Prepositions (Stellingen)

Belonging to the thesis
"Semen collection and preservation in African catfish, Clarias gariepinus"
Ana Viveiros
Wageningen, January 7th, 2002

1. In African catfish, the inhibition of the development of seminal vesicles facilitates stripping of viable sperm cells.
   This thesis

2. Cryopreservation of semen is a valuable tool for genetic conservation of fish.
   This thesis

3. The endocrine control of reproduction in fish is very comparable with that in mammalian species.

4. "It is not the strongest nor the most intelligent of the species that survive, but the one most responsive to change".
   Charles Darwin

5. Catfish individuals like to live crowded.

6. The educational curriculum of Veterinarian Sciences should include fish.

7. The creativity of the Dutch can be appreciated by the strange things they put between their bread.

8. Living in the Netherlands for 4 years, proves that the Brazilian proverb "the sun shines for everybody" is not true.

Semen collection and preservation in African catfish, *Clarias gariepinus*

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. ir. L. Speelman
in het openbaar te verdedigen
op Maandag 07 Januari 2002
des namiddags te vier uur in de Aula

Viveiros, A.T.M. 2002. Semen collection and preservation in African catfish, Clarias gariepinus.

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ISBN: 90-5808-561-9

Dedicated to Ricardo,
Giuliano,
Amanda,
Felipe

Viveiros, A.T.M. 2002. Semen collection and preservation in African catfish, Clarias gariepinus. PhD thesis, Wageningen University.

Abstract

Stock improvement using quantitative and molecular genetics is an essential part of nowadays production of farm animals and fish. To achieve this in aquaculture, germplasm of both parental sexes should be obtained in a life-saving manner. In captivity, male African catfish, Clarias gariepinus, do not release semen under abdominal massage and have to be sacrificed to obtain sperm from the macerated testes. Of course, this is regarded as a major constrains by the catfish farming sector. Against this background, the research of the present thesis had a two-pronged approach and aimed (a) to induce semen release and facilitate stripping of semen under abdominal massage, and (b) to optimize protocols for cryopreserving semen of the African catfish. To facilitate hand-stripping of semen, several maturational hormones that increase plasma gonadotropin levels and drugs that stimulate contractions of the reproductive tract, such as oxytocin, were tested. The response to some of these treatments was compared between normal males and males that possessed undeveloped seminal vesicles – a possible block of the sperm flow during abdominal massage. Based on the results, it is unlikely that catfish males kept in captivity are not strippable because of a lack of gonadotropin surge. Fertile semen was hand-stripped from males that possessed undeveloped seminal vesicles but not from normal males, suggesting that seminal vesicles actually block the sperm flow during hand-stripping. However, stripping was possible only after treatment with pituitary extract. Oxytocin may play a role in sperm transport in catfish, but more research is needed to optimize dose and latency time. To optimize protocols for semen cryopreservation, different cryoprotectors, cooling rates and temperatures at which plunging into liquid nitrogen occurred, were evaluated. Catfish semen showed good tolerance to freezing and thawing. Hatching rates similar to the fresh semen were obtained with semen frozen in 10% methanol, at a cooling rate of -2, -5 or -10°C/min to -40°C and plunged into liquid nitrogen as soon as semen temperature reached -38°C. Samples plunged into liquid nitrogen from a semen temperature above -30°C or below -50°C produced decreasing hatching rates. Post-thaw semen could be diluted at least 200 times without loosing fertilization capacity. Cryopreservation of semen is a valuable tool for selection and conservation of genetic diversity in catfish species.

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Chapter 1

General introduction

The vast majority of fish species reared in captivity exhibit some form of reproductive dysfunction. In females there is often a failure to undergo final oocyte maturation, ovulation and spawning, while in males semen production may be reduced and/or of low quality. Such reproductive dysfunctions are mainly due to the fact that fish in captivity do not experience the natural conditions of the spawning grounds and as a result the pituitary fails to release the maturational gonadotropin, luteinizing hormone (LH; Zohar and Mylonas, 2001). Reproductive hormones have been used since the 1930's (Houssay, 1930) to stimulate reproductive processes and to induce ovulation, spermiation and spawning. However, in the African catfish, while these techniques have been successfully employed with females, the response of male catfish remains poor and semen collection under abdominal massage is practically impossible. For artificial reproduction, males are commonly sacrificed and testes are macerated over the eggs. Although this method is efficient, it compromises selection and genetic improvement. The aim of this thesis was to improve the reproductive efficiency of hatchery-bred African catfish males. In order to achieve this, a two-pronged approach can be used. On the one hand, different methods to facilitate stripping of semen under abdominal massage can be developed, and on the other, successful protocols for semen cryopreservation can be assessed. Both approaches have been followed in this thesis.

The African catfish

Catfishes have a wide geographical distribution and are found in America, Africa, Eurasia, Asia and Australia. Except for two families (Ariidae and Plotosidae) with essentially marine species, catfishes are in general freshwater fishes, belonging to the order Siluriformes, and suborders Siluroidei. The Clariidae family is characterized by an elongated body with long dorsal and anal fins, a presence of a strong spine on the pectoral fin and four pairs of barbells. Most of the clariids have a suprabranchial organ formed by arborescent structures that enables them to use atmospheric air. The African Clarias gariepinus as well as the Asian C. batrachus and C. macrocephalus are the most studied species in this family and are of great importance in both fisheries and fish culture (Teugels, 1996).

Biologically, the African catfish, Clarias gariepinus (Burchell 1822; formerly C. lazera, Cuvier & Valenciennes 1840) is undoubtedly the most ideal aquaculture species. It is widely distributed; thrives in diverse environments (sub-tropical to tropical); is adapted to a wide range of water quality conditions mainly as a consequence of its breathing ability; feeds on a wide array of natural prey under diverse conditions; has a high fecundity and is easily reproduced under captive conditions (Hecht *et al.*, 1996).

The aquaculture potential of C. gariepinus in Africa was first realized by Hey (1941). Before the mid 1970's, little research on its culture potential was performed (de Kimpe and Micha, 1974; Richter, 1976), but since then, interest in African catfish research has grown and is now conducted mainly in The Netherlands, South Africa, Belgium, Central African Republic and Ivory Coast. These largely independent but parallel research initiatives have resulted in the rapid development of farming technology (Hecht *et al.*, 1996) and of techniques for reproduction and rearing (de

Leeuw, 1985; Viveen *et al.*, 1985). Currently, this species is farmed both on a commercial and subsistence basis in many African countries; the most important producers (in terms of annual tonnage) are Nigeria, South Africa, Zambia and Ghana. In Nigeria, the catfish production is currently distributed among few big and a lot of small farms. The annual production is difficult to calculate (no record in mentioned on FAO, 2001), but the country is investing in technology (such as recirculating systems) and in the coming 10 years, it will probably be the largest African catfish producer in the world, with *ca* 10-30,000 tons per year (Ir. W. Fleuren; personal communication). The expanding catfish industry in The Netherlands is producing 1500 ton yearly (FAO, 2001).

Biology of Reproduction

The brain-pituitary-gonadal axis is the most important endocrine system for the regulation of reproductive processes in vertebrates (Figure 1). Stimuli originating internally (such as the stage of somatic or gonadal development) or externally (photoperiod, temperature, behavior of conspecifics, rainfall) are transported to the brain. As a consequence, the secretion rates of the brain hormones (gonadotropin releasing hormone – GnRH, and dopamine) are changed. Release of pituitary gonadotropins (follicle-stimulating hormone – FSH, and LH) is stimulated by GnRH and inhibited by dopamine. In males, gonadotropins stimulate spermatogenesis (sperm production) and steroidogenesis (sex steroid production) in the testes. Sex steroids and other gonadal factors, such as activin and inhibin, exert both a positive and negative feedback on the brain, pituitary and the gonad itself (reviewed by de Leeuw *et al.*, 1987; Nagahama, 1994).

The reproductive organs of the male catfish are situated in the posterior end of the body cavity, just below the trunk kidney. Testes are paired structures suspended by the mesenteries. The lateral side of each testis shows a crenated border. Under light microscopy, seminiferous tubules are observed to accommodate various cysts with different stages of spermatogenic cells from spermatogonia to spermatozoa, all over the testis (Figure 1). Testes are connected to the urogenital pore (located at the end of the pointed and elongated urogenital papilla) through sperm ducts. Around the sperm ducts, accessory glands named seminal vesicles (SV) are located. The basal portion of the SV is flattened and wide, and contains the fingerlike extensions (Fishelson *et al.*, 1994). The primary function of the SV is the production of a fluid containing various active compounds including steroid glucuronides. These glucuronides act as sex pheromones that promote ovarian growth and development in pubertal females (van Weerd, 1990), and activate female responsiveness and induce ovulation, thereby improving fertilization in adults (Resink, 1988). Furthermore, SV of fish reared in laboratory contain sperm cells only in the proximal part of the lobes, while those of fish from their natural habitat contain sperm cells throughout their tubules during the breeding season (Resink, 1988).

