that ovarian growth in pubertal female *C. gariepinus* is stimulated by tactile and olfactory cues of male origin (Ch. 2; Ch. 4). We recently showed (Ch. 4) that male stimulation of female gonadal development involves shifts in plasma levels of estradiol and vitellogenin. The present results indicate that females do not stimulate, but rather tend to hamper, male gonadal development, especially through tactile cues. Effects in males are most pronounced in testicular development (GSI, *cf.* table 3A). In *S. mossambicus*, both female (Silverman, 1978a,b) and male gonadal development (Silverman, 1978b) are influenced by conspecific stimuli. In contrast, nuptial coloration of male *S. mossambicus* is not affected by conspecific stimuli (Silverman, 1978c). In *T. trichopterus*, induction of spawning readiness is more susceptible to social and environmental variables in females than in males (Cheal and Davies, 1974).

Stimulation of male gonadal development by olfactory stimuli from males is one of the more surprising findings of the present study. A somewhat similar phenomenon has been reported earlier (Stacey and Hourston, 1982), for pheromonal stimulation of male (and female) spawning behavior through milt, in *Clupea harengus pallasi*. Stimulation of testicular development of male *S. mossambicus* by conspecific stimuli has been described (Silverman, 1978b), regardless of sex of the stimulatory animals.

Regarding the possibility of mutual modulation of reproductive parameters, a feed-back model in male courting frequency of *S. mossambicus* has been suggested (Silverman, 1978a): presence of a courting male leads to changes in the chemical signals generated by a ripe female, to which changes the male responds with an elevated courting frequency. Similarly, it has been suggested (Chen and Martinich, 1975) for *B. rerio*, that less male ovulation-inducing pheromone would be secreted in the 'chemical absence' of females, also pointing to an interaction between the sexes with respect to emission of stimuli. Such a feed-back from one sex on emission of signals by the other sex, as suggested for male courting frequency of *S. mossambicus* (Silverman, 1978a), or for emission by male *B. rerio* of ovulation-inducing pheromones (Chen and Martinich, 1975), apparently does not exist in pubertal *C. gariepinus*, with respect to gonadal development.

In previous studies we have established that cues other than of tactile and olfactory nature, do not play a role in male stimulation of female gonadal development (Ch. 2; Ch. 3). The present study was conducted under the assumption that the same holds true for female stimulation of male gonadal development. We made a distinction between olfactory stimuli on the one hand, and tactile stimuli on the other hand. Tactile stimuli may actually encompass a combination of signals of varying nature, such as gustatory, tactile *s.s.* and others (electrical, sound). Gustation is generally considered to be of minor importance in the reproductive process of teleosts (Pfeiffer, 1982), although catfishes posses taste buds, notably on the barbels (Reutter, 1982). Tactile contact may be important in teleosts, but it is confined to the prelude to and the actual spawning act (in *e.g. Carassius auratus* (Partridge *et al.*, 1976) and in *B. rerio* (Lambert *et al.*, 1986)). Moreover, a contribution of the other senses during this 'butting' and 'nipping' of prospective mates has not been examined. Male *Tilapia mossambica* (syn. *S. mossambicus*) produce sounds which prime the female for oviposition (Marshall, 1972). Since it is difficult to unravel this composite tactile cue, we are not able from the present results to incriminate one or the other of these signal types, in *C. gariepinus*.

Based upon the present results, we propose the following model regarding stimulation of gonadal development by sexual interaction of pubertal *C. gariepinus*. Males stimulate ovarian development of females by both olfactory and tactile cues. In addition, males enhance gonadal development of other males through olfactory stimulation. Females tend to hamper male gonadal development and attenuate 'male-male' stimulation, the latter possibly by emission of inhibitory cues, released as a result of interaction with males. The males' ability to stimulate female gonadal development is not affected by interaction with females. This may point to the emission by males of two types of signals, which are differentially affected by interaction with females.

We mentioned above that, although male gonadal development is hampered by interaction with females, emission of olfactory cues stimulating female ovarian development is not influenced by interaction with females. This could indicate that male gonadal 'status' and emission of stimulatory cues are not related. This notion is supported by the observation, that anosmic males (which have not been included in interpretation of results because of a reduced gonad development, as mentioned earlier) are as succesful in stimulating females as their intact counterparts (comparison of treatments 2a and 4a, *cf.* fig. 1 and table 2B). In our earlier work (Ch. 2; Ch. 3; Ch. 4; Ch. 6) we speculated that steroid glucuronides of gonadal origin function as male sex-pheromones in stimulating ovarian development. Under the assumption that steroid glucuronides and/or their precursors, *i.e.* sex steroids, indeed play a role, the present results imply that either synthesis of these compounds is not proportionally related to male gonadal 'status', or that origins other than the testes and seminal vesicles are involved. With respect to the latter option, various non-gonadal organs (interrenal, Stannius corpuscles, liver and brain) are known (Fostier *et al.*, 1983), which may synthesize active sex-steroids or precursors. Also the skin of *C. gariepinus* is able to synthesize steroid glucuronides, from circulating steroids produced elsewhere (Ali *et al.*, 1987). We are currently investigating the ovarian-growth stimulating capacity of castrated male, and results of that study should clarify matters.

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Chapter 6.

GCMS-IDENTIFIED STEROIDS AND STEROID GLUCURONIDES IN OVARIAN GROWTH STIMULATING HOLDING WATER FROM ADULT AFRICAN CATFISH *CLARIAS GARIEPINUS*.

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SUMMARY.

Pubertal vitellogenic and previtellogenic female *Clarias gariepinus* were exposed to holding water from a mixed-sex population of adult conspecifics. Exposure to holding water caused an accelerated ovarian growth in both cases. Analysis of holding water by gas chromatography and mass spectrometry (GCMS) yielded the presence of several steroids and steroid glucuronides known to be produced in adult, prespawning *C. gariepinus*. The possible role of these compounds in stimulating ovarian growth is discussed in relation to earlier findings on their pheromonal properties.

INTRODUCTION.

Pheromones play an important role in reproduction of teleosts (Colombo *et al*, 1982; Liley, 1982; Liley and Stacey, 1983; Lambert *et al*., 1986; Stacey *et al*., 1986, 1987). All observations described, however, relate to advanced phases of the teleost reproductive cycle: oocyte maturation and ovulation (Van Den Hurk *et al*., 1987; Resink *et al*., 1989b), spermiation (Stacey and Sorensen, 1987) and synchronization of the two processes (Dulka *et al*., 1987; Stacey *et al*., 1989) and spawning (Resink *et al*., 1987, 1989b). In recent studies of the African catfish (*Clarias gariepinus*), we found that exposure to holding water from pubertal males and females stimulated ovarian growth of pubertal vitellogenic females (Ch. 2). A similar situation exists in adult *C. gariepinus*, when females have entered recrudescence after induced ovulation (Ch. 3). In the latter experiment, stimulation of ovarian growth did not occur in anosmic fish or by 'female' holding water, pointing to the involvement of a male pheromone detectable by olfaction. In view of our long-term aim to elucidate the chemical nature of the pheromone contained in holding water, we sought ways to collect sufficient amounts of 'active' holding water. Since in the present experiment holding water from a mixed-sex population of adult *C. gariepinus* appeared also to be able to stimulate vitellogenesis and thus to have pheromonal properties, we subjected holding water from such a population to analysis by means of gas chromatography-mass spectrometry (GCMS). The aim of this analysis was to identify in the holding water compounds which may play a pheromonal role in the observed simulation of ovarian growth. Attention was focused on steroids and steroid glucuronides, because all teleost sex pheromones identified to date are identical to or derived from sex hormones. Sorensen and Stacey (1989) and Stacey (1989) recently suggested a universal role for sex hormones or their derivatives as sex pheromones in teleosts. In the present experiment, we therefore analysed holding water for steroids and steroid glucuronides known to be produced by adult, prespawning *C. gariepinus* (Schoonen and Lambert, 1987; Resink *et al*., 1989a; Van Dam *et al*., 1989).

MATERIALS AND METHODS.

Testing of holding water.

Experimental design.

We tested the effect of holding water from adult *C. gariepinus* (donor fish) on ovarian growth of two groups of pubertal females (recipient fish). Recipients in group 1 were vitellogenic females, raised in our own hatchery; those in group 2 were previtellogenic, purchased from a commercial hatchery (Fleuren Fish Farm, Someren, The Netherlands). Recipients of group 1 were exposed to holding water for 68 days. Recipients of group 2 were exposed for a shorter period (42 days), based on results of a pilot study, designed to assess the possibility of foreshortening the response period to holding water.

Adult donor fish.

Adult 1-year old female and male *C. gariepinus* (body weight between 700g and 1000g) from our hatchery stock were used as the source of holding water. Females had a gonadosomatic index (GSI; as 100*(weight of gonads/body weight - weight of gonads)) of around 20%. Males had a GSI of 1.5-2% and a seminal vesicles somatic index of 0.5-0.7%. The fish were mature and in prespawning condition.

Pubertal recipient fish.

Females from group 1 were 171 days old, with an initial average body weight of 208 g and a GSI of 4.36 %. They were vitellogenic (yolky oocytes present), as judged macroscopically. Females of group 2 were 150 days old, with an initial average body weight of 124 g and a GSI of 0.24%. These females were previtellogenic, *i.e.* without yolky oocytes. Recipient females purchased from the commercial hatchery (group 2) were acclimated for at least 1 week in flow-through tanks upon arrival in our laboratory.

Maintenance of donor and recipient fish.

The experiment was carried out in a two-layer aquarium system (Ch. 2). Donor males and females (7 of each sex per aquarium) were housed in the top-layer, and recipient and control females in the bottom-layer (initial number 20 fish per aquarium) of the aquarium system. Aquarium volume was 70 l and water temperature was 25±0.5°C. Recipient fish received holding water through a siphon from the top-layer aquaria at a flow-rate of 2-3 l/min. They were exposed to holding water for 68 days (group 1) and 42 days (group 2). Control fish were exposed to fresh water at the same flow rate for an equivalent period of time. During the experiment, all fish were fed Trouvit trout pellets (Trouw, Putten, The Netherlands) at the optimum ration for *C. gariepinus* of 8.4 g/kg$^{0.8}$/day (Hogendoorn *et al.*, 1983).

Sampling of recipient fish and parameters used.

From group 1, a sample of 10 females was sacrificed at day 0 (initial control) and duplicate final control (12 and 17 fish) and recipient (16 and 17 fish) groups were sacrificed at day 68. In group 2, a sample of 16 females was sacrificed at day 0 (initial control), and duplicate final control (18 and 19 fish) and recipient groups (16 and 18 fish) were sacrificed at day 42. Fish were killed by an overdose of TMS (Tricaine methanesulphonate; Sandoz), to determine body weight, ovary weight and GSI. The proportion of vitellogenic females was determined by macroscopically checking ovaries for the presence of yolky oocytes, which are clearly visible to the naked eye because yolk of *C. gariepinus* is bright green in color.

Statistical analysis.

Data were tested by Student's t-test or non-parametric test of Kruskal-Wallis (Sokal and Rohlf, 1969) as described earlier (Ch. 2).

Analysis of holding water.

Collection and concentration of holding water.

To obtain holding water from donor fish, the in- and outflow of the aquaria was closed for 24 hours, during which period the fish were not fed. A total of 70 l of holding water was collected. It was filtered and stored in polyethylene bags at -20°C. Lots of 10 l of ice were crushed and subsequently freeze-dried. The temperature during freeze-drying never exceeded 15°C. The dry, powdery residues were rinsed out of the freeze-dryer trays with small amounts of distilled water and with a final wash of ethanol 100%. The ethanol fraction was reduced by evaporation to *ca.* 10 ml and added to the water fraction, resulting in a total of 2 l of concentrated holding water, which was again stored at -20°C.

Isolation of steroids and steroid glucuronides.

One l of HW-concentrate was processed according to the diagram of fig 1, largely based upon the methods of isolation of steroids and steroid glucuronides from *C. gariepinus* seminal vesicle fluid employed by Schoonen and Lambert (1987), and from *Brachydanio rerio* holding water employed by Van Den Hurk *et al.* (1987).