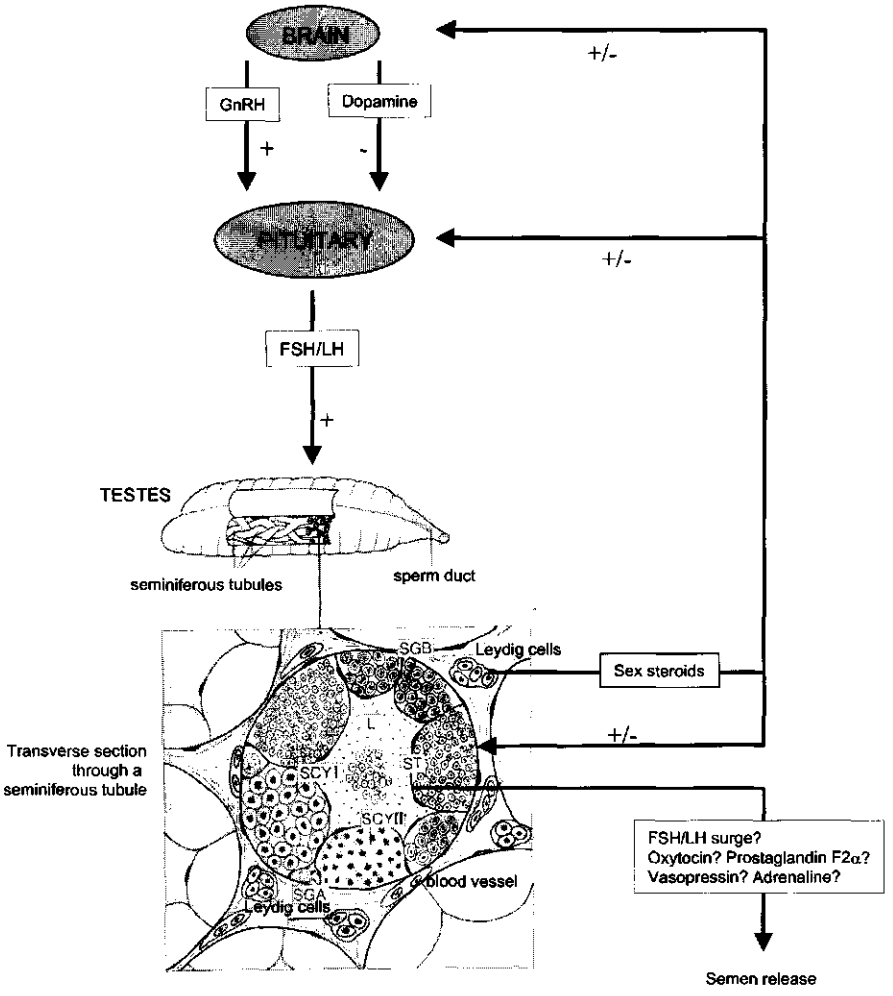

Figure 1. Diagram of the brain-pituitary-gonadal axis of the African catfish male with reference to possible ways to stimulate semen release. GnRH (gonadotropin release hormone); FSH/LH (follicle-stimulating and luteinizing hormones); SGA (spermatogonium A); SGB (spermatogonium B); SCYI (spermatocytes I); SCYII (spermatocytes II); ST (spermatids); L (lumen filled with ST and spermatozoa); +/- (positive/negative stimuli). Adapted from van Oordt *et al.* (1987).

Histological studies of the gonads and of the ultrastructure of the gonadotrophes, together with enzyme-histochemical studies of the gonadotropin content of the pituitary made it possible to divide the annual reproductive cycle of the African catfish into 3 periods (van Oordt *et al.*, 1987; van Oordt and Goos, 1987):

1. During the breeding period, the pituitary gonadotropin content reaches maximum levels. The gonadotrophes are large and densely granulated and at least once during this period, a gonadotropin surge takes place, leading to spermiation and semen release. Fish show a high but decreasing gonadosomatic index (GSI) and, because spermiation is not fully compensated by the production of new sperm cells, gametogenesis comes to an end;
2. During the resting period, pituitary gonadotrophes become smaller and show signs of internal hormone breakdown and a reduction of the gonadotropin content. GSI is relatively low, gonadal steroidogenesis is largely reduced and gametogenesis remains absent;
3. During the pre-spawning period, a recovery of the gonadotrophes and increased gonadotropin content in the pituitary is observed. This is concomitant with an increase in GSI, gonadal recrudescence with full spermatogenesis and a restoration of steroidogenesis.

While the breeding period of the African catfish can vary with location, it is correlated with periods of maximum rainfall. Spawning occurs during summer, usually in dark nights after heavy rain in recently inundated marginal areas. There is a massive aggregation of catfish before spawning, and courtship is preceded by aggressive encounters between competing males. Only victorious males form mating pairs with the females. Mating takes place between isolated pairs in shallow water amongst inundated terrestrial or semi-aquatic grasses and sedges. During courtship, male and female butt and chase each other. The male exhibits shivering motions over his body and before the actual spawning, he closely follows the female and moves ahead of her to adopt a U-shaped position around her head for several seconds. Afterwards, semen and a cloud of eggs are released. Eggs are distributed in all directions by a few vigorous beats of the female tail. The fecundity is very high, with each female capable of releasing 30,000 to 80,000 eggs. This courtship and spawning behavior, including the resting intervals, lasts from 1 to 2 hours. There is no parental protection of the young except by the careful choice of a spawning site and time (Bruton, 1996).

Reproduction in Captivity

As in most of the fish reared in captivity, the African catfish exhibits reproductive dysfunction. It is normally accepted that the absence of reproductive behavior and semen release in hatchery-bred catfish males is caused by a failure of the brain-pituitary-gonadal axis. It has also been speculated that anatomical blockage may retain the sperm flow during hand-stripping, in catfish species.

Failure of the brain-pituitary-gonadal axis

The continuity of the favorable husbandry conditions that hatchery-bred African catfish are kept in, results in the absence of a pre-spawning gonadotropin surge, and a post-spawning regression of the gonadotrophes. Although fish pituitaries store large amounts of gonadotropin, only a limited and continuous secretion of the hormone takes place. This is sufficient for a sustained spermatogenesis and gonadal steroid production, but not for spontaneous semen release and reproductive behavior (van Oordt *et al.*, 1987). The failure to release a large amount of gonadotropin is not caused by insufficient storage of the hormone in the gonadotrophes (de Leeuw, 1985). It is more likely that GnRH is not released or is prevented from eliciting its effects (Goos and Richter, 1996). Based on this fact, hormone protocols have been tested to induce stripping of semen under abdominal massage, for different catfish species.

When African catfish were captured from the wild during their natural breeding season and treated with homologous pituitary homogenates, stripping of a few drops of semen was possible. The stripped semen yielded hatching rates as high as 53% (van der Waal and Polling, 1984). For large-scale artificial propagation, however, the same authors preferred to sacrifice males after the same treatment with pituitary homogenate, and macerate the testes onto the eggs (Polling *et al.*, 1987). In the same species, treatments with carp pituitary suspension (Hogendoorn, 1979), homologous pituitary homogenate and human chorionic gonadotropin (Hecht *et al.*, 1982) failed to induce stripping of semen. Treatment with the mammalian LHRH (which has the same biological activity as the fish GnRH) induced stripping of a few drops of semen from Blue catfish, Ictalurus furcatus, but the sperm cells lost their fertilizing capacity within 5 *min* (Dunham, 1993).

Anatomical blockage of the sperm flow

Several authors have stressed that the difficulty of stripping semen from catfish species may be caused by anatomical blockage. Testes are located deep in the body cavity surrounded by other organs (e.g. gut and stomach). During stripping, most of the hand-pressure is applied to these other organs. In addition, the ripe sperm gathers along the convex lobular edge of the testis rather than leaving it through the sperm duct. Furthermore, a thick interstitial tissue surrounds the spermatogenic cell area of the testis and SV, possibly blocking sperm flow during abdominal massage (Tan Fermin *et al.*, 1997).

The sperm ducts are surrounded by up to 50 fingerlike extensions of the SV (Fishelson *et al.*, 1994). Richter (1976) suggested that these SV extensions may retain sperm flow when pressure is applied to the abdomen. This hypothesis, as far as we know, has never been tested, but is supported by preliminary results obtained in our laboratory (Eding *et al.*, 1999). In an experiment to determine the effects of 17α-methyltestosterone (MT), 17β-estradiol and 11-ketoandrostenedione on gonad development and sex differentiation in African catfish, hormones were fed to larvae at 50 ppm for 13-40 days post hatch. All treatments failed to induce permanent and complete sex reversal, but, surprisingly, 13 out of 68 MT-treated males sampled at 6 months post hatch could be stripped with abdominal massage, yielding a few drops of

fluid. Upon dissection, all MT-treated males possessed incomplete SV fingerlike extensions. Partial inhibition of male gonad development and associated ducts are in agreement with the observed feminizing action of MT and other testosterone derivatives on the gonad development in clariid African catfish (van den Hurk *et al.*, 1989) and in ictalurid Channel catfish I. punctatus (Davis *et al.*, 1990).

The effects of muscle-contractors

In mammals, some muscle contractors are used to stimulate ejaculation and increase the number of sperm cells in the ejaculate. In rats, prostaglandins, mainly F2α, modulate the contractility of seminiferous tubules and mediate the sperm transport from the testis (Farr and Ellis, 1980). Oxytocin plays an important role in gamete transport, by activating smooth muscle contractions of the reproductive tract. In sheep (Assinder *et al.*, 2000) and in bulls (Berndtson and Igboeli, 1988), oxytocin has a stimulatory effect on contractility of the seminiferous tubules during ejaculation, increasing semen volume and concentration in the ejaculate. The related peptide vasopressin also increases epididymal contractions in rams (Knight, 1974) and rabbits (Kihlstrom and Agmo, 1974), although higher doses of vasopressin are required to produce an effect similar to oxytocin. The contractions of the ductus deferens, which cause the emission of semen during ejaculation, are stimulated by catecholamines released from the sympathetic nerve endings (Cross and Glover, 1958). In female brook trout, Salvelinus fontinalis, adrenaline can stimulate *in vitro* both ovulation and contraction of the follicles (Goetz and Bradley, 1994). Adrenaline has stimulated testis contraction during *in vitro* experiments with Channel catfish (Dr. R. Dunham, personal communication). To the best of our knowledge, these hormones (including isotocin, the fish oxytocin-like peptide) have never been evaluated as semen release-inducer in catfish species.

Semen Cryopreservation

Since the first attempt to preserve semen (Spallanzani, 1776), there have been numerous improvements in conservation technology (e.g. discovery of cryoprotectants, development of semen extender, progress of reproductive technology) with a number of detailed reviews of cryopreservation of both mammalian (Watson, 1995; Woelders, 1997; Yoshida, 2000) and fish spermatozoa (McAndrew *et al.*, 1993).

The importance of preserving genetic resources for the future is widely recognized, and the conservation of semen would be a major contribution with great potential applications in agriculture, biotechnology, species conservation and clinical medicine (Yoshida, 2000). In aquaculture, the major benefits resulting from fish gamete preservation include (Lubzens *et al.*, 1997):

- Potentially greater efficiency in selective breeding through storage of gametes from genetically improved fish stocks obtained by classical selective techniques, or by genetic manipulation (e.g., triploids, transgenic fish), through cross-fertilization between related species with non-overlapping breeding seasons, through the use of self-fertilization in protandric hermaphrodites (e.g., gilthead seabream, Sparus aurata) and through an extension of the breeding season in fish;

- Increased protection of stocks from diseases by allowing the introduction of new genetic lines with reduced danger of transmitting unknown pathogens to cultured fish;
- A continuous supply of gametes for optimum utilization of hatchery facilities or for experimentation;
- Economy of maintenance of brood stock and safety against accidental loss of genetically improved lines.

It has been estimated that semen from 200 fish species have been cryopreserved, including some endangered species (Steyn and van Vuren, 1991; Gwo *et al.*, 1999; David *et al.*, 2000; Mongkonpunya *et al.*, 2000; Tiersch *et al.*, 2000). There has been also considerable research on the development of gene banks for aquatic species (McAndrew *et al.*, 1993). The reported post-thaw viability, even for the same species, is highly variable. Most of the work carried out so far is empirical, based on a trial and error approach, leading to heterogeneous results.

To develop an efficient freezing protocol, some background information on cryobiology is needed, especially as to the possible causes of freezing injury. It is well known that freezing injuries can be caused by slower- or faster-than-optimal cooling and thawing rates, and that the level of cryoinjury can be affected by the choice of cryoprotectants and the composition of the freezing medium (extenders).

Cooling velocity (or cooling rate) is one of the major factors which determines whether or not viable cells can be frozen to temperatures that permit indefinite storage. Cooling either slowly or too rapidly tends to be damaging. When cells are cooled between -5 and -15°C, ice forms in the external medium, but cell contents remain unfrozen and supercooled (Figure 2). The latent heat of crystallization raises the temperature to the freezing (or melting) point of the medium, e.g. to -5°C. As part of the water exits the extracellular medium as it becomes ice, the residual solute concentrates. This residual brine is hyperosmotic to the unfrozen cells. The supercooled water in the cells then flow out. The subsequent physical events in the cell depend on the cooling rate. If cooling is sufficiently slow, the cell is able to lose water rapidly, dehydrate and shrink. However, prolonged exposure of the unfrozen cells to the increasing hyperosmotic residual medium will lead to excessive dehydration, shrinkage and a very high intra- and extracellular solute concentration. The effects of these events is referred to as "solution effects" (Mazur, 1970). On the other hand, if the cell is cooled too fast, it is not able to lose water fast enough to maintain equilibrium. Water becomes increasingly supercooled and eventually freezes internally, forming intracellular ice (IIF). During thawing, these ice crystals may recrystallize to larger crystals and disrupt cell membranes. A clear line between damages caused by IIF and solution effects cannot be drawn, mainly because solution effects can happen throughout the entire freezing process. The optimal cooling rate, then, is slow enough to prevent IIF but yet rapid enough to minimize the length of time during which cells are exposed to solution effects. The critical rate varies among different types of cells, depending on surface area, membrane permeability to water and the activation energy. Yeast, for example, will display IIF when cooled faster than -10°C/min, whereas red blood cells will display IIF only when cooled at rates well above -1000°C/min (Mazur, 1977).

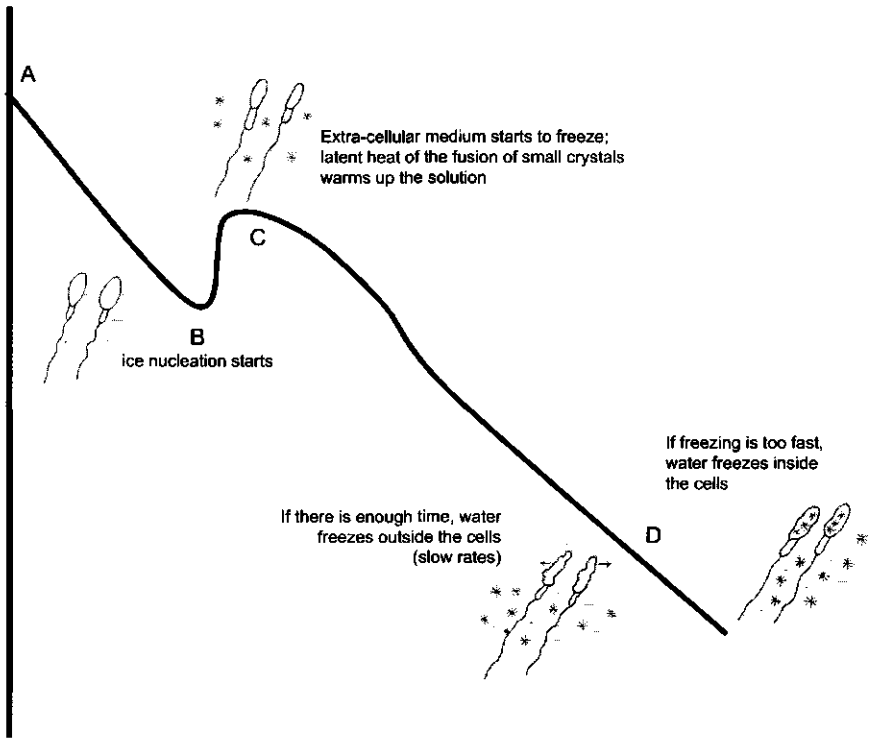

Figure 2. Freezing process of a simple aqueous solution with reference to the effects of cooling rates on sperm cells. A-B (supercooling); B (spontaneous ice nucleation occurs); B-C (latent heat is released); C-D (efflux of water from the cells causing dehydration); D (intracellular medium is frozen). Adapted from McAndrew *et al.* (1993).

The warming rate during thawing of the semen is also of importance. The effects of warming rate on cell survival will depend on the cooling history. Cells cooled much too rapid will have large intracellular ice crystals and will be dead. Cells cooled more slowly, but still relatively fast could have very small intracellular ice crystals. During slow warming these crystals could grow in a process called recrystallization, which could hurt the cells. These cells could therefore be rescued by rapid thawing. When cells are cooled very slowly the IIF could be insignificant, and a high warming rate may not be needed. In fact, it has been proposed that when cells are cooled very slowly cells will be very much dehydrated and shrunk and could be damaged by rapid thawing because of a too rapid rehydration (Leibo, 1976).

Even though the survival of the cells during freezing and thawing can be maximized by optimizing the freezing and warming protocols, the post-thaw recovery will be

unacceptably low if cryoprotectants are not added to the freezing medium. The discovery that glycerol could prevent the damaging effects of slow cooling and thawing on spermatozoa and red blood cells (Polge *et al.*, 1949), renewed interest in banking living cells and tissues at low temperatures in a state of anhydrobiosis or cryptobiosis. Since then, many other compounds have been found to protect living cells against some of the damage caused by freezing. A range of permeating cryoprotectants have been used but the most common ones for freezing fish semen are dimethyl sulphoxide (DMSO), glycerol, methanol and dimethyl acetamide (DMA). These cryoprotectants depress the freezing point of the extracellular medium, ameliorate the detrimental effects of ice crystals and regulate the rate of cell dehydration, reducing the damages caused by high solute concentration during slow cooling rates (the solution effects). However, at high concentration and/or during prolonged equilibration time (period of time between adding the cells to the freezing medium plus cryoprotectant, and freezing), cryoprotectants denature cellular proteins and this can reduce pre-freezing viability (Farrant, 1970). Non-permeating compounds like sugars (glucose, trehalose, sucrose) and proteins (milk powder, egg yolk, glycoproteins) are frequently added to the freezing medium and are known for their membrane-stabilizing activity (de Leeuw *et al.*, 1993). Sugars have shown a strong protective action in cryopreservation of bull semen at too high cooling rates, and an almost insignificant one at optimal cooling rates (Woelders, 1997).

Finally, the efficacy of cryopreservation can be greatly enhanced if the pre-cooled semen is diluted in a suitable freezing medium. The composition and osmolality of a freezing medium are usually based on saline or sucrose solutions, mostly similar to blood or seminal plasma. A good medium for fish semen should keep spermatozoa immotile and stable during storage. Semen is mostly stored on liquid nitrogen (LN_2) at -196°C, in vials or straws. At this temperature, cell viability can be stored in a genetically stable form and is affected only by background radiation (Stoss, 1983).

African catfish semen was first successfully cryopreserved by Steyn *et al.* (1985). Since then, different freezing protocols have been developed for this species. The most widely used cryoprotectant is glycerol, often in combination with glucose, but DMSO and DMA have also yielded good survival (Horvath and Urbanyi, 2000). Semen can be cooled at a fast rate when placed in LN_2 vapor (Steyn *et al.*, 1985), or a slow rate when working with fixed cooling rates in a programmable freezer (Steyn, 1993; van der Walt *et al.*, 1993). However in most cases, sperm quality is only evaluated in terms of motility after thawing. When fertilization is included in the post-thaw evaluation, sperm:egg ratios are not optimized and often excessive (e.g. Steyn and van Vuren, 1987). Using an excess of sperm cells for fertilization obviously masks the quality of cryopreserved semen, making comparison of protocols difficult. Recently, Rurangwa *et al.* (2001) have described an objective method to calculate post-thaw motility by using computer-assisted sperm analysis (CASA) and used a standardized fertilization ratio of 101,400 spermatozoa per egg.

Semen collection and preservation in African catfish, Clarias gariepinus, kept under husbandry conditions (outline of the thesis)

The aim of this thesis was to develop techniques to enhance the reproductive efficiency of African catfish males kept in captivity (Figure 3). The first section of the thesis concentrates on the study of different methods to facilitate stripping of semen under abdominal massage. We then developed suitable protocols for semen cryopreservation, in this species.