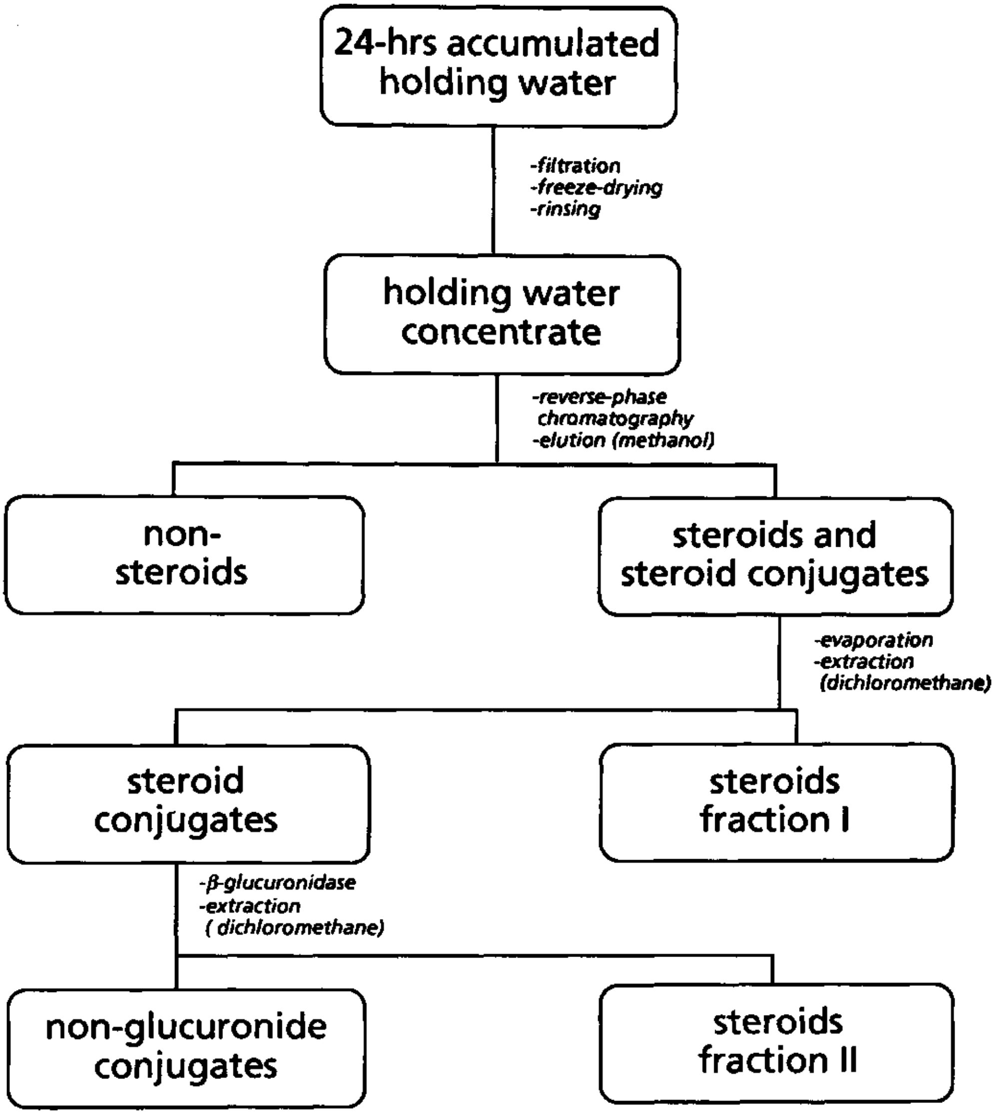


Figure 1.

Processing of holding water, yielding fraction I containing steroids, originally present as free steroids, and fraction II, originally present as steroid glucuronides.

Table 1.

Steroids and steroid glucuronides demonstrated in male seminal vesicle fluid and female blood plasma of adult *C. gariepinus* in prespawning condition. The data were used as a reference for the analysis of holding water.

Demonstrated in adult *Clarias gariepinus*[a]	Detected in holding water as			
	Free steroid		Steroid gluc.	
	FS	SIM	FS	SIM
Progestagens[b] :				
5ß-Pregnane-3α,17α,20α-triol	-	-	-	+
5ß-Pregnane-3α,17α,20ß-triol	-	-	-	+
5ß-Pregnane-3α,17α-diol-20-one	-	-	-	+
Androgens[b] :				
5ß-Androstane-3ß,17ß-diol	-	-	-	-
5ß-Androstane-3α,17ß-diol	-	-	+	+
Etiocholanolone	+	+	+	+
5ß-Androstane-3α,17ß-diol-11-one	+	+	-	+
5ß-Dihydrotestosterone	-	-	-	-
5ß-Androstane-3α,11ß-diol-17-one	-	-	-	-
Testosterone	-	-	-	-
Estrogens[c] :				
Estradiol-17ß	-	+	-	+
Estrone	+	+	+	+
Cholesterol	-	+	-	+

[a] Data from Schoonen and Lambert (1987), Resink *et al.* (1989a) and Van Dam *et al.* (1989).

[b] as steroid glucuronide

[c] as free steroid

gluc. = glucuronide

FS = full spectrum

SIM = selected ion monitoring.

The procedure yielded two steroid fractions, one originally present as free steroids (fraction I), the other as steroid glucuronides (fraction II). Efficiency of the isolation procedure, determined for steroid glucuronides by adding ^{3}H-estrone-glucuronide to the HW-concentrate, was over 90%.

Derivatization.

Attention was focused on steroids, demonstrated as free steroids or steroid glucuronides in adult *C. gariepinus* (table 1). As a standard, a mixture was prepared of all the steroids mentioned in table 1. Cholesterol was included in the mixture since it has been demonstrated in seminal vesicle fluid of feral *C. gariepinus* (Schoonen and Lambert, 1987) and was demonstrated in holding water from male *B. rerio* (Van Den Hurk *et al.*, 1987). 5α-Androstane-3ß-ol was added as the internal standard. Trimethylsilyl (TMS) and oxime-trimethylsilyl (OTMS) derivatives were made according to Van Dam *et al.* (1989) and Schoonen *et al.* (1989), with a slight modification in the silylation procedure (1 hr at 100°C).

Capillary gas chromatography-mass spectrometry.

GCMS-conditions were similar to those used by Schoonen and Lambert (1987), Schoonen *et al.* (1989) and Van Dam *et al.* (1989). A Hewlett-Packard 5992 B gas chromatograph-mass spectrometer was used. The injection port temperature was 250°C. The oven temperature was set at 160°C, and made to increase 1 min after injection at a rate of 15°C/min to 190°C. Half a minute after this temperature had been reached, the temperature was made to increase further at 2°C/min to 235°C. For total ion monitoring with a scan reach of 200-600 m/z, the multiplier detector was set at 1800 V and for selected ion monitoring (SIM) at 2600 V. The mass spectrometer was optimized for the mid range area (m/z 414) and the obtained mass spectra were non-normalized.

Spectra comparison - similarity index.

Spectra from steroids of standard mixture and holding water fractions were compared by calculating the similarity index (SI) after both spectra had been reduced to 10 peaks, selected on the base of the highest mass times the abundance values. An $SI \geq 0.8$ was arbitrarily considered as indicative of a sufficient correlation (Schoonen and Lambert, 1987; Schoonen *et al.*, 1989).
The similarity index is defined as (Schoonen and Lambert, 1987; Schoonen *et al.*, 1989):

$$SI = \frac{\sum_{m=1}^{k} A_m . a_m}{\sqrt{\sum_{m=1}^{k} A_m^2 . \sum_{m=1}^{k} A_m^2}}$$

where SI = similarity index ($0 \leq SI \leq 1$)(0=totally different; 1=identical)
A_m = abundance of the ion at mass m in unknown spectrum
a_m = abundance of the ion at mass m in reference spectrum
k = total number of different ions.

RESULTS.

Response of pubertal fish to holding water from adult fish.

Body weight.

In both groups 1 and 2, mean body weight of recipient and control groups had increased significantly ($P<0.01$) from the initial situation, but differences between mean final body weight of recipient and control groups were never significant. In group 1 body weight increased from 208 g to an average of 270 g; in group 2, from 124 g to an average of 190 g.

Ovarian development.

Fig. 2 shows mean GSI values at each sampling date. Exposure to holding water significantly affected GSIs. All final GSI-values differed significantly from initial values, except in the final controls of group 1. Significant differences in GSI between controls and recipients groups were established for both groups. Exposure of vitellogenic females to holding water resulted in significant differences in GSI between controls and recipients ($P<0.01$ and $P<0.001$) after 68 days (group 1). The duplicate recipients had significantly different GSIs ($P<0.01$). Exposure of previtellogenic females to holding water for 42 days also yielded a significant difference ($p<0.01$) in GSI between recipients and controls (group 2). In group 2, the final proportion of fish having yolky oocytes was higher in recipients (50-55%) than in controls (0-10%). In the vitellogenic females of group 1, no change occurred in the course of the experiment in this respect.

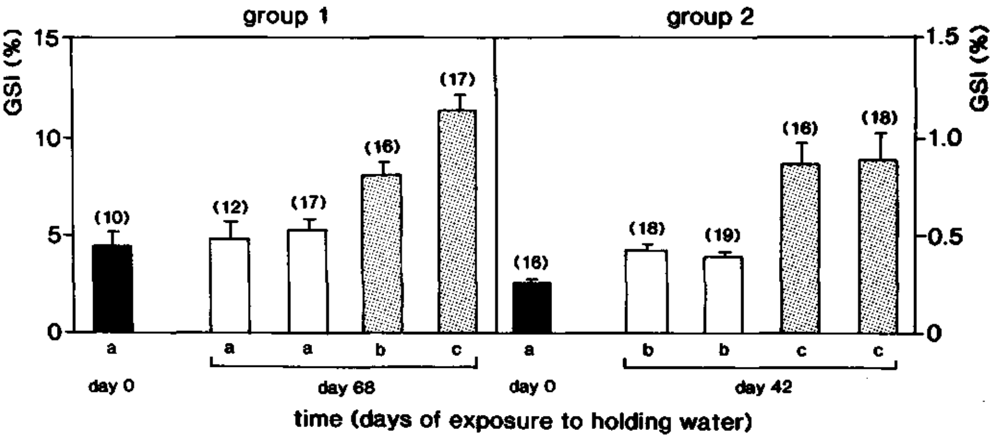


Figure 2.

Mean GSI of pubertal female *C. gariepinus* exposed to holding water from prespawning adult conspecifics. Values are means+SEM. In brackets, number of fish. Values with the same subscript do not differ significantly.

Group 1: a-b<0.01; a-c<0.001; b-c<0.01

Group 2: a-b<0.0001; a-c<0.0001; b-c<0.01

Initial controls: closed bars; controls: open bars; recipients: hatched bars.

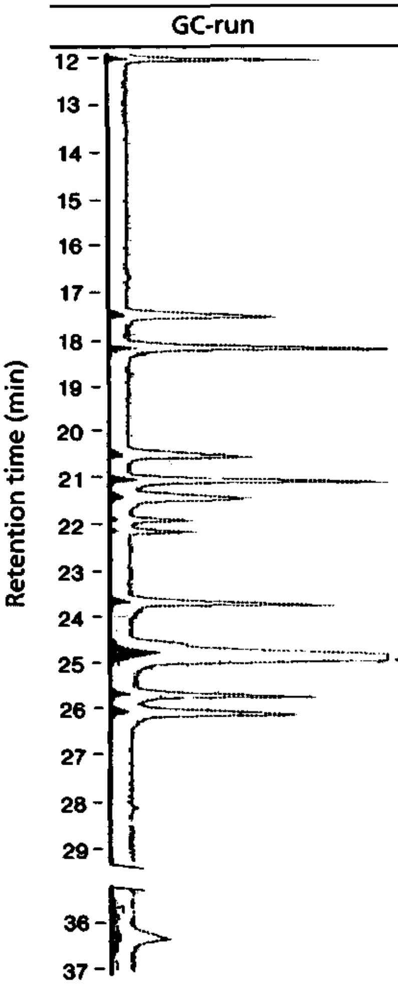
GCMS-analysis of holding water.

Separation of steroids by gas chromatography.

Fig. 3 shows results of a standard gas chromatographic run of the mixture of standard steroid derivatives. 5ß-androstane-3α,11ß-diol-17-one (oxime-triTMS), 5ß-pregnane-3α,17α,20ß-triol (triTMS) and testosterone (cis and trans) (oxime-diTMS) had nearly similar retention times, causing their peaks to coincide.

Identification by GC-MS.

In table 2, compounds which were demonstrated by SIM-analysis in the free steroid fraction or the steroid glucuronide fraction of the holding water are listed. The choice of mass fragments used in SIM-analysis is largely based on the work of



Min	Steroid derivative
11.9	5α-Androstane-3β-ol(TMS) - internal standard
17.5	5β-Androstane-3β, 17β-diol (diTMS)
18.0	5β-Androstane-3α, 17β-diol (diTMS)
20.5	Etiocholanolone (oxime-diTMS)
21.0	Estradiol-17β(diTMS)
21.4	5β-Androstane-3α, 17β-diol-11-one(diTMS)
21.9	5β-Dihydrotestosterone(cis)(oxime-diTMS)
22.1	5β-Dihydrotestosterone(trans)(oxime-diTMS)
23.6	Estrone(oxime- diTMS)
24.8	5β-Androstane-3α,11β-diol-17-one(oxime-triTMS)
24.8	5β-Pregnane-3α,17α,20β-triol(triTMS)
24.9	Testosterone(cis)(oxime-diTMS)
25.2	Testosterone(trans)(oxime-diTMS)
25.8	5β-Pregnane-3α,17α,20α-triol(triTMS)
26.0	5β-Pregnane-3α,17α-diol-20-one(oxime-triTMS)
36.4	Cholesterol(TMS)

Figure 3.

Capillary gas chromatogram of derivatives of internal standard and standard steroids (50-100 ng per injection).

Table 2.

Results of SIM-analysis of steroid derivatives present in standard mixture of steroids, and in free steroid and steroid glucuronide fractions of holding water from adult *C. gariepinus*. Molecular fragments (M^{+}) in bold script.