Initially, different maturational hormones, such as GnRH with or without a dopamine antagonist, and crude pituitary extracts were tested to induce semen release and facilitate hand-stripping of semen (**Chapter 2**). We then focused on developing techniques to inhibit the seminal vesicle development with a dietary 17α-methyltestosterone treatment during larval stages. After sexual maturation, these males received a hormone treatment defined in the previous chapter, to facilitate hand-stripping of semen (**Chapter 3**). Finally, we compared the effects of pituitary extracts and muscle contractors on the semen release response of normal males and of males without seminal vesicles (**Chapter 4**).

In the second part of this thesis, a suitable method for freezing semen was developed. We first tested, under standardized conditions, different cryoprotectant agents, different cooling rates and different sperm:egg dilution ratios (**Chapter 5**). Then, using a low sperm:egg ratio, we studied the effects of semen temperature measured inside a vial during the freezing process, on semen survival (**Chapter 6**). In **Chapter 7,** the overall results and possible implications of these results are discussed.

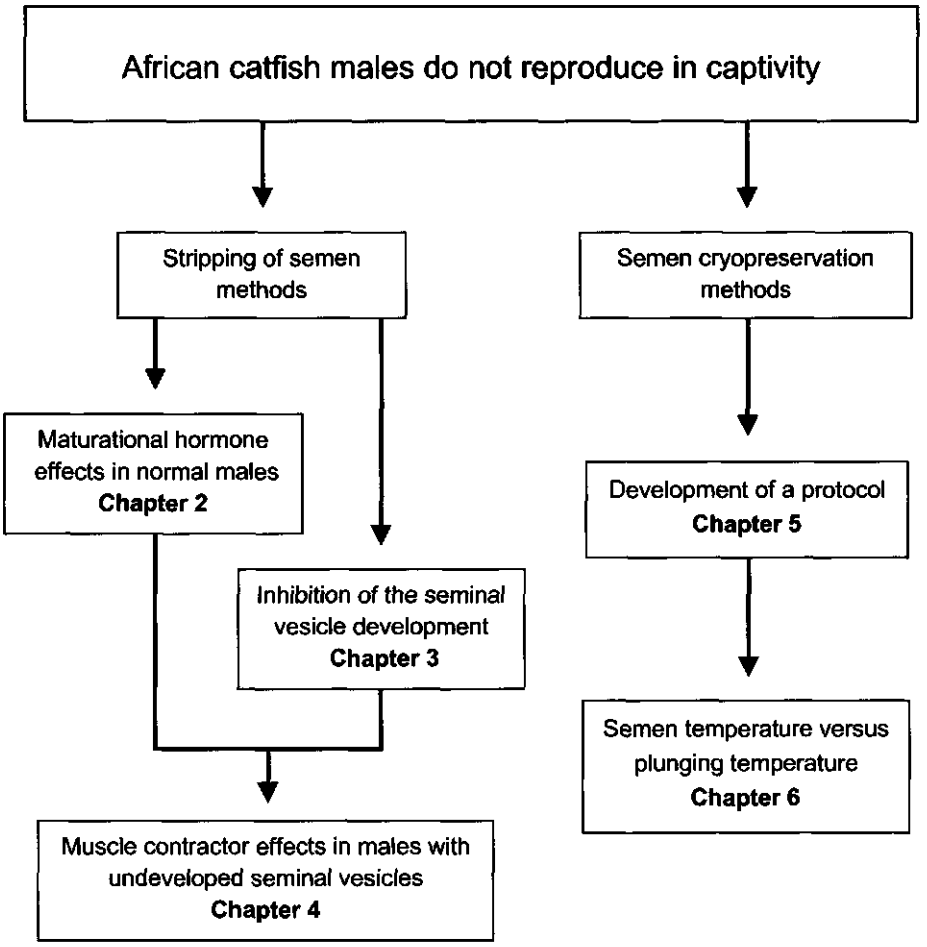

Figure 3. Schematic representation of the experiments carried out with African catfish males in this thesis and a reference to the specific Chapter.

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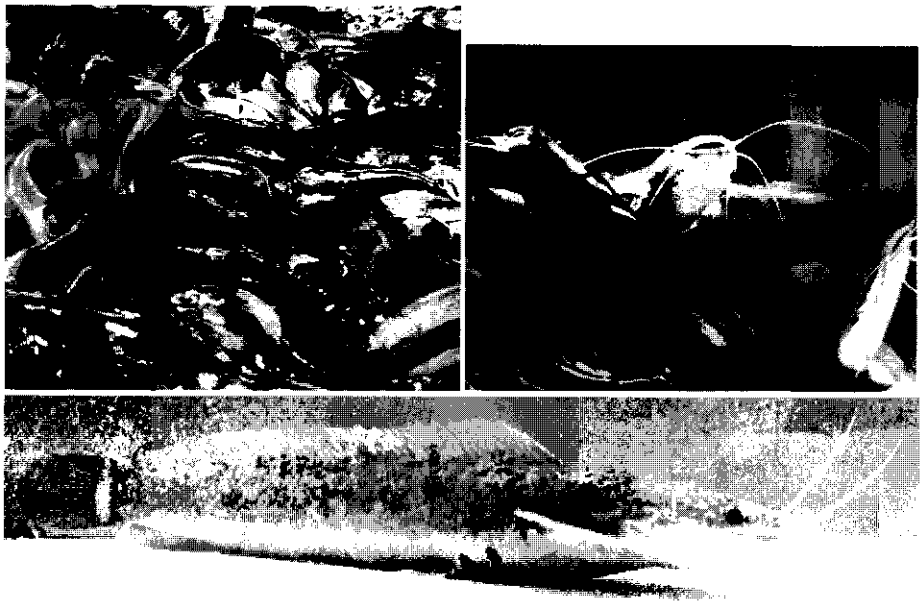

Picture 1. The African catfish, Clarias gariepinus.

Chapter 2

Hand-stripping of semen and semen quality after maturational hormone treatments, in African catfish Clarias gariepinus

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Submitted to Aquaculture

Abstract

In the hatchery-bred African catfish, Clarias gariepinus, spontaneous semen release does not occur and hand-stripping of semen is practically impossible. This reproductive dysfunction may be due to a lack of a pre-spawning gonadotropin (luteinizing hormone – LH) surge. To test this hypothesis, the effects of drugs that increase plasma LH levels were analyzed. LH-releasing hormone analogue (LHRHa), LHRHa plus a dopamine antagonist (LHRHa-PIM), ovaprim (salmon gonadotropin releasing hormone analogue plus a dopamine antagonist), carp pituitary suspension (cPS), Clarias pituitary suspension and combinations of cPS and ovaprim were tested. Stripped fluid, when present, was compared to intratesticular semen, 12 or 24 h after injection (latency time). Plasma LH levels increased ($P<0.05$) 2 h after injection in all hormone treatments, compared to control fish. Stripping of a few drops of fluid, containing some viable spermatozoa, was possible in 4 out of 5 males treated with two injections of cPS, sampled 12 h later, and in 13 out of 24 males treated with combinations of cPS and ovaprim. Spermatocrit, sperm concentration and hatching rates obtained with stripped fluid, however, were very low compared to those obtained with intratesticular semen, from the same males. The number of sperm cells collected per kg body weight increased only in fish treated with two consecutive injections of cPS and a 12-h latency time. Treatments using single injections of pituitary suspensions and treatments with LHRHa, LHRHa-PIM or ovaprim, did not facilitate hand-stripping of viable sperm cells, nor did they increase the number of sperm cells collected per kg. Based on these results, it is unlikely that hatchery-bred catfish males are not strippable because of a lack of the gonadotropin surge.

Introduction

In nature, the African catfish has a discontinuous annual reproductive cycle with alternate periods of resting, pre-spawning and breeding, regulated by cyclically active gonadotrophes (van Oordt and Goos, 1987). The breeding season correlates with periods of maximal rainfall and a pre-spawning LH surge takes place at least once during this period (van Oordt *et al.*, 1987). Spawning occurs usually during the scotophase, after rain in recently inundated marginal areas. When catfish males were captured from the wild during the natural breeding season, stripping of 3-5 drops of a cloudy fluid was possible, 24 h after treatment with homogenized pituitary (van der Waal and Polling, 1984). The fluid was stripped over the eggs and produced 53% of hatching; intra-testicular (IT) semen obtained from macerated testes produced 78% of hatching. For a reliable large-scale artificial propagation, however, the same authors preferred to repeat the homogenized pituitary treatment, sacrifice the males and use IT semen from macerated testes (Polling *et al.*, 1987).

In captivity, catfish are kept under constant environmental conditions. Throughout the year, their pituitaries contain large and densely granulated gonadotrophes, storing large amounts of LH (de Leeuw *et al.*, 1987). Their gonads show a continuous cycle

with numerous ripe sperm cells at all seasons (Richter *et al.*, 1987b). However, the pituitary fails to release the pre-spawning LH surge and males do not release semen spontaneously. Under laboratory and fish-farming conditions, the natural cues are difficult to mimic. To be able to reliably induce year-round reproduction artificially, hormonal treatments have been tested, but the results were mostly disappointing. Treatments with cPS (Hogendoorn, 1979), Clarias pituitary homogenate and human chorionic gonadotropin (hCG; Hecht *et al.*, 1982) failed to induce stripping of semen in African catfish. Treatment with LHRHa induced stripping of few drops of semen in Blue catfish Ictalurus furcatus, but sperm lost their fertilizing capacity 5 min after collection (Dunham, 1993). In an attempt to optimize the use of each male, techniques for surgical removal of part of the testis were described for Blue catfish, Channel catfish, I. punctatus (Bart and Dunham, 1990) and African catfish, Heterobranchus longifilis (Nguenga *et al.*, 1996), but expensive veterinarian costs are involved. For artificial reproduction, therefore, males are killed, testes are removed and macerated, and IT semen is spread over the eggs. Although sacrificing males to collect IT semen is effective, it compromises attempts to selection and genetic improvement.