Steroid divative	RT (min)		abundance
		ion	255.3
5β-Pregnane-3α, 17α, 20α-triol	25.8	standard	100
(triTMS)		glucuronide	100
		ion	255.3
5β-Pregnane-3α, 17α, 20β-triol	24.8	standard	100
(triTMS)		glucuronide	100
		ion	246.1
5β-Pregnane-3α, 17α-diol-20-one	26.0	standard	49
(oxime-triTMS)		glucuronide	60
		ion	241.1
5β-Androstane-3α, 17β-diol	18.0	standard	65
(diTMS)		glucuronide	63
		ion	270.2
Etiocholanolone	20.5	standard	100
(oxime-diTMS)		free steroid	100
		glucuronide	100
		ion	306.2
5β-Androstane-3α, 17β-diol-11-one	21.4	standard	90
(diTMS)		free steroid	87
		glucuronide	84
		ion	232.1
Estradiol-17β	21.4	standard	12
(diTMS)		free steroid	15
		glucuronide	11
		ion	340.2
Estrone	23.6	standard	100
(oxime-diTMS)		free steroid	100
		glucuronide	100
		ion	329.3
Cholesterol	36.4	standard	100
(TMS)		free steroid	100
		glucuronide	100

RT = retention time, in minutes

(%) of selected mass fragments (ions)				
256.2	345.3	435.3	436.2	437.2
22	16	80	31	11
27	22	86	32	15
256.2	345.3	435.3	436.2	437.2
22	26	89	35	13
24	27	87	35	18
422.1	423.1	476.2	477.2	**565.3**
100	38	65	27	14
100	40	68	28	15
256.2	331.2	346.3	347.3	**436.1**
100	7	51	15	22
100	7	52	15	21
271.3	360.3	361.3	**449.3**	
29	76	36	16	
21	74	23	23	
20	76	23	16	
307.3	345.3	360.2	361.3	**450.2**
22	79	100	29	35
21	85	100	30	36
20	81	100	28	32
285.2	286.1	**416.2**	417.2	418.2
58	17	100	38	13
56	18	100	41	17
56	16	100	40	20
341.2	414.0	**429.1**	430.1	431.1
50	27	88	33	20
34	20	97	40	15
38	19	93	39	16
330.3	353.3	368.4	**458.2**	459.3
24	41	100	45	17
27	43	96	54	21
26	42	95	51	19

Schoonen and Lambert (1987) and Schoonen *et al.* (1989).
In some cases, SIM-identification was supported by a full spectrum (see below). Identification results are illustrated by two GCMS-printouts (5ß-Pregnane-3α,17α-diol-20-one and 5ß-Androstane-3α,17ß-diol-11-one; see figs 4 and 5).

- **5ß-Pregnane-3α,17α,20α-triol.**
  The retention time of standard 5ß-pregnane-3α,17α,20α-triol (triTMS) was 25.8 min. The mass spectrum was characterized by mass fragments with m/z 255.3, 256.2, 345.3, 435.3, 436.2 and 437.2, in an abundance ratio of 100:22:16:80:31:11. It was not possible to find a full spectrum of this compound in neither the free steroid fraction nor the steroid glucuronide fraction of the holding water. SIM analysis revealed the presence in the steroid glucuronide fraction of the above-mentioned mass fragments in an abundance ratio of 100:27:22:86:32:15 at the standard retention time.
- **5ß-Pregnane-3α,17α,20ß-triol.**
  The retention time of standard 5ß-pregnane-3α,17α,20ß-triol (triTMS) was 24.8 min. The mass spectrum was characterized by mass fragments with m/z 255.3, 256.2, 345.3, 435.3, 436.2 and 437.2, in an abundance ratio of 100:22:26:89:35:13. It was not possible to find a full spectrum of this compound in neither the free steroid fraction nor the steroid glucuronide fraction of the holding water. SIM analysis revealed the presence in the steroid glucuronide fraction of the above-mentioned mass fragments in an abundance ratio of 100:24:27:87:35:18 at the standard retention time.
- **5ß-Pregnane-3α,17α-diol-20-one.**
  The retention time of standard 5ß-pregnane-3α,17α-diol-20-one (oxime-triTMS) was 26.0 min. The mass spectrum was characterized by mass fragments with m/z 246.1, 422.1, 423.1, 476.2, 477.2 and 565.3 (molecular ion, M^+) (see also fig. 4A), in an abundance ratio of 49:100:38:65:27:14. It was not possible to find a full spectrum of this compound in neither the free steroid fraction nor the steroid glucuronide fraction of the holding water. SIM analysis did reveal the presence in the steroid glucuronide fraction of the above mentioned mass fragments (see also fig. 4B) in an abundance ratio of 60:100:40:68:28:15 at the standard retention time.
- **5ß-Androstane-3α,17ß-diol.**
  The retention time of standard 5ß-Androstane-3α,17ß-diol (diTMS) was 18.0 min. The mass spectrum was characterized by mass fragments with m/z 241.1, 256.2, 331.2, 346.3, 347.3 and 436.1 (M^+) in an abundance ratio of 65:100:7:51:15:22. SIM analysis revealed the presence of this compound in the steroid glucuronide fraction with the above-mentioned mass fragments present in an abundance ratio 63:100:7:52:15:21. Moreover, a full spectrum was obtained for the steroid glucuronide fraction, with an SI of 0.93.

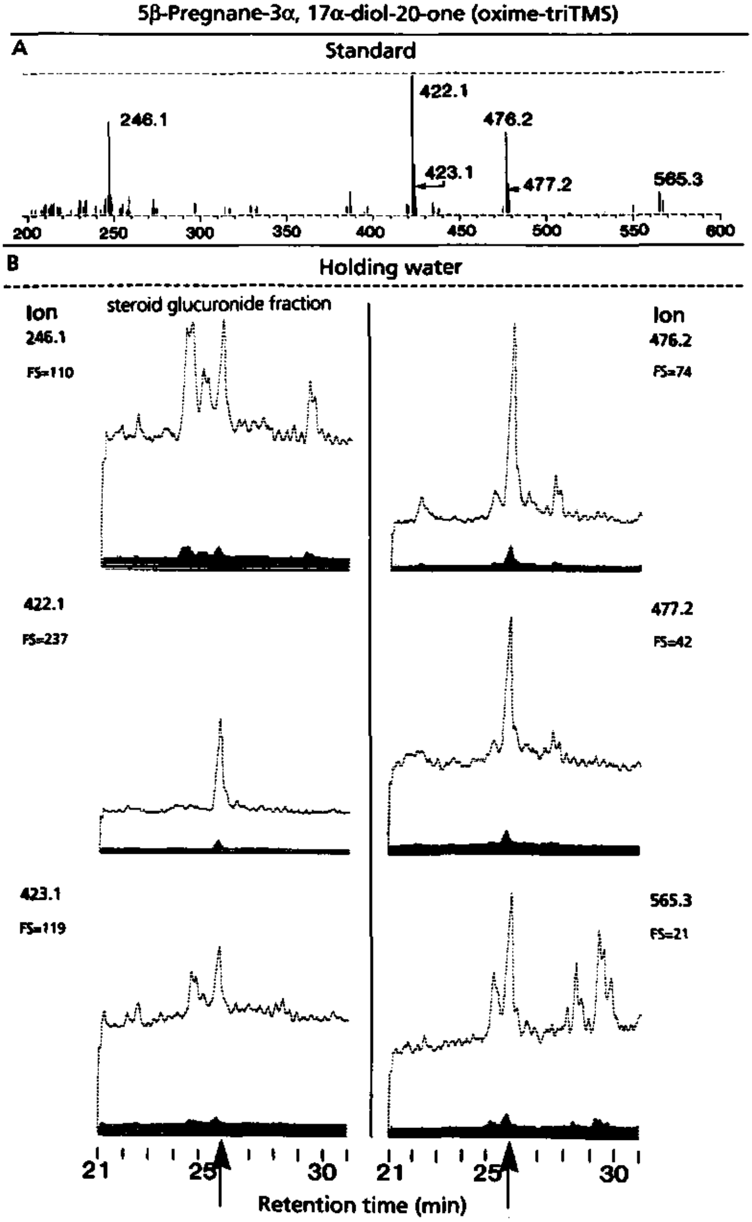


Figure 4.

A. Mass spectrum (non-normalized) of 5β-Pregnane-3α,17α-diol-20-one (oxime-triTMS) standard at a retention time of 26.0 min. The characteristic ions are the mass fragments m/z 246.1, 422.1, 423.1, 476.2, 477.2 and the molecular ion 565.3.

B. SIM analysis of the derivatized steroid glucuronide fraction of the holding water of *C. gariepinus* between 21 and 31 min of the GC run. The characteristic ions were present at a retention time of 26.0 min.

FS = full scale.

- **Etiocholanolone.**
  The retention time of standard etiocholanolone (oxime-diTMS) was 20.5 min. The mass spectrum was characterized by mass fragments with m/z 270.2, 271.3, 360.3, 361.3 and 449.3 (M^+) in an abundance ratio of 100:29:76:36:16. SIM analysis revealed the presence of this compound in both the free steroid fraction and the steroid glucuronide fraction, with abundance ratios of 100:21:74:23:23 and 100:20:76:23:16, respectively. In addition, a full spectrum was obtained from both fractions, with an SI of 0.93 in the free steroid fraction and of 0.97 in the steroid glucuronide fraction.
- **5ß-Androstane-3α,17ß-diol-11-one.**
  The retention of the diTMS derivative of standard 5ß-Androstane-3α,17ß-diol-11-one was 21.4 min. The mass spectrum was characterized by mass fragments with m/z 306.2, 307.3, 345.3, 360.2, 361.3 and 450.2 (M^+) (see also fig. 5A), in an abundance ratio of 90:22:79:100:29:35. A full spectrum with an SI of 0.95 was obtained for the free steroid fraction (fig. 5B). The compound was

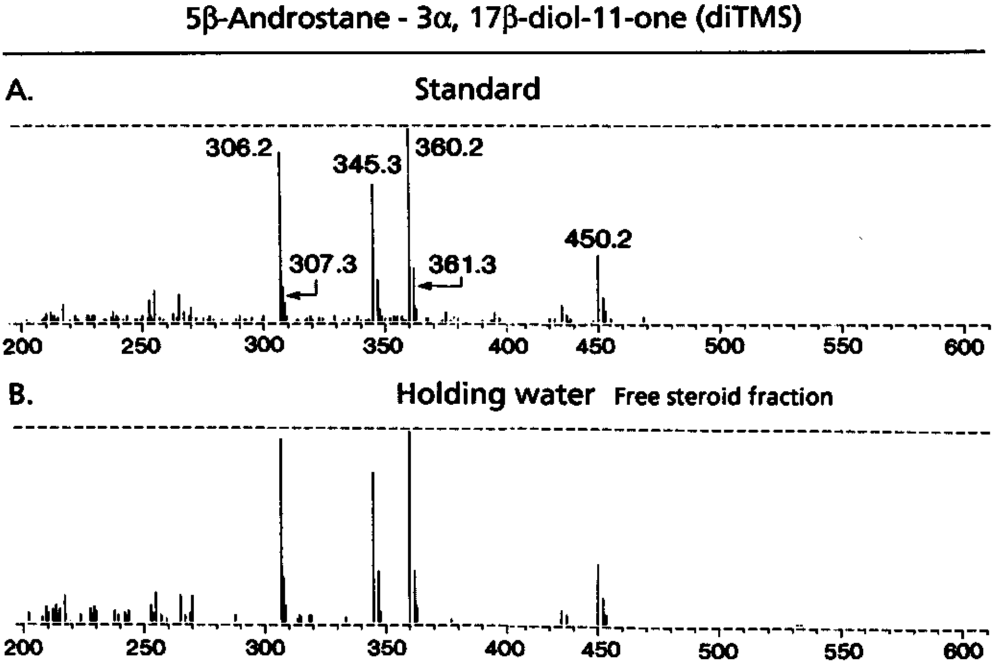


Figure 5.

A. Mass spectrum (non-normalized) of 5ß-Androstane-3α,17ß-diol-11-one (diTMS) standard at a retention time of 21.4 min. The characteristic ions are the mass fragments m/z 306.2, 307.3, 345.3, 360.2, 361.3 and the molecular ion 450.2.

B. Mass spectrum (non-normalized) of the derivatized free steroid fraction of the holding water of *C. gariepinus* at the expected retention time of 5ß-Androstane-3α,17ß-diol-11-one (diTMS).

demonstrated in the free steroid fraction and in the steroid glucuronide fraction by SIM analysis, with abundance ratios of the above mentioned mass fragments of 87:21:85:100:30:36 and 84:20:81:100:28:32, respectively.

- **Estradiol-17ß.**
  The retention time of standard estradiol-17ß (diTMS) was 21.4 min. The mass spectrum was characterized by mass fragments with m/z 232.1, 285.2, 286.1, 416.2 (M+), 417.2 and 418.2 with an abundance ratio of 12:58:17:100:38:13. It was not possible to find a full spectrum of this compound in neither the free steroid fraction nor the steroid glucuronide fraction of the holding water. SIM analysis revealed the presence of estradiol-17ß in both the free steroid and the steroid glucuronide fractions, with the above-mentioned mass fragments in abundance ratios of 15:56:18:100:41:17 and 11:56:16:100:40:20, respectively.

- **Estrone.**
  The oxime-diTMS derivative of standard estrone had a retention time of 23.6 min. The mass spectrum was characterized by mass fragments with m/z 340.2, 341.2, 414.0, 429.1 (M+), 430.1 and 431.1, with an abundance ratio 100:50:27:88:33:20. SIM analysis revealed the presence of this compound in both the free steroid fraction and the steroid glucuronide fraction with abundance ratios of 100:34:20:97:40:15 and 100:38:19:93:39:16, respectively. Full spectra were also obtained in both fractions, with an SI of 0.80 and 0.96, respectively.

- **Cholesterol.**
  The retention time of standard cholesterol (TMS) was 36.4 min. The mass spectrum was characterized by mass fragments with m/z 329.3, 330.3, 353.2, 368.4, 458.2 (M+) and 459.3, with an abundance ratio of 100:24:41:100:45:17. It was not possible to find a full spectrum of cholesterol in neither the free steroid fraction nor the steroid glucuronide fraction of the holding water. SIM analysis revealed the presence of the compound in both fractions, with the above-mentioned mass fragments in a ratio of 100:27:43:96:54:21 and 100:26:42:95:51:19, respectively.

The above-mentioned results are summarized in table 1. The androgens 5ß-Androstane-3ß,17ß-diol, 5ß-Dihydrotestosterone, 5ß-Androstane-3α,11ß-diol-17-one and testosterone could not be demonstrated in either fraction (free steroid or steroid glucuronide) of the holding water. They were thus absent or present in amounts below the detection threshold for GCMS-analysis (ranging from 100 pg to 3.98 ng/injection; Schoonen *et al.*, 1989), corresponding to a concentration of less than 10^{-11}-10^{-13} M in the holding water.
The steroids which were demonstrated can thus be assumed to have been originally present in concentrations above 10^{-11}-10^{-13} M.

DISCUSSION.

The present experiment consisted of two parts. The first part dealt with the effect of holding water from a mixed-sex population of adult *C. gariepinus* on ovarian development of pubertal females, to verify earlier findings (Ch. 2; Ch. 3). The effect on ovarian growth was determined in both vitellogenic and previtellogenic females. The second part of the experiment dealt with identification of steroids and steroid glucuronides in this holding water by means of gas chromatography-mass spectrometry.

Results indicate that holding water from adult *C. gariepinus* is effective in enhancing ovarian growth. Both vitellogenic and previtellogenic pubertal females, the former already having yolky oocytes (group 1), the latter about to undergo exogenous vitellogenesis (group 2), responded to holding water from adult prespawning male and females. Exposure at the beginning of or during exogenous vitellogenesis can thus induce an acceleration of ovarian growth. Ovarian growth in relation to pheromonal stimulation in adult female *C. gariepinus* during recrudescence depends on contributions of two mechanisms (Ch. 3). Vitellogenic oocytes reach a larger average size, but also the number of oocytes taking part in vitellogenesis increases. The fact that in the present experiment ovarian development can be boosted by exposure to holding water, not only in already vitellogenic females but also in previtellogenic females, also seems to suggest that both processes are involved.

To our knowledge, the present paper is the first report of chemical stimulation of puberty by adult conspecifics, in teleosts. The phenomenon has been described for several mammals, where adult males produce a primer pheromone, which accelerates puberty of females (reviews by Izard, 1983 and Vandenbergh, 1983, 1988).

In spite of strong indications for the existence of a pheromone of male origin (Ch. 2; Ch. 3), we continued to use holding water from mixed-sex groups of donor fish as we did in our earlier studies, because not only male stimulation of female development (Ch. 2) but also the reverse situation may exist, with possible implications for the male's pheromonal production capability. The possibility of such an interaction was also recognized in a comparable context by *e.g.* Chen and Martinich (1975) who suggested that male *B. rerio* might secrete less sex pheromone in the 'chemical absence' of females. As a consequence, a definite conclusion regarding the contribution of the two sexes to the effect of holding water in the present experiment awaits experimentation with holding water from monosex origin.

In the second part of the present study, a qualitative analysis was carried out of holding water for the presence of compounds which could be implicated in the observed stimulation of ovarian growth. As mentioned earlier, all teleost sex pheromones identified to date are sex hormones or their derivatives and prostaglandins, of which table 3 gives a summary. In all species investigated, their function is related to advanced phases of the reproductive cycle: oocyte maturation and ovulation, spermiation and attraction of spawning partners. Some of these pheromones have also been identified in holding water from *e.g. B. rerio* (Van Den Hurk *et al.*, 1987) and *Carassius auratus* (Sorensen *et al.*, 1988; Stacey *et al.*, 1989). Non-steroid teleost pheromones are involved in other processes. For instance, a proteinaceous aggression-suppressing pheromone exists in *Ictalurus nebulosus* (Carr and Carr, 1986) and the free amino acid fraction from skin extracts attracts immature *C. auratus* (Saglio and Blanc, 1989). All steroids and steroid glucuronides in holding water of the present experiment are also present and probably produced by the gonads of adult prespawning *C. gariepinus* (table 1). Nevertheless, other organs may have contributed. The interrenal, Stannius-corpuscles, the liver and the brain are examples of non-gonadal (peripheral) sources of sex-steroids in teleosts (Fostier *et al.*, 1983). The skin of male *C. gariepinus* has a strong capability for steroid glucuronide synthesis (Ali *et al.*, 1987) and also the teleost liver produces 5ß-reduced steroids and glucuronides as part of an inactivation mechanism (Kime, 1987). In particular cholesterol and its glucuronide, although synthesized in seminal vesicles of feral male *C. gariepinus*, is probably for the major part of hepatic origin, as was also assumed for this substance in holding water from male *B. rerio* (Van Den Hurk *et al.*, 1987).

As discussed above, the donor females may have intensified the donor males' steroid(glucuronide) production in a quantitative or qualitative sense. However, also the females themselves will have contributed to the steroid-spectrum found in the holding water. Indeed, the estrogens estrone and estradiol-17ß and their glucuronides in holding water (table 1) can be assumed to be of female origin, as is the case for the glucuronides of the progestagens 5ß-pregnane-3α,17α,20α-triol and of 5ß-pregnane-3α,17α,20ß-triol. Of presumed male origin are 5ß-pregnane-3α,17α-diol-20-one-glucuronide, etiocholanolone and its glucuronide, 5ß-androstane-3α,17ß-diol-11-one and its glucuronide, and 5ß-androstane-3α,17ß-diol-glucuronide. The glucuronides of four androgens produced by adult males (table 1), could not be detected in holding water. These were glucuronides of 5ß-androstane-3ß,17ß-diol, 5ß-dihydrotestosterone, 5ß-androstane-3α,11ß-diol-17-one and testosterone.

Resink *et al.* (1989c) investigated the sensitivity of the olfactory epithelium of female *C. gariepinus* to steroid glucuronides from the male's seminal vesicle. The most potent odorant proved to be 5ß-pregnane-3α,17α-diol-20-one-3α-glucuronide with a detection threshold of 10^{-11} M, as measured by electro-olfactogram. Other steroids glucuronidated at the 3α position were less potent (5ß-androstane-3α,11ß-diol-17-one, 5ß-androstane-3α,17ß-diol and etiocholanolone, with detection thresholds of 10^{-9}-10^{-7}M), as were steroids glucuronidated at the 17ß position (5ß-dihydrotestosterone, testosterone, 5ß-androstane-3α,17ß-diol; 10^{-7}M) and at the 3ß position (5ß-androstane-3ß-ol-17-

Table 3.

Identified reproductive pheromones in the gonads of teleosts, their source and function.

Species	Compound(source)	Function	Author
Brachydanio rerio	steroid glucuronides (testes)	induction of ovulation	Van Den Hurk *et al.*, 1987.
Brachydanio rerio	steroid glucuronide mixture containing estradiol-17ß (ovary)	attraction of male	Van Den Hurk and Lambert, 1983; Lambert *et al.*, 1986.
Carassius auratus	17α,20ß-dihydroxy-4-pregnen-3-one (ovary)	raising milt volume in male	Stacey and Sorensen, 1987; Stacey *et al.*, 1989.
		induction of ovulation in female	Sorensen and Stacey, 1987.
Carassius auratus	prostaglandins (ovary)	attraction of male	Sorensen *et al.*, 1988.
Clarias gariepinus	steroid glucuronides (seminal vesicle)	attraction of ovulated female	Lambert *et al.*, 1986. Resink *et al.*, 1987,1989a,c
Gobius jozo	etiocholanolone glucuronide (mesorchial gland)	attraction of gravid female	Colombo *et al.*, 1980.
Poecilia reticulata	free and glucuronidated estradiol-17ß	attraction of male	Johansen, 1985.

one, 5ß-androstane-3ß,17ß-diol; 10^{-6}M). Some of the steroid glucuronides mentioned above were found in holding water from the present experiment (table 1). It is worth noting at this point that a provisional quantification -by comparison of SIM ion abundancies of known amounts of the standards with those of holding water fractions- indicate that only the estimated concentration of 5ß-pregnane-3α,17α-diol-20-one-glucuronide in holding water approached that of its detection threshold as determined by Resink *et al.* (1989c). Other glucuronides found in the present experiment, of which olfactory detection thresholds have been determined (Resink *et al.*, 1989c), were below this threshold.

Holding water from adult *C. gariepinus* has pheromonal properties in stimulating the early phases of ovarian development (this experiment). Since all sex pheromones are steroidal in nature, except prostaglandins (Sorensen and Stacey, 1989; Stacey, 1989), it may be hypothesized that the steroid glucuronides in holding water of the present experiment are responsible for the observed stimulation of ovarian growth.

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Chapter 7.

PLASMA ANDROGEN LEVELS IN CASTRATED ADULT MALE AFRICAN CATFISH, *CLARIAS GARIEPINUS*, IN RELATION TO PHEROMONAL STIMULATION OF OVARIAN GROWTH IN PUBERTAL CONSPECIFICS.

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(submitted in condensed form as short communication to
Aquaculture)

SUMMARY.

An attempt was made to establish the origin of male olfactory stimuli which influence ovarian development of female *Clarias gariepinus*. Pubertal females (recipients) were exposed for 62 days to holding water from intact, completely castrated, seminal vesicles-extirpated or testes-extirpated adult males or from intact adult females (donors). At the end of the experiment, the average GSI (gonado somatic index) and SVSI (seminal vesicles somatic index) was significantly lower in operated male donor groups, but only a few fish completely lacked the extirpated organs. Male donor plasma levels of 6 androgens were determined by RIA. Levels of all androgens were lowest in testes-extirpated and completely castrated males. Significant correlations were found between GSI, but not SVSI, and androgen levels, suggesting that the testes are the main androgen source. However, even complete absence of the testes (GSI 0%) did not eliminate circulating androgens. Male holding water was effective in enhancing ovarian development in recipient females, irrespective of the male donor group. Holding water from female donors was ineffective, comparable to controls (fresh water). Thus, the effect of holding water is linked to the male gender, and the persistence of the effect after extirpation of male gonadal parts may be explained by circulating androgens.

INTRODUCTION.

Ovarian growth in female *Clarias gariepinus* is enhanced by tactile and chemical cues from males. The chemical stimuli are perceived by olfaction. Holding water from a mixed-sex population is effective, whereas that from a female population is not. The effect of holding water is mediated through the hypothalamus-pituitary-gonad axis and we concluded that sex-pheromones of male origin affect ovarian development through stimulation of the process of vitellogenesis (Ch. 2; Ch. 3; Ch. 4; Ch. 5; Ch. 6). Thus, an involvement of the male gonad is likely and this is supported by the observations that 'adult male' (with mature gonads) holding water has a stronger enhancing effect than 'pubertal male' holding water (Ch. 8). Moreover, stimulation of vitellogenesis in pubertal females becomes apparent in unison with the appearance of the seminal vesicles in pubertal males (Ch. 4).
In *C. gariepinus*, the male gonad consists of the testes and of the seminal vesicles, with differing capacities for steroid synthesis (Schoonen *et al.*, 1987a,b). The seminal vesicles in particular produce steroid glucuronides (Schoonen and Lambert, 1987, Schoonen *et al.*, 1987a, 1988), but also the testes (Resink *et al.*, 1987b). Steroid glucuronides act as releaser pheromones, not only in *C. gariepinus* (Resink *et al.*, 1987a, 1989a,b) but also in other teleosts (for review, see *e.g.* Stacey, 1989). An alternative source of steroid glucuronides in teleosts in general is the liver (Kime, 1978, 1987), and Ali *et al.* (1987) demonstrated that skin tissue of *C. gariepinus* is also able to synthesize steroid glucuronides. However, since epithelial

cells lack the key-enzymes for steroidogenesis, glucuronidation in the skin depends on circulating steroids as precursors (Ali *et al.*, 1987). In holding water from adult males and females, which stimulated ovarian development of prepubertal and pubertal *C. gariepinus*, we recently demonstrated steroid glucuronides (Ch. 6). However, their role as primer pheromones affecting vitellogenesis awaits confirmation.

The present study concerns the possible role as primer pheromones of steroid glucuronides in stimulation of ovarian development of *C. gariepinus*. After having shown pheromonal stimulation through holding water (Ch. 2; Ch. 3) and the presence of steroid glucuronides in 'vitellogenesis-stimulating' holding water (Ch. 6, see above), we sought to confirm in the present study the involvement of the male gonadal system in secretion of ovarian-growth-stimulating substances. Through extirpation of testes, seminal vesicles, or both, we attempted to elucidate the role of these tissues in emission of compounds to the holding water.