It has been speculated that catfish under favorable husbandry conditions either do not release the gonadotropin releasing hormone (GnRH), or GnRH is prevented from eliciting its effects (Goos and Richter, 1996). If this is true, then GnRH treatment, or GnRH plus a dopamine antagonist (like domperidone or pimozide) treatment should overcome this failure, induce LH surge, stimulate semen release and facilitate hand-stripping of semen. To investigate this hypothesis, the effects of hypothalamic hormones (LHRHa, LHRHa plus pimozide and salmon GnRHa plus domperidone - ovaprim), homologous and heterologous crude pituitary suspensions (Clarias-PS and cPS) and the combination of both hypothalamic hormones and crude pituitary extracts (cPS plus ovaprim) on African catfish males were evaluated. Plasma LH was quantified to confirm the occurrence of the LH surge after treatments with hypothalamic hormones.

Material and Methods

All experiments were carried out at the hatchery "De Haar Vissen" of the Fish Culture and Fisheries Group, Wageningen University and Research Center (WUR), The Netherlands. In experiment 1, fish were bought from a private farm (Aquafish, Leekerseweg 4, Venhorst, The Netherlands) two months before starting the experiment, when fish were about 7 months old. In experiments 2, 3 and 4, different progeny groups of the "De Haar Vissen" catfish were used. These fish were 12-14 months old.

During the pre-experimental period, males were kept in groups of 10-15 males plus one sexually mature female. These females had fully developed gonads, with post-vitellogenic eggs. After receiving the hormone treatment, males were individually housed in 120-L tanks to avoid aggressive interaction and to maximize care during the experimental period. All tanks were connected to a recirculating system equipped with a biofiltration unit and received a constant flow of well-aerated water at 25.0 ± 0.2°C, and at 4000 µS conductivity to prevent fungal infection. NH_4^+ and NO_2 were kept below 1

ppm and pH was kept at 6.5. Fish were hand-fed with commercial dry feed twice a day, ad libitum and daily monitored.

Hormones

Homologous catfish pituitary suspension (Clarias-PS; obtained from a commercial farmer - Ir. Fleuren, Someren, The Netherlands), cPS (Crescent Research Chemicals, Phoenix, Arizona, USA), LHRHa (Sigma Chemical, Steinheim, Germany) and pimozide (PIM; Sigma Chemical, Steinheim, Germany), were each dissolved in 0.9% NaCl (w/v) in such a concentration that each fish received the same volume of 0.5 mL/kg body weight (bw). Ovaprim (Syndel, Vancouver, Canada), containing 20 μg salmon GnRHa (sGnRHa) and 10 mg domperidone per mL, was injected at either 0.5 or 1.0 mL/kg bw. Control fish received a constant volume of 0.9% NaCl (w/v) at 0.5 mL/kg bw. All hormones and saline were injected intra-muscularly.

Experimental design

Four experiments were designed to increase plasma LH levels and, consequently, induce hand-stripping of semen. All experiments were approved by the Ethical Committee judging Animal Experiments (DEC) of the Wageningen University, number 87.a.

Firstly, the effects of exogenous and endogenous LH on sperm quality were compared. Twenty fish were randomly divided over 4 groups. Each group received one of the following treatments (/kg bw): Clarias-PS 8 mg (the double concentration used for females according to normal practice in our hatchery); LHRHa 60 μg (doses varying from 1-100 μg induce LH release in several fish species); LHRHa 20 μg plus pimozide 5 mg (LHRHa-PIM; dopamine antagonist potentiates the LHRHa-induced LH surge in African catfish and cyprinids, so that a reduced LHRHa dose was used); or saline only (control). All fish were sampled 24 h after injection.

In experiment 2, we repeated all treatments with another set of 30 fish, with the following changes: cPS was included for comparison with Clarias-PS, and LHRHa plus pimozide was substituted by sGnRHa plus domperidone (ovaprim). Thirty fish were randomly divided over 6 groups and each group received one of the following treatments (/kg bw): Clarias-PS 8 mg, cPS 8 mg, LHRHa 60 μg, ovaprim 0.5 mL (according to the manufacturer recommendations), ovaprim 1.0 mL, or saline only. All fish were sampled 24 h after injection.

In experiment 3, the effects of repeated administration of cPS were investigated. Ten fish received 2 consecutive injections of cPS at 8 and 10 mg/kg bw respectively, with a 48-h interval. Simultaneously, 6 fish received 2 injections of saline only. Five cPS-treated fish and 3 control fish were randomly sampled 12 h after the last injection (latency time; LT). The remaining treated and control fish were sampled after 24-h LT.

In experiment 4, the effects of cPS on semen as observed in experiment 3, were analyzed in combination with ovaprim. Two groups of 12 fish each, first received an

injection with cPS at 8 mg/kg bw. After 48 h, one group received a second injection of cPS at 5 mg/kg bw, while the other group received ovaprim at 0.5 mL/kg bw. Twenty-four h later, all fish received a third injection with ovaprim at 0.5 mL/kg bw (cPS-cPS-ovp and cPS-ovp-ovp groups, respectively). Simultaneously, control fish (n=6) were injected 3 times with saline only. Six fish from each treatment and 3 control fish were sampled after 12-h LT. The remaining fish were sampled after 24-h LT.

Sampling

After the corresponding LT, males were sacrificed in tricaine methanesulphonate (TMS; Crescent Research Chemicals, Phoenix, Arizona, USA) at 0.8 g/L of tap water for 40 min. Males were weighed, genital area was dried and hand pressure was applied midway between pectoral and pelvic fins, moving posteriorly right down to the urogenital papilla (van der Waal and Polling, 1984). This stripping process was repeated 10-15 times for each male. The stripped fluid, when present, was collected in a test tube where volume was immediately measured. Then, males were dissected and testes and seminal vesicles (SV) were carefully removed and weighed. Gonado- (GSI) and SV-somatic index (SVSI) were calculated as percentage of testes and SV weight, respectively, relative to total bw. Testes were slit, IT semen was squeezed out and volume was measured. IT semen and stripped fluid were kept in crushed ice during all analyses.

All qualitative and quantitative analyses were carried out in duplicate for each sample of IT semen or stripped fluid. Spermatocrit determination was performed in capillary tubes in a micro-hematocrit IEC MB centrifuge equipped with a capillary tube rotor at 10,000 G for 3 min, at room temperature. The white packed volume was calculated as percentage of total volume. For microscopic sperm cell counting and spectrophotometric measurement, IT semen and stripped fluid were diluted 1000 times with 0.9% NaCl (w/v). A double Burker Türk counting chamber (W. Schreck, Hofheim TS.) was used to count sperm cells and determine sperm concentration per mL of semen. Absorbance was measured with a Beckman DU-64 spectrophotometer at a wavelength of 505 nm in polystyrene disposable cuvettes, according to Ciereszko and Dabrowsky (1993). A solution of 0.9% NaCl (w/v) served as blank. The number of sperm cells collected per kg bw was calculated based on IT semen volume and concentration. Sperm motility was assessed by mixing 15 µL of IT semen or stripped fluid in 30 µL of tap water under microscope, at 200x magnification. Motility was subjectively scored according to the percentage of moving cells, as: 0, when no movement was observed; 1, when up to 25% cells were moving; 2, when up to 50% cells were moving; 3, when up to 75% cells were moving and; 4, when more than 75% cells were moving.

IT and stripped samples of males from experiment 4 were also tested for fertility. One female catfish was treated with cPS at 4 mg/kg bw and stripped of eggs 12 h later (25°C). For fertilization, we followed the standardized method described previously (Viveiros *et al.*, 2000). Each portion of 0.2 g eggs was fertilized with 100 µL of IT semen (previously diluted 200 times with 0.9% NaCl, w/v) or stripped fluid (undiluted). The fertilization trial was carried out in duplicate for each semen sample. The

percentage of hatched larvae from the total number of eggs in contact with sperm, was calculated 24 h after fertilization (30°C).

Radio-immuno assay (RIA)

To confirm that treatments with hypothalamic hormones alone or in combination with a dopamine antagonist induced LH surge and that the LH content of pituitary suspension increased plasma LH levels, 18 fish were divided over 6 groups. Each group was injected intra-muscularly with one of the following hormones (/kg bw): LHRHa 60 µg, LHRHa 20 µg plus PIM 5 mg, ovaprim 0.5 mL, ovaprim 1.0 mL, cPS 8 mg, or saline. Blood was sampled 2 and 8 h after hormone injections. These time points were chosen based on plasma LH profiles obtained after hypothalamic hormone injections described in Schulz *et al.* (1993) and Goos *et al.* (1987) for males, and in Richter *et al.* (1987a) and van Asselt *et al.* (1988) for females catfish. Blood was collected in Eppendorf tubes containing 6% sodium citrate and 0.9% NaCl (w/w), centrifuged at 300 G for 10 min at 4°C. Plasma was stored at -20°C. RIA for LH quantification was performed as described previously (Schulz *et al.*, 1995).

Statistical analyses

For each treatment, data from all males and duplicate measurements for IT semen quality were pooled per parameter to calculate mean and standard deviation. When control fish were sampled at different LT, as in experiments 3 and 4, data were only pooled after checking for any significant difference between LT groups. Stripped fluid was not analyzed statistically due to low number of strippable fish per treatment. The presence of blood in stripped fluid samples disturbed the spermatocrit reading, thus we preferred to refer to it as cell packed volume. All statistical analyses were carried out using the SAS 6.11 package (SAS Institute Inc., 1990). Each parameter was tested for significant differences by ANOVA using the parametric general linear model procedure, followed by Duncan's multiple range test. For GSI, SVSI, spermatocrit values and hatching rates, the residues of each model were tested for normality using the univariate procedure. Data on spermatocrit and optical density of IT samples from all 4 experiments were regressed on IT sperm concentration per mL, to obtain a linear equation. Correlation was expressed as Pearson correlation coefficient (r). Data on plasma LH level was processed after logarithmic transformation. Means were tested for significant differences by ANOVA, followed by Duncan's multiple range test. P-values <0.05 were regarded significant.