The effects were assessed by measurement of the gonadosomatic index (GSI) of pubertal female *C. gariepinus* in response to exposure to holding water. The completeness of the operation was checked by inspection of tissue remnants. Furthermore, plasma levels of the androgens 11-keto-testosterone (OT), testosterone (T), androstenedione (A), 11ß-hydroxy-testosterone (OHT), 11ß-hydroxy-androstenedione (OHA) and 11-keto-androstenedione (OA) were quantified (by RIA) in donor males. These steroids present a rather complete androgen profile in teleost fish (Mayer *et al.*, 1990a).

MATERIALS AND METHODS.

General.

The effect of holding water from adult *C. gariepinus* (donor fish) on ovarian growth of pubertal females (recipient fish) was tested by exposing the latter to holding water for a 62-day period. Recipient and donor fish were purchased from a commercial hatchery (Aquafish, Venhorst, The Netherlands). Upon arrival in our hatchery the fish were acclimated for 1 month in a flow-through basin.

Adult donor fish.

The body weight of these fish ranged from 450 to 760 g, having an age of approximately one year. Female gonadosomatic index (GSI) was *ca.* 5 %, male GSI *ca.* 1.5% and male seminal vesicle somatic index (SVSI) *ca.* 0.5%. Indices are calculated as: 100*(weight of organ/(bodyweight-weight of organ)). Three weeks before the start of the experiment, fish were sexed.

Forty males were randomly assigned to 4 different groups of 10 males each. One donor group was not operated (M), whereas the other three groups were destined for operation. Operation was performed under anaesthesia with 0.1 g/l TMS (Tricainemethanesulfonate; Sandoz) and resulted in 3 donor groups of completely castrated (M-t-sv), seminal vesicles-extirpated (M-sv) and testes-extirpated (M-t) males. Furthermore, ten females were assigned to a female donor group (F). The fish were allowed to recover for 3 weeks.

Pubertal recipient fish.

The weight of these females ranged from 215 to 450 g, having an age of 7 months. Their average GSI was 1.0%.

Experimental design.

From male and female donor groups, 6-8 specimens were randomly selected and stocked per group in 70-l aquaria. Holding water from each donor group was supplied to duplicate groups of pubertal recipient females (n=15 per replicate). Pubertal recipient females receiving fresh water served as controls (C).
The experiment was carried out in a two-layer aquarium system (Ch. 2). Donor and recipient fish received water of a temperature of 25±1 °C at a flow rate of 2-3 l/min. Fish were fed Trouvit trout pellets (Trouw, Putten, The Netherlands) at the optimum ration for *C. gariepinus* of 8.4 g/kg$^{0.8}$/day (Hogendoorn *et al.*, 1983).

Sampling of fish and parameters used.

Adult donor fish.

At the end of the experiment, donor fish were sacrificed to assess gonadal development and check for remnants of gonadal and seminal vesicle tissue in operated males (M-t-sv, M-sv and M-t groups).
Of males, blood samples (1-3 ml) were collected before sacrifice and mixed with 20-60 µl of Na-citrate (4%) and centrifuged. Plasma was stored at -20 °C until further use. Plasma was analyzed for OT, T, A, OHT, OHA and OA, by radioimmunoassay (RIA). The assay is decribed in Schulz (1985).
The following modifications were made. Plasma aliquots of 1 ml were mixed with an equal volume of RIA-buffer, heated for 1 hr at 80 °C, and centrifuged to precipitate denatured plasma proteins. Samples were then extracted three times with ether, and the combined ether phase was evaporated. The dry residue was reconstituted in 1 ml RIA-buffer. Extraction efficiency was checked by adding recovery tracer to a selected number of samples. Of each sample, volumes of 100 and 20 µl were measured, to test for parallelism. Sensitivity of the assay was 7.8 pg/tube.

Pubertal recipient fish.

An initial sample of 12 females was sacrificed at the beginning of the experiment. At the end of the experiment, all recipient groups were sacrificed.
At sacrifice, fish were killed by an overdose of TMS and individual body weight and gonad weight were taken. Gonad indices (GSI) were calculated as mentioned above.

Statistical analysis.

Donor fish.

The body weight, ovary weight, testes weight, seminal vesicles weight, GSI (males and females), SVSI (males) and plasma levels of androgens (males) were compared between groups by the non-parametric Mann-Whitney test. The correlation between GSI and SVSI, respectively, and plasma levels of androgens was calculated, by linear regression analysis. Similarly, in males without testes (GSI 0%), the correlation between plasma levels of the six androgens were calculated by linear regression analysis.

Recipient fish.

The body weight, ovary weight and GSI were also compared by Mann-Whitney test. Results of duplicate treatments were combined if not statistically different. P-values ≤0.05 are considered significant.
All statistical analysis were performed with the BMDP statistical software (Dixon *et al.*, 1988).

RESULTS.

General.

During the 62-days experimental period, mortality was absent in donor groups. In recipient groups, mortality was low, with maxima of 3.3 %.
In the RIA for OHT and OHA, a slight non-parallelism was found between 100 and 20 µl samples. For calculations, the average value was used, in view of the comparative nature of the investigations.

Adult donor fish.

Final average body weight of adult female donor fish (F) was 870 g. Their GSI had not increased from the initial value of *ca.* 5% (final average GSI was 4.3%). Final average body weight of adult males did not differ significantly between groups, and ranged from 725 to 871 g.

Fig. 1 shows final average GSI and SVSI of male donor fish. In intact males (M),

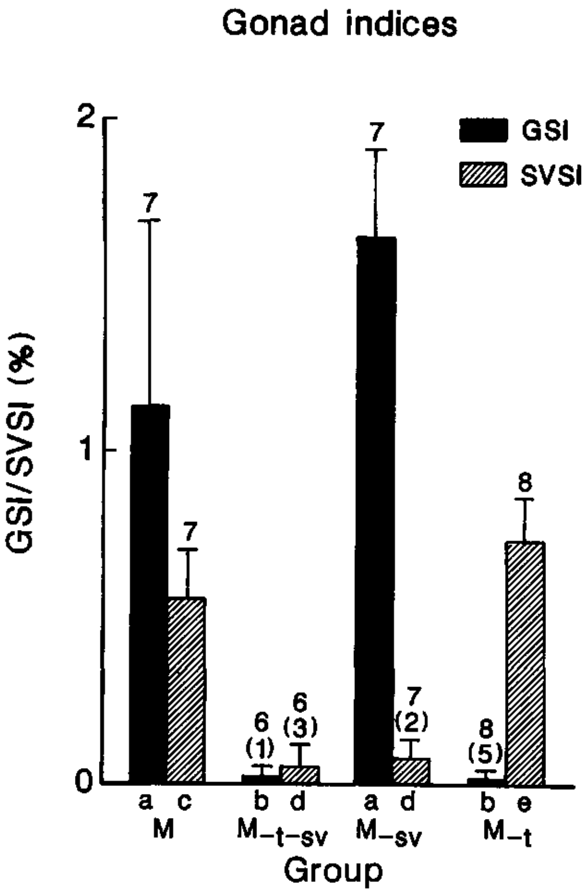

Fig. 1. Final average GSI ($\bar{X}$+SD) and SVSI ($\bar{X}$+SD) of male donor *C. gariepinus*.

M = intact males
M-t-sv = completely castrated males
M-sv = seminal vesicles-extirpated males
M-t = testes-extirpated males.

Indices a-b indicate significance of differences in GSI; indices c-e indicate significance of differences in SVSI. Same indices indicate absence of significant difference.

Number above bar indicates number of fish; number in brackets indicate number of fish without testes and/or seminal vesicles (GSI/SVSI 0%).

GSI and SVSI had hardly changed from the initial value of 1.5% and 0.5%, respectively. Final values were 1.3% (GSI) and 0.6% (SVSI). Average GSI and/or SVSI were significantly lower in operated groups, but only a few males completely lacked the extirpated organs, eighty-three days after the operation (3 weeks post-operative recuperation and 62 days experimental period). Their number is indicated in fig 1. In testes-extirpated males, the final SVSI is significantly higher than in intact males (0.77 vs 0.56%).

Fig. 2 shows plasma levels of androgens in male donor fish. In intact males highest levels are found of OT and T, and lowest levels of A, OHT, OHA and OA.

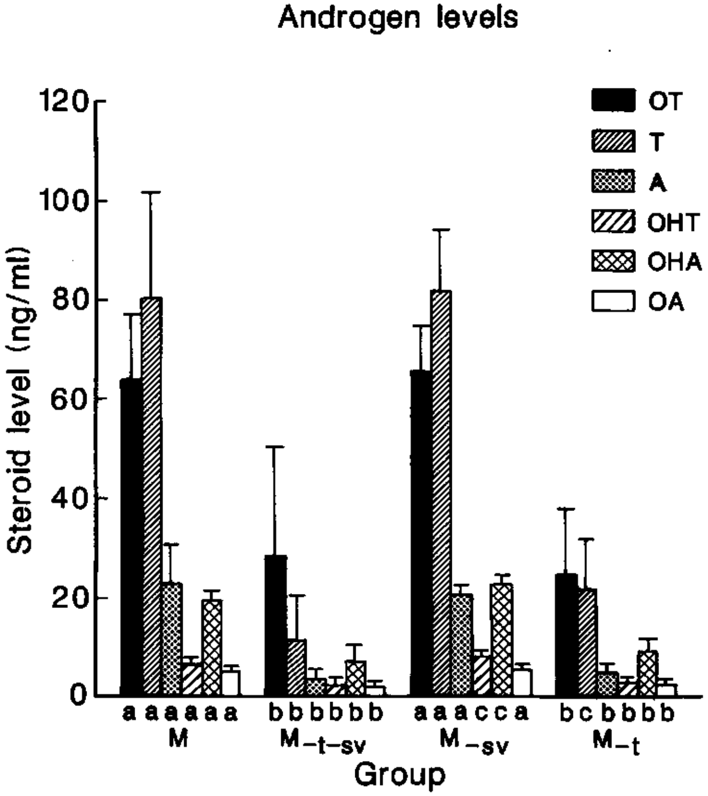

Fig. 2. Plasma levels of androgens ($\bar{X}$+SD) of male donor *C. gariepinus*.

M = intact males

M-t-sv = completely castrated males

M-sv = seminal vesicles-extirpated males

M-t = testes-extirpated males.

Statistical analysis was conducted separately for each steroid, between groups. Same indices indicate absence of significant difference.

Androgen levels are significantly reduced in males without testes, both in M-t-sv and M-t groups. In these males, levels of T and OT are most reduced (15-25% of the levels of intact males) and those of A, OHT, OHA and OA are least reduced (30-45% of the levels of intact males). Androgen levels were not significantly reduced when only the seminal vesicles had been removed. In these males, all levels are about 100% of the levels of intact males.

Highly significant (P<0.005) correlations exist between GSI and androgen levels (fig 3), when all data are combined regardless of donor group. The strongest

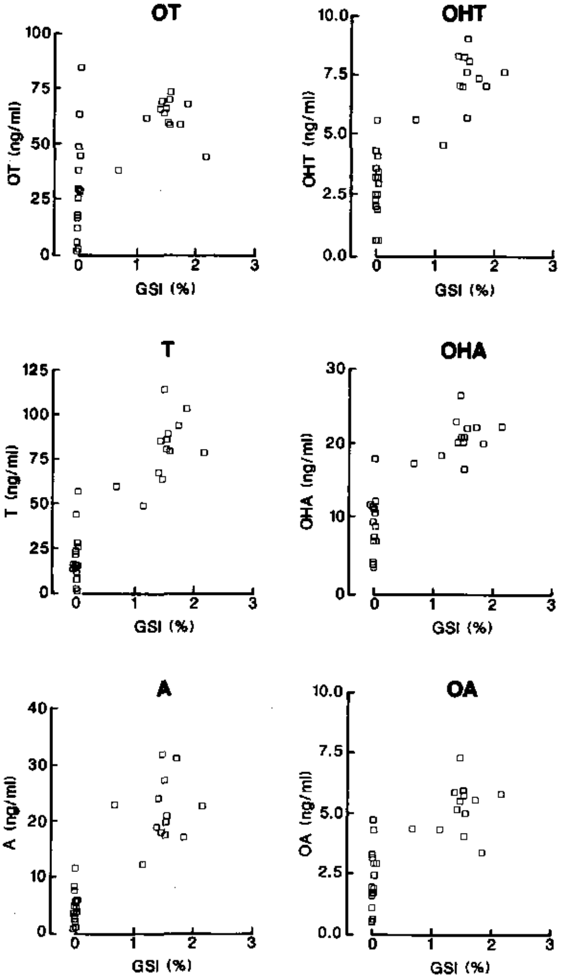

Fig. 3. Correlation between GSI and plasma levels of OT, T, A, OHT, OHA and OA, of male donor *C. gariepinus*.

Correlation coefficients (R^2, based upon 28 observations; all are significant with P<0.005):

OT 0.43; T 0.82; A 0.76; OHT 0.77; OHA 0.76; OA 0.60.

correlation is found for T (R^2=0.82) and the weakest for OT (R^2=0.43). Even when the GSI is 0 or nearly so, androgens are detectable, with the largest variation in the case of OT. No significant correlations were found between SVSI and androgen levels.
Significant ($P<0.05$) correlations were found in donor males without testes (GSI 0%), between the plasma levels of the 6 androgens measured ($R^2 \geq 0.9$).

Pubertal recipient females.

Final average body weight of duplicate groups of recipient females did not differ significantly. Body weight had increased significantly from the initial value of 336 g to values ranging from 420 g to 641 g.
Final average GSI values did not differ between duplicate groups, and therefore, pooled results are presented in fig. 4. All final GSI values were significantly higher than the initial value of 1.0%.

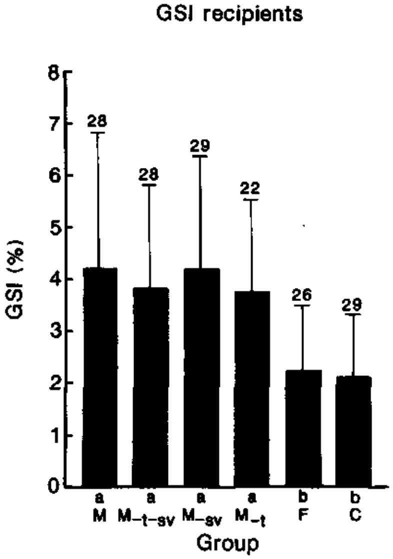

Fig. 4. Final average GSI ($\bar{X}$+SD) of pubertal female recipient *C. gariepinus*.

M = holding water from intact males
M-t-sv = holding water from completely castrated males
M-sv = holding water from seminal vesicles extirpated males
M-t = holding water from testes extirpated males
F = holding water from intact females
C = control (fresh water)
Same indices indicate absence of significant difference.
Numbers above bars indicate number of fish

Lowest final GSIs were found in control females (C) (2.1%) and in females having received holding water from female donor fish (F) (2.3%). These values did not differ significantly.

Highest final GSIs, significantly different from control females, were found in females having received holding water from male donor fish. Among these, the highest GSI response (4.2%) was found in recipients of holding water from intact males (M) and from seminal vesicles-extirpated males (M-sv) (4.3%). The response in recipients of holding water from completely castrated males (M-t-sv) was slightly lower (4.0%), whereas the response in recipients of holding water from testes-extirpated males (M-t) was lowest (3.8%). Differences between final mean GSI values of recipient groups exposed to holding water from the various male donor groups, however, were not significant.

DISCUSSION.

Demonstration of the presence of an ovarian growth-stimulating factor in holding water from adult *C. gariepinus* males is in agreement with earlier observations (Ch. 3; Ch. 5). This implies a role for the male gonad in emission of the factor, unless other organs or tissues synthesizing specific male compounds exist. In earlier studies with *C. gariepinus*, we speculated about a role for steroid glucuronides, which can be produced in, notably, the seminal vesicles (Schoonen and Lambert, 1987; Schoonen *et al.*, 1988) but also in the testes (Resink *et al.*, 1987b) or in the skin from gonadal precursors (Ali *et al.*, 1987). These compounds exert pheromonal action, albeit during later stages of the reproductive cycle, not only in *C. gariepinus* (Resink *et al.*, 1987a; 1989a,b), but in a variety of other teleosts as well (review by Stacey, 1989). They have recently been identified in ovarian growth-stimulating holding water from *C. gariepinus* (Ch. 6). The possibility exists that the compounds of male origin responsible for enhanced vitellogenesis of pubertal female *C. gariepinus* are non-steroidal, in spite of an involvement of the male gonad in their emission. Non-steroid teleost pheromones have been identified but they have not been brought in connection with reproductive processes (Colombo *et al.*, 1982; Stacey, 1989). For example, a proteinaceous pheromone of *Ictalurus nebulosus* suppresses aggression (Carr and Carr, 1986) and the free amino acid fraction from skin extracts attracts immature *Carassius auratus* (Saglio and Blanc, 1989).

Extirpation of the testes stimulated growth of the seminal vesicles, as follows from the significantly higher SVSI in testes-extirpated males as compared to intact males. This effect has been noted earlier by Resink *et al.* (1987), and was attributed to a removal of the normally occurring negative feed-back of aromatizable testicular androgens on secretion of pituitary GTH, responsible for seminal vesicle growth. Seminal vesicle extirpation on the other hand, did not cause a significant weight

gain of the testes, when compared to the situation in intact males. In experiments conducted by Resink *et al.* (1987) with *C. gariepinus*, an enlarged seminal vesicle after extirpation of testes caused an increased attraction response of ovulated females. This indicates a testes-independent role of the seminal vesicles as the site of synthesis of releaser pheromones. In the present experiment, however, enlarged seminal vesicles in testes-extirpated donor males do not alter the effect of holding water. Possibly, the testes are indispensible in providing steroids for consecutive conversion to primer ("vitellogenic") pheromones through glucuronidation in the seminal vesicles (and indeed the response of recipient females to holding water from testes-extirpated or completely castrated males was slightly, but not significantly, lower than to holding water from intact or seminal vesicles-extirpated males, *cf.* fig. 4). Another possibility is that testicular steroid glucuronides, in spite of the fact that they are much less readily synthesized than those from the seminal vesicles (Schoonen, 1987), do play a role in stimulation of ovarian development. The latter option was suggested by Resink *et al.* (1987b), who observed testicular synthesis of steroid glucuronides and polar steroids during the period of gonadal recrudescence in feral *C. gariepinus*.
The levels of androgens found in intact male donor *C. gariepinus* of the present study are much higher than those reported earlier. De Leeuw *et al.* (1986) found in sham-operated males levels of T of 8.2-11.3 ng/ml and of A of 2.6-4.4 ng/ml. Likewise, Peute *et al.* (1989) found levels of 8 (OT), 3 (T) and *ca.* 1.5 (OHT, OHA, OA) ng/ml in sham-operated males. The age of the experimental animals or the holding conditions may be responsible for this variation. For example, Schoonen *et al.* (1988) found much higher levels of steroid glucuronides in seminal vesicle fluid from feral males than from captive males, allthough both were in non-spawning condition.

As mentioned above, castration in *C. gariepinus* did not result in elimination of androgens from the circulation. The level of androgens is strongly correlated to GSI but not SVSI, providing evidence for the testes being the major androgen source, in *C. gariepinus*. However, also in males without testicular tissue (GSI 0%), androgens were still detectable (*cf.* fig. 3). and this result corresponds with findings of Peute *et al.* (1989), who were able to detect OT, T, OHT, OHA and OA in plasma of castrated male *C. gariepinus*, 6 weeks after the operation. Mayer *et al.* (1990a) found still detectable plasma levels of the androgens OT, T, OHT, OHA, OA and of 17α-hydroxy-20ß-dihydroprogesterone after castration of male *Salmo salar* parr, and of the same androgens in *Gasterosteus aculeatus* (Mayer *et al.*, 1990b). Also in *Oncorhynchus mykiss*, castration reduced but did not eliminate plasma androgen levels (*e.g.* Schulz and Blüm, 1987), and extragonadal sources of biologically active androgens have been proposed for maintenance of an organism's steroid balance (the liver, Kime, 1978; the red blood cells, Schulz, 1986, Schulz and Blüm, in press; the interrenal, Schreck *et al.*, 1989). However, a contribution of an extragonadal source of androgens, playing a role in the present experiment with *C. gariepinus*, is unlikely as mentioned earlier, in view of the observation that female holding water lacks the stimulating compound. We therefore wish to maintain the hypothesis that, in *C. gariepinus* male gonadal pheromones, or pheromones synthesized in non-gonadal tissue from gonadal precursors, be responsible for the observed effects. Even low levels of circulating androgens would then be efficiently processed into active compounds (in the case of steroid glucuronides, in the liver, Kime, 1978, 1987; in the skin, Ali *et al.*, 1987; in the seminal vesicles, Schoonen and Lambert, 1987, Schoonen *et al.*, 1987a).

Holding water from male donor *C. gariepinus* in the present gonad extirpation experiment was effective, regardless the 'gonadal status' of the males. Johns and Liley (1970) observed persistent androgen-dependent nest-building and sexual behavior in some castrated *Trichogaster trichopterus*, which was attributed to either an unidentified fragment of regenerated testicular tissue or an extragonadal source of androgen. Incomplete extirpation or regeneration of the gonad in the present experiment may have contributed significantly to the continued production of pheromonal substances from circulating steroidal precursors (see above) and may be largely responsible for the seeming indepence of holding water effect and 'gonadal status' of the donor. This is the more plausible in view of the low detection threshold (10^{-11}M) of pheromonal steroid glucuronides in female *C. gariepinus* (Resink *et al.*, 1989a).
It is recommended that, in future, castrated male *C. gariepinus* be screened for low circulating androgen levels, before use as holding water donors. The strong correlation between plasma levels of the various androgens in males without testes indicate that measurement of all six androgens may not be necessary.

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Chapter 8.

PHEROMONES AND OVARIAN GROWTH IN AFRICAN CATFISH *CLARIAS GARIEPINUS*.

A discussion of results.

ANALYSIS OF THE NATURE OF SEX STIMULI.

In juvenile *Clarias gariepinus*, male donor fish stimulate the ovarian development of female recipients (Ch. 2, 5). Chemical and tactile stimuli are relevant to the process, whereas other cues investigated are not (visual, auditory, electric) (Ch. 2). Visual and auditory cues have been shown to influence interspawning intervals, possibly as a consequence of enhanced ovarian development, in cichlids (*Pterophyllum scalare*, Chien, 1973; *Oreochromis mossambicus*, Marshall, 1972, Silverman, 1987a,b). A function of electroreception in reproduction has not been described for teleosts, although *C. gariepinus* also possesses electroreceptive sense organs (Teunis, 1990). Tactile cues may actually encompass a combination of signals of different types, such as gustatory or mechanical. Catfishes possess taste buds, notably on the barbels (Reutter, 1982), but gustation is generally considered to be of minor importance in teleost reproduction (Pfeiffer, 1982). Moreover, it would only be useful when fish are in close contact. Mechanical contact may be important in teleosts, but it is confined to the prelude to spawning. For example, spawning male *Carassius auratus*, by 'butting' a female's genital region, cause release of urine from the bladder. The urine contains pheromones (as in the plaice *Pleuronectes platessa*; Canario and Scott, 1989) signalling the female's reproductive 'state' to the male (Stacey *et al.*, 1987). However, in our experiments we did not observe any specific form of physical contact between the sexes.

Moreover, in adult female recipient *C. gariepinus*, ovarian development after induced maturation, ovulation and stripping of eggs (recrudescence) is influenced by stimuli from male donors (Ch. 3). Chemical cues from donor males, conveyed via the medium of transport (holding water) to the recipient females, are perceived by the sense of smell, since anosmic females do not respond. Metabolites which are known to negatively influence ovarian growth (Billard *et al.*, 1981; Gerking, 1982; Sumpter *et al.*, 1987) and which are inevitably present in holding water, did not affect the female response in *C. gariepinus* (Ch. 3, 5). Hence, the chemical cues are indeed pheromones, and their male origin is strongly suggested by the fact that 'female holding water' has no effect.

Male tactile stimuli do not play a role in ovarian development of adult *C. gariepinus* (Ch. 3, 4). The situation with respect to the importance of tactile stimuli from male donors may be different in pubertal and adult *C. gariepinus*. These cues were consistently found to be of importance in pubertal *C. gariepinus* (Ch. 2, 4, 5), but unimportant in adults (Ch. 3, 4). The importance of this finding is not understood.

Pheromones are involved in teleost reproduction, but to date, observations are confined to advanced reproductive phases (Colombo *et al.*, 1982; Lam, 1983; Liley and Stacey, 1983; Lambert *et al.*, 1986; Stacey *et al.*, 1986, 1987; Stacey, 1989), also in *C. gariepinus* (Resink, 1988). To our knowledge, the present thesis provides the first evidence of pheromonal stimulation of vitellogenesis in teleosts.

MONITORING OF STIMULATION OF OVARIAN DEVELOPMENT.

Ovarian development is under the control of the endocrine system, through the hypothalamic-pituitary-gonad axis. In pubertal female *C. gariepinus*, ovarian growth is parallelled by changes in plasma levels of estradiol-17ß (E_2) and vitellogenin (VTG), and is reflected in increases in diameter and relative proportion of vitellogenic oocytes within the ovary. The effects were less clear in adult females during recrudescence, presumably as a consequence of their short gonadal cycle (Ch. 4). Stimuli from male donor fish cause changes in levels of E_2, VTG and oocyte development, and temporal shifts in the pattern of vitellogenesis of recipients. These are most pronounced in pubertal *C. gariepinus* (Ch. 4), and are comparable to results obtained by other workers who used day-length manipulations to alter the reproductive cycle in *Oncorhynchus mykiss* (e.g. Bromage *et al.*, 1982; Duston and Bromage, 1986, 1987; Bromage and Cumaranatunga, 1988; Cyr *et al.*, 1988) and the sea bass *Dicentrarchus labrax* (Zanyu *et al.*, 1989). It seems likely that male donor stimuli trigger these changes in plasma levels and temporal shifts in pattern through the olfactory-brain relay. This pathway was demonstrated in adult *C. gariepinus* by Resink (1988).