Results

Experiment 1

Stripping of semen was not possible after treatments with Clarias-PS, LHRHa or LHRHa-PIM. Internal inspection of the fish revealed that testes were still small (only 9.5 ± 1.7 g), despite an age of 9 months and a mean bw of 1.5 ± 0.2 kg. IT semen

volume was only 3.6 ± 1.3 mL (Table 1). As these fish originally came from a commercial farm, the immaturity of their gonads possibly resulted from selection programs for fast growth and delayed sexual maturity. Clarias-PS treatment increased ($P<0.05$) GSI and semen volume, compared to LHRHa-PIM treatment, and decreased ($P<0.05$) sperm concentration and spermatocrit, compared to both control and LHRHa-PIM treated group. GSI, SVSI, semen volume and the number of sperm cells collected per kg were not affected by any treatment, compared to control (Table 2).

Experiment 2

Mean bw of all 30 fish was 3.5 ± 0.4 kg and testes weight in control fish was 29.2 ± 10.8 g. Stripping of a fluid was possible after treatments with Clarias-PS (n=1), ovaprim at 0.5 mL/kg (n=2) and ovaprim at 1 mL/kg (n=3). The stripped fluid, however, was watery and bloody and there were no moving sperm cells after addition of water (score 0; Table 1). Determination of sperm concentration was, therefore, not meaningful. GSI, IT semen volume, sperm concentration and sperm cells/kg were not affected by any treatment, compared to control (Table 3). Clarias-PS and cPS treatments increased ($P<0.05$) SVSI compared to LHRHa and control groups. CPS treatment increased ($P<0.05$) both GSI and semen volume, compared to LHRHa treatment, and decreased ($P<0.05$) spermatocrit, compared to LHRHa, ovaprim at 0.5 mL/kg and control groups.

Experiment 3

Mean bw of all 16 fish was 3.3 ± 0.4 kg and testes weight in control fish was 19.4 ± 10.0 g. Stripping was possible after treatment with two cPS injections, but only after 12-h LT (n=4). The stripped fluid was watery and had a mean volume of 0.9 mL, a mean cell packed volume of 2.0% and a mean sperm concentration of 0.55 x 10^9 cells/mL. Motility was very low, with a maximum score equal to 1 (Table 1). Treated fish sampled after 24-h LT were not strippable. CPS treatment increased ($P<0.05$) GSI and IT semen volume, and decreased ($P<0.05$) spermatocrit values, compared to control fish. Three times more sperm cells/kg were collected from treated fish sampled after 12-h LT, compared with control fish (Table 4).

Experiment 4

Mean bw of all 30 fish was 3.3 ± 0.7 kg and testes weight in control fish was 32.1 ± 19.9 g. Stripping was possible in some fish after all combinations of cPS and ovaprim. Mean stripped volume was larger than 1 mL with at least 0.69 x 10^9 sperm cells/mL in all treatments. However, the stripped fluid was watery, sometimes bloody and motility was generally low (maximum score 1; Table 1). Hatching rates were very poor with a maximum of 4.3% produced with stripped fluid from cPS-cPS-ovp treated fish, sampled after 24-h LT. Stripped samples from the other treatments produced 0% of hatching. GSI, SVSI, IT semen volume, sperm cells/kg and hatching rates produced with IT semen were not affected by any treatment, compared to control. CPS-cPS-ovp treatment decreased ($P<0.05$) spermatocrit and sperm concentration in fish sampled after 12-h LT, compared to control. CPS-ovp-ovp treatment decreased ($P<0.05$) spermatocrit and

Table 1 - Semen release response and stripped fluid quality (mean ± SD) in African catfish treated with Clarias pituitary suspension (Clarias-PS), carp pituitary suspension (cPS), LHRHa, LHRHa plus pimozide (LHRHa-PIM) or ovaprim, and sampled after a 12- or 24-h latency time (LT).

Exp.	Treatment	LT (h)	Treated fish (n)	Strippable fish (n)	Volume (mL)	Packed cell volume (%) [1]	Cells/mL ($x10^9$)	Motility [2] (0-4)
1	Clarias-PS	24	5	0				
	LHRHa	24	5	0				
	LHRHa-PIM	24	5	0				
	Control	24	5	0				
2	Clarias-PS	24	5	1	1.3	11.5	N	0
	CPS	24	5	0				
	LHRHa	24	5	0				
	Ovaprim 0.5 mL	24	5	2	0.7 ± 0.3	11.5 ± 10.1	N	0
	Ovaprim 1.0 mL	24	5	3	1.0 ± 0.6	10.3 ± 8.9	N	0
	Control	24	5	0				
3	cPS-cPS [3]	12	5	4	0.9 ± 0.8	2.0 ± 2.1	0.55 ± 0.36	0.8 ± 0.5
	cPS-cPS [3]	24	5	0				
	Control	12/24	6	0				
4	cPS-cPS-ovp [4]	12	6	4	1.5 ± 0.9	5.8 ± 4.1	0.69 ± 0.13	0.3 ± 0.5
	cPS-cPS-ovp [4]	24	6	3	1.7 ± 1.2	8.3 ± 4.5	1.60 ± 0.87	0.7 ± 0.6
	cPS-ovp-ovp [5]	12	6	2	1.7 ± 1.1	3.7 ± 4.2	0.94 ± 0.23	0.5 ± 0.5
	cPS-ovp-ovp [5]	24	6	4	1.1 ± 0.3	5.6 ± 1.5	0.72 ± 0.34	0.5 ± 0.5
	Control	12/24	6	0				

[1] Because stripped fluid were mostly contaminated with blood, we prefer to refer to the spermatocrit reading as the percentage of cell packed volume. [2] Motility scored as: 0 (no moving cells), 1 (1-25%), 2 (25-50%), 3 (50-75%) and 4 (75-100% moving cells). [3] Two cPS injections given with a 48-h interval. [4] Two cPS injections given with a 48-h interval, plus one ovaprim (ovp) injection 24 h later. [5] One cPS injection, after 48-h interval, two ovaprim injections given with a 24-h interval. Ovaprim contains salmon GnRHa and domperidone. N - parameters not measured, due to the absence of sperm motility.

Table 2. Intra-testicular semen quality (mean ± SD) of African catfish treated with Clarias pituitary suspension (Clarias-PS), LHRHa or LHRHa plus pimozide (LHRHa-PIM) and sampled after a 24-h latency time (Experiment 1).

Treatment	GSI (%)	SVSI (%)	Volume (mL)	Spermatocrit (%)	Cells/mL ($x10^9$)	Cells/kg ($x10^9$)
Clarias-PS	0.75 ± 0.27^{a}	0.19 ± 0.06^{a}	6.1 ± 3.5^{a}	16.2 ± 2.0^{b}	2.8 ± 0.7^{b}	11.8 ± 7.3^{a}
LHRHa	0.37 ± 0.14^{b}	0.13 ± 0.10^{a}	3.2 ± 2.1^{ab}	36.3 ± 17.2^{ab}	6.5 ± 3.3^{ab}	11.0 ± 2.9^{a}
LHRHa-PIM	0.40 ± 0.09^{b}	0.14 ± 0.06^{a}	2.1 ± 1.1^{b}	54.1 ± 24.6^{a}	9.2 ± 3.8^{a}	11.6 ± 6.6^{a}
Control	0.57 ± 0.08^{ab}	0.16 ± 0.04^{a}	3.6 ± 1.3^{ab}	41.8 ± 16.7^{a}	8.5 ± 3.7^{a}	17.1 ± 6.8^{a}

[a,b] Means followed by the same superscript are not significantly different (Duncan; $P<0.05$).

Table 3 - Intra-testicular semen quality (mean ± SD) of African catfish treated with Clarias pituitary suspension (Clarias-PS), carp pituitary suspension (cPS), LHRHa or ovaprim either at 0.5 or at 1.0 mL/kg, and sampled after a 24-h latency time (Experiment 2).

Treatment	GSI (%)	SVSI (%)	Volume (mL)	Spermatocrit (%)	Cells/mL ($x10^9$)	Cells/kg ($x10^9$)
Clarias-PS	1.08 ± 0.24^{ab}	0.75 ± 0.07^{a}	25.1 ± 10.8^{a}	27.7 ± 14.9^{ab}	7.2 ± 2.8^{a}	46.1 ± 14.5^{a}
cPS	1.14 ± 0.33^{a}	0.81 ± 0.20^{a}	21.9 ± 8.2^{a}	16.8 ± 11.3^{b}	5.6 ± 4.9^{a}	29.7 ± 20.9^{a}
LHRHa	0.81 ± 0.24^{bc}	0.50 ± 0.13^{c}	12.2 ± 4.5^{b}	48.3 ± 18.5^{a}	10.3 ± 3.6^{a}	38.6 ± 23.8^{a}
Ovaprim 0.5 mL	0.67 ± 0.20^{c}	0.68 ± 0.17^{abc}	14.8 ± 6.0^{ab}	43.1 ± 28.9^{a}	9.2 ± 6.1^{a}	29.5 ± 15.1^{a}
Ovaprim 1.0 mL	0.81 ± 0.17^{bc}	0.72 ± 0.19^{ab}	16.2 ± 6.3^{ab}	35.9 ± 12.1^{ab}	7.8 ± 3.5^{a}	34.0 ± 15.9^{a}
Control	0.84 ± 0.24^{abc}	0.57 ± 0.13^{bc}	18.8 ± 14.1^{ab}	42.8 ± 15.3^{a}	9.6 ± 5.1^{a}	41.1 ± 21.6^{a}

[a-c] Means followed by the same superscript are not significantly different (Duncan; $P<0.05$).

Table 4. Intra-testicular semen quality (mean ± SD) of African catfish treated with two cPS injections at 48-h interval and sampled after a 12- or 24-h latency time (LT; Experiment 3).

Treatment	LT (h)	GSI (%)	SVSI (%)	Volume (mL)	Spermatocrit (%)	Cells/mL ($x10^9$)	Cells/kg ($x10^9$)
cPS-cPS	12	1.37 ± 0.41^a	0.47 ± 0.09^b	27.7 ± 16.1^a	20.5 ± 6.7^b	7.5 ± 3.5^a	53.9 ± 23.7^a
cPS-cPS	24	1.56 ± 0.51^a	0.72 ± 0.24^a	31.0 ± 15.8^a	11.7 ± 8.2^b	4.9 ± 4.4^a	38.9 ± 25.5^{ab}
Control [1]	12/24	0.55 ± 0.25^b	0.48 ± 0.09^b	7.3 ± 4.5^b	47.0 ± 25.9^a	10.1 ± 8.0^a	15.8 ± 15.4^b

[a,b] Means followed by the same superscript are not significantly different (Duncan; $P<0.05$).
[1] Because values from control fish sampled at 12- or 24-h of LT were not significantly different, data was pooled.

Table 5. Intra-testicular semen quality (mean ± SD) of African catfish treated with triple hormone injections: first cPS, after 48-h interval, either cPS or ovaprim and finally after 24-h interval, ovaprim. Fish were sampled after a 12- or 24-h latency time (LT; Experiment 4).

Treatment	LT (h)	GSI (%)	SVSI (%)	Volume (mL)	Spermatocrit (%)	Cells/mL ($x10^9$)	Cells/kg ($x10^9$)	Hatching Rates (%)
cPS-cPS-ovp	12	1.61 ± 0.59^a	0.79 ± 0.32^a	32.5 ± 14.1^a	8.8 ± 2.4^b	2.2 ± 1.5^b	21.2 ± 15.8^a	80.3 ± 6.2^a
cPS-cPS- ovp	24	1.07 ± 0.71^a	0.76 ± 0.30^a	20.9 ± 11.9^a	28.9 ± 25.3^{ab}	6.7 ± 5.9^{ab}	29.2 ± 19.0^a	78.7 ± 18.2^a
cPS-ovp-ovp	12	1.41 ± 0.68^a	0.80 ± 0.27^a	29.7 ± 27.7^a	19.8 ± 15.8^b	5.7 ± 5.6^{ab}	33.4 ± 27.7^a	79.4 ± 36.9^a
cPS-ovp-ovp	24	1.51 ± 0.24^a	0.61 ± 0.18^a	31.8 ± 10.8^a	11.0 ± 6.1^b	2.4 ± 1.6^b	22.5 ± 15.8^a	88.5 ± 3.2^a
Control [1]	12/24	1.03 ± 0.50^a	0.89 ± 0.18^a	17.9 ± 12.4^a	52.6 ± 40.0^a	10.8 ± 9.2^a	26.9 ± 15.3^a	66.0 ± 22.1^a

[a,b] Means followed by the same superscript are not significantly different (Duncan; $P<0.05$).
[1] Because values from control fish sampled after a 12- or 24-h latency time were not significantly different, data was pooled.

sperm concentration in fish sampled after 24-h LT, and decreased ($P<0.05$) only spermatocrit in fish sampled in 12-h LT, compared to control (Table 5).

The sperm motility test showed more than 75% moving cells (score 4) in all IT samples of both hormone treated and control fish, from all 4 experiments. A high correlation was found between sperm concentration and both optical density ($r = 0.936$) and spermatocrit ($r = 0.841$), when data from all 96 IT semen samples were pooled (Table 6).

Table 6. Relationship between sperm concentration and either spermatocrit or optical density (absorbance 505 nm), in African catfish.

Exp.	Samples	Spermatocrit		Optical density	
	(n)	Correlation (r)	Y = a x + b	Correlation (r)	Y = a x + b
1	20	0.918	N	0.967	N
2	30	0.765	N	0.939	N
3	16	0.707	N	0.981	N
4	30	0.947	N	0.998	N
Total	96	0.841	0.20 x + 1.06	0.936	42.05 x – 1.04

N – parameter calculated only with data of all fish.
All Pearson correlation were significant at $P<0.05$.

Radio-immuno assay (RIA)

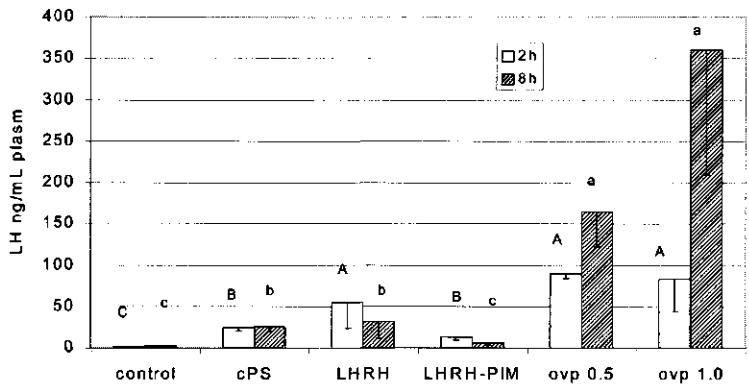

Figure 1. Plasma gonadotropin (LH) levels in African catfish males, 2 (open column, capital letters) and 8 (closed column, lowercase letters) hours after treatments with carp pituitary suspension (cPS), LHRHa, LHRHa plus pimozide, ovaprim at 0.5 mL/kg, ovaprim at 1.0mL/kg or saline (control). Vertical bars represent standard deviation of triplicate measurements. Means followed by the same letter do not differ significantly (Duncan; $P<0.05$).

All hormone treatments increased ($P<0.05$) plasma LH levels 2 h after injection, compared to control fish. After 8 h, the LH levels of LHRH-PIM treated fish decreased to basal levels, while in the other treated groups it remained high. The highest LH values were observed at both 2 and 8 h after treatment with ovaprim (Figure 1).

Discussion

Effects of hypothalamic hormones

Previous studies (Schulz *et al.*, 1993) and the present data show that unstimulated plasma LH levels in male African catfish are rather low (2.4 ng/mL plasma). All hypothalamic hormone treatments tested here clearly stimulated LH secretion. Several drugs with anti-dopamine properties show a potentiating effect of LHRHa treatment in this species (Goos *et al.*, 1987). In female, LHRHa-PIM induced a sharp increase in LH from basal levels in control fish to 72.1 ng/mL plasma (Richter *et al.*, 1987a) and 68 ng/ml plasma (van Asselt *et al.*, 1988) 8 h after treatment. In both studies, ovulation was recorded in 100% of the treated fish. Assuming that female response to LHRH treatments is similar to male, 360.1 ng LH/mL plasma observed 8 h after ovaprim treatment should be sufficient to induce semen release and facilitate stripping of semen in males. Instead, the sperm cells present in the few drops of stripped fluid obtained after single injections of ovaprim were immotile. In Blue catfish, LHRHa treatment induced stripping of 2-30 mL of a clear liquid containing some viable sperm. This fluid gave a fertilization rate of 50% when directly stripped onto eggs. However, when this fluid was first diluted in extender and then used for fertilization of 2,000 eggs 5 min later, only 3 embryos hatched (Dunham, 1993). Perhaps, catfish males need a sustained LH surge to stimulate the sperm release from the testes and facilitate hand-stripping of larger amounts of semen. Weekly injections of the hypothalamic hormones or a sustained-release GnRH delivery system results in high LH levels for at least 8 weeks and induces both spermatogenesis and spermiation in salmonids (Weil and Crim, 1983), white bass, Morone chrysops (Mylonas *et al.*, 1997), European seabass Dicentrarchus labrax (Sorbera *et al.*, 1996) and other species.

An increased seminal fluid production as a result of testicular hydration after treatment with hypothalamic hormones was not observed in this study, but was reported for Asian catfish, C. macrocephalus after ovaprim treatment (Tambasen Cheong *et al.*, 1995), for rabbitfish, Siganus guttatus, after LHRHa treatment (Garcia, 1991) and for carp, Cyprinus carpio, after combinations of LHRHa and pimozide (Billard *et al.*, 1987).

Effects of crude pituitary extracts

To compare the effects of endogenous with exogenous LH, fish were also injected with suspensions of crude pituitary extracts. The induced plasma LH level, measured 2 h after injection, was higher in cPS-treated fish than in control fish, but lower than the plasma LH levels observed after LHRHa and ovaprim treatments.

A single cPS injection induced a slight testicular hydration response. When a longer-term cPS treatment was used (2 injections of cPS), a significant testicular hydration was observed, with increased GSI, SVSI and IT semen volume and decreased spermatocrit and sperm concentration. It has been described that the seminal fluid production as a result of testicular hydration is the earliest effect of a brief elevation in plasma LH (Mylonas *et al.*, 1997). Because all treatments tested in the present study elevated plasma LH levels but testis hydration was observed only when pituitary extracts were used, it suggests that this process was not caused by a brief elevation in plasma LH. A recent study in African catfish showed that the fingerlike extensions of the seminal vesicle (SV) expressed high levels of the FSH receptor gene (Bogerd *et al.*, 2001). Since the secretory epithelium of the SV is considered to be homologous to FSH receptor expressing Sertoli cells (Loir *et al.*, 1989), it is possible that the relatively high activity of crude pituitary extracts to increase testis and SV weight, possible mainly due to hydration, is related to compounds in the pituitary extract that are able to activate fluid production by testicular Sertoli cells and by the SV epithelium. After all, production of the tubular fluid is a typical Sertoli cell function in mammals (Jegou, 1993). Furthermore, treatment with 2 cPS injections was the only one that increased the number of sperm cells collected per kg bw. As spermatozoa production is a long-term process, the increased number of sperm cells collected was possibly caused by the large amount of seminal fluid produced by the seminiferous tubules that washed out the sperm cells present in the lumen, rather than stimulation of spermatogenesis. Hecht *et al.* (1982) treated the same catfish species with pituitary extract and hCG. Although stripping of semen was not possible, both treatments induced testis hydration and facilitated semen collection from the testis, compared to untreated males. In Asian green catfish, Mystus nemurus, cPS treatment induced increased volume and sperm cells/fish, but did not affect spermatocrit or sperm concentration (Christianus *et al.*, 1996). European catfish, Silurus glanis, after 7 days of continuous semen sampling, also had more sperm cells collected per kg after stimulation with cPS than after GnRH implant or control (Linhart and Billard, 1994). Although not quantified, South American catfish, Rhamdia sapo, males injected with Prochilodus pituitary extracts showed increased volume and decreased sperm concentration within a few hours (Espinach Ros *et al.*, 1984).