The effect of stimulation of ovarian development is seen in an increase in both the diameter and the relative proportion of exogenous vitellogenic oocytes. These phenomena have also been reported for *e.g. O. mykiss* (Bourlier and Billard, 1984), the Indian catfish *Heteropneustes fossilis* (Sundararaj and Anand, 1972; Sundararaj *et al.*, 1972) and the goby *Gobius niger* (LeMenn and Burzawa-Gérard, 1985). In *C. gariepinus*, a comparable situation exists (Ch. 3, 4), in that ovarian growth is caused by enhanced vitellogenesis, leading to larger oocytes, and by increased recruitment of oocytes into the stage of vitellogenesis.

In *C. gariepinus*, monitoring the effect of stimulation by donors on the process of vitellogenesis in recipients does not lead to earlier detection of actual stimulation than with the use of gonadosomatic index (GSI) or oocyte development (Ch. 4). However, it may make sacrifice of fish unnecessary.

INTERACTION BETWEEN SEXES IN EMISSION OF CUES.

In pubertal *C. gariepinus*, the interaction between donors with respect to the emission of stimuli enhancing gonadal development was investigated (Ch. 5). The interpretation of results from this study was seriously hampered by the fact that anosmia in males depressed testis and seminal vesicle development, making it impossible for us to study some of the potential interactive pathways. Not only were the tactile and olfactory stimuli from male donor fish on ovarian growth, demonstrated earlier (Ch. 2), confirmed, but an effect of male olfactory stimuli on gonadal development of male recipients was also observed. Tactile stimuli from female donors do not influence, or perhaps even tend to curb gonadal development of male recipients, suggesting an inhibitory female cue (Ch. 5). The emission of 'female-directed' olfactory stimuli by male donors is not modified by females, but the emission of 'male-directed' olfactory stimuli is, suggesting two types of male olfactory cues, differentially influenced (Ch. 5). Thus, in pubertal *C. gariepinus* there is bisexual stimulation by a monosex cue, in that both male and female gonadal development is stimulated by male olfactory cues. A finding comparable with the latter result was reported by Stacey and Hourston (1982), who observed induction of spawning behavior in both sexes of the Pacific herring *Clupea harengus pallasi* by a pheromone contained in milt (Stacey and Hourston, 1982). Silverman (1987b) describes the reverse situation, in which stimulation of testicular development of male *Sarotherodon mossambicus* (monosexual stimulation) is caused by conspecific cues from both sexes (bisexual cues). In addition, in pubertal *C. gariepinus*, females inhibit male gonadal development and the emission of 'male-directed' male olfactory stimuli (Ch. 5), *i.e.* there is monosexual inhibition (in males) by a monosex cue (from females). General social inhibition of maturation (gonadal development) was described for *e.g.* platyfish *Xiphophorus spp.* (Sohn, 1977; Borowsky, 1978), the guppy *Poecilia reticulata* (Dahlgren, 1979) and the cichlid *Haplochromis burtoni* (Fraley and Fernald, 1982), but gender-related inhibition of gonadal development has not been described for teleosts, to our knowledge.

THE FUNCTIONAL RELATIONSHIP BETWEEN TESTES AND SEMINAL VESICLES, WITH RESPECT TO EMISSION OF PHEROMONES.

The synthesis or presence of steroid glucuronides has been demonstrated in gonads of a variety of teleosts, of both sexes, including *C. gariepinus* (Schoonen, 1987). Adult sexually mature male *C. gariepinus* secrete steroid glucuronides, which function as releaser pheromones in attracting ovulated female conspecifics (Resink, 1988). In this phase of sexual development, the testes are barely able to synthesize steroid glucuronides, unlike the seminal vesicles (Schoonen, 1987). The skin of male *C. gariepinus* is also able to synthesize steroid glucuronides (Ali *et al.*, 1987). Since testes-extirpated males with enlarged seminal vesicles are more attractive to ovulated females than intact males (Resink, 1988), the seminal vesicles function independently from the testes in synthesizing the releaser pheromone. Adult feral male *C. gariepinus* are able to synthesize polar steroids and steroid glucuronides in the testes during the period of gonadal recrudescence, and it has been suggested that these, as primer pheromones, are involved in stimulation of vitellogenesis (Resink, 1988). In pubertal recipient *C. gariepinus*, ovarian development (GSI, E_2) becomes noticeable in conjunction with the appearance of the seminal vesicles in pubertal male donors (Ch. 4). There is a possibility that the seminal vesicles synthesize steroid glucuronides with this primer pheromone function from testicular steroid precursors, as has also been suggested for the skin (Ali *et al.*, 1987).

THE MALE GONAD AS POSSIBLE CENTER FOR EMISSION OF PHEROMONES.

Several of the experiments described in this thesis suggest that the male gonad is involved, directly or indirectly, in the ovarian growth-stimulating effect of donor holding water, since holding water from males, but not from females, is effective. Moreover, unpublished results indicate that holding water from adult males has a stronger enhancing effect on ovarian growth than holding water from pubertal males. In the experiment described in Ch. 7, holding water was effective in stimulating ovarian development of pubertal recipient females over control (and female donor holding water) levels when donor males were intact, testes-extirpated, seminal vesicles extirpated, or completely castrated. However, extirpation did not result in complete absence of gonads or seminal vesicles in every individual male within operated donor groups, although the average gonadosomatic index (GSI) and seminal vesicles somatic index (SVSI) was greatly reduced. Androgen levels were measured in plasma of donor males of this study (Ch. 7) to assess success of the castration, and because of their role as substrate for glucuronidation, in the liver (Kime, 1978, 1987), skin (Ali *et al.*, 1987), seminal vesicles (Schoonen, 1987) or testes (Resink, 1988). Strong correlations were found between GSI and androgen levels, but not between SVSI and androgen levels, providing evidence for the testes being the major androgen source. Surprisingly, even an apparent total absence of testes did not always eliminate circulating androgens, possibly because of an extra-gonadal source (Kime, 1978; Schulz, 1986; Schreck *et al.*, 1989). However, because female holding water does not affect ovarian growth in pubertal (Ch. 5) or adult female *C. gariepinus* (Ch. 3), an extra-gonadal source without a gonadal relay does not seem likely. We tentatively conclude that the still circulating androgens may have been effectively converted to pheromonal steroid glucuronides in levels within the holding water above the recipients' detection threshold, which is easily surpassed. In the case of *C. gariepinus*, detection thresholds for pheromonal steroid glucuronides may be as low as 10^{-11}M (Resink, 1988).

PRESENCE OF STEROID GLUCURONIDES IN HOLDING WATER.

Non-steroid pheromones exist in teleosts, but these are not sex pheromones (Colombo *et al.*, 1982). Sex pheromones are invariably of a steroidal nature, with the exception of prostaglandins (Stacey, 1989). This was the reason for us to strongly suspect that the sex pheromones, the existence of which we demonstrated earlier (Ch. 2, 3, 4), are of a steroidal nature. Analysis of the medium of transport (holding water from a mixed-sex group of adult male and female *C. gariepinus*) by gas chromatography and mass spectrometry revealed the presence of several steroids and steroid glucuronides, known to act as pheromones in later stages of the reproductive cycle in *C. gariepinus* and other teleosts (Ch. 6). In view of the above-mentioned interaction between sexes in emission of cues (Ch. 5), it was desirable to analyze holding water from interacting adult male and female *C. gariepinus*. The presence in 'active' holding water of pheromonal steroid glucuronides was also demonstrated for *Brachydanio rerio* (Van den Hurk *et al.*, 1987) and *C. auratus* (Van Der Kraak *et al.*, 1989).

PERCEPTION OF STEROID GLUCURONIDES FROM HOLDING WATER.

A provisional calculation indicated that the concentration in holding water (Ch. 6) of the glucuronide of one steroid in particular (5ß-pregnane-3α,17α-diol-20-one) approached the detection threshold of the olfactory epithelium found by Resink (1988). This compound is part of the pheromone mixture from the seminal vesicles with releaser effects in *C. gariepinus*. It has been suggested that this compound also has a 'vitellogenic' function (Resink, 1988)(see above). However, in the case of *C. gariepinus*, the actual involvement of steroid glucuronides present in 'active' holding water in stimulation of ovarian development awaits experimental confirmation (see below).

ECOLOGICAL IMPLICATIONS OF PHEROMONAL COMMUNICATION.

It is not difficult to envisage the ecological significance of the mechanism of ovarian development stimulation which *C. gariepinus* display in captivity, in both the pubertal and the adult stage. Field studies (Thomas, 1966; Holl, 1968; Clay, 1979) indicate that juvenile *C. gariepinus* live in shallows of lakes or the like, and retreat to deeper waters when advanced stages of gonadal development have been reached. Thus relatively high densities may coincide with the stage of active vitellogenesis, under natural conditions. In such a situation, an ecological function of pheromonal (and perhaps tactile) stimulation of ovarian development in pubertal *C. gariepinus* is plausible. Spawning in *C. gariepinus* is triggered by the onset of the monsoon rains, and adult fish leave their 'summer retreats' to spawn in flood-plains of tribituaries (Rinne and Wanjala, 1983; Bruton, 1987). In the Hula reserve in Israel, spawning is observed throughout the breeding period, but spent females with obvious oocyte atresia cannot be found before August (Van Den Hurk *et al.*, 1984-1985), indicating multiple spawning (Viveen *et al.*, 1985). Multiple spawning entails rapid recrudescence, a capacity which adult female *C. gariepinus* exhibit in captivity (Hogendoorn and Vismans, 1980). Since *C. gariepinus* generally assemble in dense populations during the breeding season, it would be possible for vitellogenic pheromones to exert their action especially during this period.

The ecological significance of the interactive pathways discussed above (Ch. 5) is not understood. The male stimulation of male development, and the inhibitory action of females on male gonadal development and the emission of 'male-directed' male olfactory cues, may constitute artifacts caused by captivity. As an example, we mention findings by Schoonen (1987), who found distinct differences in testicular and seminal vesicle steroid metabolism between feral and pond-raised adult *C. gariepinus*. Alternatively, the inhibitory pathways present in captivity may be of an as yet unrecognized significance under natural circumstances.

Stacey (1989) and Sorensen and Stacey (1989) distinguish several consecutive phases in the evolution of pheromonal communication. In the most advanced phase, both donor and recipient benefit from a pheromone-based interaction: 'the signalling phase'. In an earlier phase, however, only an adaptive response on the part of the recipient to the adventitious release of chemical substances by the donor is evident. In this so-called 'spying phase', there is no clear benefit for the donor. Releaser pheromones influencing spawning behavior or serving to attract prospective mates (as in *C. gariepinus*; Resink, 1988) fall within the 'signalling' category. Male primer pheromones influencing ovarian development in *C. gariepinus* (this thesis) belong to the 'spying' category, since there is no direct benefit for the individual. The long-term effect, however, will be advantageous for the whole population, resulting in a synchronous attainment of maturity.

FINAL CONSIDERATIONS.

Recently, much research has been devoted to pheromonal communication in teleosts (*e.g.* Schoonen, 1987; Van Oordt, 1987; Resink, 1988; Canario and Scott, 1989; Stacey, 1989). Applications are found at the fundamental level and the practical level. For research, Stacey (1989) mentions the use of the primer pheromone 17α,20ß-dihydroxy-4-pregnen-3-one in *C. auratus* to study the mechanism of release of gonadotropic hormone (GTH). Application of the pheromone predictably triggers release of GTH, without the need for invasive treatment, *e.g.* with hormones. For application in aquaculture, pheromones offer the prospect of manageable stimulation of gametogenesis (this thesis) or ovulation in order to obtain gametes (Resink, 1987; Van Oordt, 1987; Stacey, 1989).

Major challenges for research at the fundamental and applied level have also been posed by experience with the intensive culture of eel (*Anguilla anguilla*) in the Netherlands, where olfactory interactions seem to play a decisive role in controlling somatic and gonadal growth, and possibly in sex differentiation.

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SUMMARY

Pheromones are defined as 'substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction'. In teleost fish, pheromones play a role in a variety of social interactions. Sex pheromones are involved in reproduction of several teleosts species, including the African catfish *Clarias gariepinus*. They regulate spawning behavior and endocrine events leading to reproduction. Most, if not all reports in literature describing the action of sex pheromones in teleosts pertain to advanced phases of the reproductive cycle. In those phases, sex pheromones influence oocyte maturation and ovulation in females, and spermiation in males. Furthermore, they play a role in mediating attraction of prospective mates and in triggering spawning behavior and release of gametes.

To date, all teleost sex pheromones identified are steroids and steroid glucuronides, derivatives of reproductive hormones, with the exception of prostaglandins. In *C. gariepinus* the presence of males enhances ovarian growth in pubertal females under hatchery conditions and in wild-caught adult females kept in ponds. This phenomenon led to the assumption that sex pheromones are also involved in the early phases of the reproductive cycle of *C. gariepinus*, *i.e.* ovarian development and possibly also of the testes and seminal vesicles. Experiments described in the present thesis were carried out to validate this hypothesis, the rationale being that findings might be applied to other fish species which do not or rarely reach the stage of ovarian growth in captivity.

In juvenile *C. gariepinus*, males stimulate ovarian development of females. Chemical and tactile stimuli are relevant to the process, whereas the other cues investigated are not (visual, auditory, electric). Also in adult female *C. gariepinus* which had entered the stage of recrudescence, *i.e.* ovarian development after induced maturation, ovulation and stripping of eggs, is influenced by stimuli from males. Chemical cues in holding water are perceived by the sense of smell, since females deprived of their sense of smell (anosmia) do not respond. Metabolites which are known to negatively influence ovarian growth and which are inevitably present in holding water, do not affect the female response.

Hence, the chemical cues are indeed pheromones, and their male origin is strongly suggested by the fact that female holding water has no effect. Male tactile stimuli do not play a role in ovarian development of adult *C. gariepinus*. The importance of tactile stimuli, which may actually encompass a combination of signals of different types (gustatory, mechanical) may be different in pubertal and adult *C. gariepinus*.

In pubertal female *C. gariepinus*, ovarian growth is parallelled by changes in plasma levels of estradiol-17ß (the female sex steroid) and vitellogenin (the female-specific precursor of yolk proteins) and is reflected in increases in diameter and

relative proportion of vitellogenic oocytes within the ovary. The effects were less clear in adult females during recrudescence, presumably as a consequence of their short gonadal cycle. Male stimuli cause changes in levels of estradiol-17ß, vitellogenin, and ovarian development, and temporal shifts in the pattern of vitellogenesis. These are most pronounced in pubertal *C. gariepinus*. It seems likely, that male stimuli trigger these changes through the olfactory-brain-pituitary-gonad axis. The effect of stimulation of ovarian development is seen in an increase in both the diameter and the relative proportion of exogenous vitellogenic oocytes, indicating both an enhanced vitellogenesis and an increased recruitment of oocytes into the stage of vitellogenesis. Monitoring the effect of stimulation on the basis of the parameters mentioned above does not lead to earlier detection of actual stimulation than with the use of gonadosomatic index or oocyte development. However, it may make sacrifice of fish unnecessary.

An experiment aiming at investigating the interaction between the sexes with respect to emission of stimuli was hampered by the fact that anosmia in males depressed development of testes and seminal vesicles. Nevertheless, the experiment indicated that not only male tactile and olfactory cues stimulate ovarian development, but that also male olfactory cues stimulate gonadal development of other males. Tactile stimuli from females do not influence, or perhaps even tend to curb gonadal development of males, suggesting the existence of a female inhibitory cue. The emission of 'female-directed' olfactory cues by males is not modified by females, but the emission of 'male-directed' olfactory cues is, suggesting two types of male olfactory cues, differentially influenced.

Several of the experiments suggest that the male gonad is involved, directly or indirectly, in the ovarian growth stimulating effect of holding water, since male, but not female holding water is effective. Extirpation of testes, seminal vesicles or both, however, did not abolish the effect of holding water. Still-circulating androgens in operated fish may have been effectively converted to pheromonal steroid glucuronides, leading to holding water concentrations above the detection threshold of females.

Holding water was analyzed by gas chromatography and mass spectrometry and was found to contain several of the steroids and steroid glucuronides known to act as pheromones in later stages of the reproductive cycle of *C. gariepinus*. This holding water originated from a mixed-sex group of adult male and female *C. gariepinus*. A provisional calculation learned that the concentration in holding water of the glucuronide of one steroid in particular, 5ß-pregnane-3α,17α-diol-20-one, of presumed male origin, approached the detection threshold of the olfactory epithelium.

It is concluded that in C. *gariepinus*, pheromones of male origin stimulate ovarian development, through the olfactory-brain-pituitary-gonad axis. Emission of pheromones and gonadal development are modulated by interaction between the sexes. The question whether male pheromones are of gonadal origin could not be answered conclusively. Holding water with the capacity to stimulate ovarian development contains steroids and steroid glucuronides. The actual involvement in stimulation of ovarian development of these compounds, however, awaits experimental confirmation.

SAMENVATTING.

Feromonen zijn stoffen die, wanneer ze worden uitgescheiden door een individu en worden ontvangen door een ander individu van dezelfde soort, in dat andere individu een specifieke reactie oproepen. Bij teleosten spelen feromonen een belangrijke rol in tal van sociale interacties. Geslachtsferomonen spelen een rol bij de voortplanting van een aantal vissoorten, waaronder de afrikaanse meerval (*Clarias gariepinus*). Deze stoffen hebben een regulerende werking op het paaigedrag en induceren endocriene processen die leiden tot voortplanting. Wanneer in de literatuur de werking van geslachtsferomonen wordt beschreven, dan gaat het in de meeste, zoniet alle, gevallen om gevorderde fasen in de voortplantingscyclus. Zo beïnvloeden geslachtsferomonen bij wijfjes de rijping en ovulatie van oöcyten en bij mannetjes de spermiatie. Ze spelen bovendien een rol bij het aantrekken van mogelijke partners, bij het op gang brengen van paaigedrag en bij de afgifte van gameten. Met uitzondering van prostaglandinen blijken alle geslachtsferomonen die tot op heden zijn geïdentificeerd bij teleosten, steroïden en steroïdglucuroniden te zijn, afgeleid van geslachtshormonen. Bij vrouwelijke *C. gariepinus* wordt door de aanwezigheid van mannetjes de ovariumgroei bevorderd, zowel bij puberale vrouwtjes onder broedhuisomstandigheden als bij adulte 'wildvang' vrouwtjes die in vijvers worden gehouden. Dit verschijnsel gaf aanleiding tot de aanname dat geslachtsferomonen eveneens betrokken zijn bij de vroege fasen van de voortplantingscyclus, dwz. ontwikkeling van het ovarium bij vrouwtjes en mogelijk ook de ontwikkeling van testis en zaadblaas bij mannetjes. De experimenten beschreven in dit proefschrift werden uitgevoerd om deze hypothese te toetsen, met als achtergrond de gedachte dat de resultaten misschien toegepast zouden kunnen worden bij andere vissoorten die in gevangenschap niet of nauwelijks het stadium van ovariële groei bereiken.

De ovariumontwikkeling van juveniele *C. gariepinus* wordt gestimuleerd door mannetjes. Hierbij zijn chemische en tactiele prikkels van belang, terwijl de andere onderzochte prikkels (visuele, auditieve, electrische) irrelevant bleken. Ook de ovariumontwikkeling van adulte vrouwtjes tijdens de zogenaamde recrudescentie, dwz. na geïnduceerde maturatie en ovulatie en afstrijken van eieren, wordt beïnvloed door mannelijke prikkels. De chemische prikkels in verblijfswater worden door het reukvermogen waargenomen, aangezien vrouwtjes waarbij het reukvermogen was uitgeschakeld (anosmia), niet reageerden. De respons van vrouwtjes wordt niet beïnvloed door de metabolieten in het verblijfswater. Van deze stoffen is bekend dat ze de ovariumgroei negatief kunnen beïnvloeden, en ze zijn onvermijdelijk aanwezig in verblijfswater. De chemische prikkels zijn derhalve inderdaad feromonen. Dat ze hun oorsprong vinden in mannetjes wordt sterk gesuggereerd door het feit dat verblijfswater afkomstig van vrouwtjes géén effect heeft. Tactiele prikkels van mannetjes spelen geen rol bij de ovariumontwikkeling van adulte *C. gariepinus*. Met betrekking tot het belang van tactiele prikkels, die uit verschillende signalen (smaak, mechanisch) opgebouwd gedacht kunnen worden, zou er een verschil kunnen bestaan tussen puberale en adulte *C. gariepinus*.

De ovariumgroei van puberale vrouwelijke *C. gariepinus* loopt parallel met veranderingen in de concentratie oestradiol-17ß (het vrouwelijke geslachtssteroïd) en vitellogenine (de specifiek vrouwelijke voorloper van dooiereiwit) in het bloedplasma en wordt weerspiegeld in de toename van de diameter en de relatieve proportie van oocyten binnen het ovarium. Mannelijke stimuli veroorzaken veranderingen in de concentratie oestradiol-17ß en vitellogenine in het bloedplasma en in de mate van ovariumontwikkeling. Bovendien treden er verschuivingen van het vitellogenese patroon op. Deze effecten zijn het duidelijkst bij puberale *C. gariepinus*. Het lijkt waarschijnlijk dat de veranderingen op gang worden gebracht door mannelijke prikkels die via de reuk-hersen-hypofyse-gonade as hun uitwerking hebben op het ovarium. Stimulatie resulteert in een toename van de gemiddelde diameter en van het relatieve aantal oöcyten dat overgaat tot exogene vitellogenese. Het vervolgen van de stimulatie aan de hand van het gehalte in het bloed aan oestradiol-17ß en vitellogenine resulteert niet in een vroegere detectie van het effect dan op basis van de gonadosomatische index of de ontwikkeling van oöcyten verwacht kan worden. Wél zou de 'vroege' detectiemethode het opofferen van vissen overbodig kunnen maken.

Een experiment, met als doel het onderzoeken van de interactie tussen de geslachten met betrekking tot de afgifte van feromonale prikkels, werd belemmerd door het feit dat anosmia bij mannetjes de ontwikkeling van testes en zaadblaas onderdrukt. Desalniettemin bevestigde het experiment de eerdere waarneming dat de ovariumontwikkeling van vrouwtjes wordt gestimuleerd door mannelijke tactiele en olfactorische prikkels. Andersom bleek dat de gonadale ontwikkeling van mannetjes niet wordt beïnvloed door vrouwelijke prikkels, maar veeleer wordt belemmerd (door met name tactiele prikkels afkomstig van vrouwtjes). Dit suggereert een remmende prikkel, afkomstig van vrouwtjes. De gonadale ontwikkeling van mannetjes wordt wél gestimuleerd door olfactorische prikkels afkomstig van andere mannetjes. De afgifte van mannelijke prikkels, gericht op vrouwtjes, wordt niet gemoduleerd door interactie met vrouwtjes, terwijl de afgifte van mannelijke prikkels, gericht op mannetjes, dat wél wordt. Dit geeft aan dat er twee soorten mannelijke olfactorische prikkels bestaan, die op verschillende wijze worden beïnvloed.

Verscheidene experimenten tonen aan dat de mannelijke gonade direct of indirect is betrokken bij het stimuleren van de ovariumgroei, aangezien alleen verblijfswater afkomstig van mannetjes effect heeft. Het effect werd echter niet opgeheven door extirpatie van testes, zaadblaas, of beide. Mogelijk werden de - ondanks het geheel of nagenoeg afwezig zijn van de gonaden - nog steeds in het plasma meetbare androgenen effectief omgezet in feromonale steroïdglucuroniden, zodat in het verblijfswater concentraties boven de vrouwelijke detectiedrempel werden bereikt.

Uit analyse met behulp van gas chromatografie en massa spectrometrie blijkt dat verblijfswater afkomstig van een gemengde groep adulte mannelijke en vrouwelijke *C. gariepinus* verschillende steroïden en steroïdglucuroniden bevat, waarvan bekend is dat ze als feromoon fungeren in latere fasen van de voortplantingscyclus. Uit een voorlopige berekening blijkt dat de concentratie in het verblijfswater van het glucuronide van één steroïd in het bijzonder - 5ß-pregnaan-3α,17α-diol-20-on - de waarnemingsdrempel van het reukepitheel benadert.

Geconcludeerd kan worden dat de ovariumontwikkeling van *C. gariepinus* wordt gestimuleerd door feromonen van mannelijke oorsprong, via de reuk-hersen-hypofyse-gonade as. Afgifte van feromonen en ontwikkeling van de gonade worden gemoduleerd door interactie tussen de sexen. Hoewel niet eenduidig aangetoond, zijn mannelijke feromonen waarschijnlijk van gonadale oorsprong. De feitelijke betrokkenheid van de in verblijfswater aangetroffen steroïdglucuroniden bij stimulatie van de ovariumontwikkeling bij *C. gariepinus* moet nog experimenteel worden bevestigd.

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CURRICULUM VITAE.

Jan Hendrik van Weerd werd op 29 december 1955 geboren te Arnhem. In 1973 behaalde hij het Gymnasium-ß diploma aan de Chr. Scholengemeenschap 'Melanchton' te Rotterdam. In datzelfde jaar werd begonnen met de studie Biologie aan de Landbouwuniversiteit te Wageningen. In januari 1981 behaalde hij 'cum laude' het doctoraal diploma. Hoofdvakken waren Visteelt en Fysiologie der Dieren. Een bijvak Vispathologie werd aan de Rijksuniversiteit van Utrecht gevolgd. In oktober 1981 trad hij in dienst van het raadgevend ingenieursbureau Euroconsult BV te Arnhem en hield zich gedurende een vijftal jaar als aquaculturist in binnen- en buitenland bezig met studies en projecten betreffende aquacultuur en ontwikkelingssamenwerking. Van november 1986 tot november 1990 was hij in dienst van de Stichting Technische Wetenschappen (STW) van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), en deed bij de Vakgroep Visteelt en Visserij van de Landbouwuniversiteit te Wageningen het in dit proefschrift beschreven onderzoek. Begin 1991 hoopt hij te vertrekken naar Malang, Indonesië, in het kader van een door de Vakgroep Visteelt en Visserij uitgevoerd DGIS/NUFFIC universitair samenwerkingsproject.