Hand-stripping of few viable sperm cells was possible only after 2 injections of cPS and combinations of cPS and ovaprim. Crude pituitary extracts contain all hormones, factors etc produced or stored in the pituitary. One of these hormones, besides LH, may have acted on testicular functionality. It has been demonstrated that high levels of isotocin (the fish oxytocin-like peptide) are present in catfish brain and pituitary (Goos *et al.*, 1997) and that the paraventricular nucleus of the hypothalamus, which uses oxytocin as a neuro-transmitter, has a key role in sexual function in mammals (Mckenna, 1999). Oxytocin levels in the circulation and in the cerebrospinal fluid are greatly increased by sexual arousal, especially during ejaculation. Oxytocin modulates the contractility of reproductive tubules and mediates the sperm transport from the testis, increasing sperm concentration in bulls (Berndtson and Igboeli, 1988) and rams (Assinder *et al.*, 2000). Thus, it is possible that the isotocin present in the pituitary extracts was responsible for facilitating the hand-stripping of semen observed in this study.

Finally, it should be emphasized that the failure to hand-strip African catfish males can also be caused by an anatomical blockage. Sperm ducts in the African catfish are surrounded by up to 50 fingerlike extensions of the SV that may retain semen flow when pressure is applied on male abdomen (Richter, 1976). Recently, we found that 10 of 19 males previously fed 17α-methyltestosterone diet were strippable after cPS treatment. Under dissection, all males from this group possessed undeveloped SV fingerlike extensions (Viveiros *et al.*, 2001).

Acknowledgements

This research was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) - Brazil and is part of the first author's Ph.D. project. We thank Mohamed Yagoub, Erik-Jan Lock and Ronald Booms for assistance on experiments.

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Chapter 3

Effects of 17α-methyltestosterone on seminal vesicle development and semen release response in the African catfish, Clarias gariepinus

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Reproduction (2001), 122:817-827

Abstract

The effects of 17α-methyltestosterone on the seminal vesicle development in African catfish, Clarias gariepinus, were investigated with a view to improving semen collection from this species. Treatment of larvae with dietary 17α-methyltestosterone at 50 ppm for 12-33 or 12-40 days post hatch or at 20 ppm for 12-26, 12-33, 12-40 or 12-47 days post hatch, resulted in inhibition of the seminal vesicle fingerlike extensions in males, but no change in the sex ratio was observed. The minimum effective dose and period to inhibit the seminal vesicle development in all treated males was 20 ppm 17α-methyltestosterone for 12-40 days. Males from this treatment developed normal testes that contained, in some cases, few oocytes that tended to disappear before sexual maturation. After sexual maturation, the semen release response in males with incomplete seminal vesicle was evaluated. Fluid with viable spermatozoa was obtained following two consecutive injections of carp pituitary suspension from 10 out of 19 males previously fed 20 ppm 17α-methyltestosterone for 12-40 or 12-47 days, but from only 4 out of 15 males which had not received any dietary steroid. Intra-testicular semen quality was not affected by 17α-methyltestosterone treatment. These results demonstrate that the 17α-methyltestosterone-induced absence of seminal vesicle extensions facilitated the collection of semen by stripping from this species of fish.

Introduction

The African catfish, Clarias gariepinus (formerly C. lazera; Fishelson *et al.*, 1994), are well adapted to captivity and, at 25°C and 12 h light per day, their pituitary stores large amounts of gonadotropin and shows a limited, continuous secretion of the hormone. This is sufficient for a sustained gametogenesis and gonadal steroid production, but not for spontaneous semen release and male reproductive behavior (van Oordt *et al.*, 1987). For artificial reproduction, males are killed, testes are removed and macerated, and intra-testicular (IT) semen is spread over the eggs.

In a previous study (Chapter 2 in this thesis), we evaluated the effects of drugs that are known to increase gonadotropin plasma levels, on semen output and stripping of semen under abdomen massage, in African catfish. We tested both exogenous gonadotropin by injecting males with Clarias- or carp pituitary suspensions (cPS), and endogenous gonadotropin by stimulating fish pituitary with luteinizing hormone releasing hormone analogue (LHRHa), or LHRHa plus a dopamine antagonist, or ovaprim (salmon gonadotropin releasing hormone analogue plus a dopamine antagonist). Semen production per kg body weight, assessed by IT semen volume and sperm concentration, was improved only after two consecutive injections of cPS. Stripping of a few drops of fluid with some viable spermatozoa was possible after two consecutive injections of cPS, or after combinations of cPS followed by ovaprim injections. Semen volume, spermatocrit and sperm concentration was always lower in the stripped fluid, than in the IT semen from the same group of males. Similar inconclusive results on semen release response and sperm production, after maturational

hormone treatment, were described for Asian catfish, C. macrocephalus (Tan Fermin *et al.*, 1999), Asian green catfish, Mystus nemurus (U. Schneiderat, personal communication) and Blue catfish, Ictalurus furcatus (Dunham, 1993). All together, these results suggest that catfish males kept in captivity are not strippable because of other reasons than the lack of gonadotropin surge, as normally accepted.

Several authors tried to explain the difficulty in stripping of semen in catfishes by stressing that testes are located deep in the body cavity and are covered by other organs (e.g. gut and stomach). During stripping, most of the pressure is then applied on these other organs. Besides, the ripe milt gathers along the convex lobular edge of the testes rather than leaving through the sperm ducts. This condition has been described for the African catfish C. gariepinus (Hogendoorn, 1979), for another African catfish, Heterobranchus longifilis (Oteme *et al.*, 1996), for Channel catfish, I. punctatus (Legendre, 1986) and for Asian green catfish (Christianus *et al.*, 1998). Moreover, a thick interstitial tissue surrounds the spermatogenic cell area in parts of the testis and seminal vesicle (SV; also called glandular testis), possibly blocking sperm flow during abdominal massage (Tan Fermin *et al.*, 1999). And finally, sperm ducts are surrounded by up to 50 fingerlike extensions of the SV (Fishelson *et al.*, 1994) that may retain sperm flow when pressure is applied on the abdomen (Richter, 1976). Preliminary results obtained in our laboratory support the latter hypothesis. We investigated the effects of 17α-methyltestosterone (MT), 17β-estradiol and 11-ketoandrostenedione treatments on gonad development and sex differentiation in African catfish. Hormones were fed to larvae at 50 ppm for 13-40 days post hatch (dph). All treatments failed to induce permanent and complete sex reversal, but, to our surprise, 13 out of 68 MT-treated males sampled at 6 months post hatch (mph) could be stripped with abdominal massage, yielding a few drops of fluid. Upon dissection, all MT-treated males possessed an incomplete development of the SV fingerlike extensions (Eding *et al.*, 1999). Partial inhibition of male gonad development and associated ducts are in agreement with the observed feminizing action of MT and other testosterone derivatives on gonad development in African catfish (Clariidae; van den Hurk *et al.*, 1989) and in Channel catfish (Ictaluridae; Davis *et al.*, 1990).

In this paper, we evaluated the inhibitory effects of 17α-methyltestosterone at different doses and periods of treatment, on the development of the SV fingerlike extensions. Then, we evaluated the semen release response (stripping ability) in males with incomplete SV, after hormonal stimulation.

Material and Methods

These experiments were approved by the Ethical Committee judging Animal Experiments (DEC) of the Wageningen University, number 98046.b.

Husbandry of brood stock and larvae production

The brood stock of African catfish, C. gariepinus, originally came from the Central Africa Republic, and has been bred for several generations in the Hatchery of Fish

Culture and Fisheries Group at Wageningen University, The Netherlands. Three progeny groups were produced, each from a different set of male and female catfish. Females were injected with cPS at 4 mg/kg body weight and stripped 12 h later (25°C). Eggs were fertilized with IT semen obtained from a freshly killed male. After hatching, larvae were reared at a constant temperature (27 ± 0.5°C) and photoperiod (14 h of light per day), in tanks connected to a recirculating system with a flow rate of 1-2 L per minute per tank. Larvae were fed Artemia *ad libitum* for 2-10 dph. Between 7 and 10 dph, Artemia was gradually substituted by a commercial dry feed (Nutra, Trouw France, S.A). From 11 dph onwards, larvae received only dry feed.

Experimental diets.

The experimental diets were prepared by the alcohol evaporation method (Guerrero, 1975). Twenty or 50 mg 17α-methyltestosterone (MT; Sigma Chemical CO. St. Louis. USA) was dissolved in 300 ml ethanol 99.6% and carefully mixed with 1 kg dry feed (MT-diet at 20 or 50 ppm, respectively). Diets were dried at room temperature in an airflow cabinet for 2 days and stored at 4°C. Control-diets were mixed with ethanol only. Diets were prepared every 3 weeks and fed to the fish according to the recommendations of the manufacturer.

During MT treatment, tanks receiving MT-diet were disconnected from the water recirculating system and put on flow through, so that hormone residue could not contaminate the water supply. One week after the end of MT treatments, the treated tanks were reconnected to the water system.

Experimental design

1. 17α-methyltestosterone experiments

A summary of the experimental design is presented in Table 1. In total, three experiments were performed, each with a different progeny group.

Table 1. Experimental design.

Experiments	MT dose (ppm)	period of treatment (days post hatch)	Sampling time (months post hatch)
1	50	12 to 40	4
2	50	12 to 33	4
3	20	12 to 26	4 and 10
		12 to 33	4 and 10
		12 to 40	4, 10 and 14[1]
		12 to 47	4, 10 and 14[1]

[1] At 14 months post hatch, only males were sampled

The aim of the first two experiments was only to confirm the MT inhibitory effects on SV development, described earlier (Eding *et al.*, 1999). In experiment 1, two replicate groups of 500 fish were fed the MT-diet at the same dose of 50 ppm and over the same period of 12-40 dph as tested earlier, while two other groups of 500 fish were fed the control-diet for the same period. In experiment 2, fish were fed the MT-diet at 50 ppm but for a shorter period of 12-33 dph. Due to low numbers of fish, only one treatment and one control group could be compared (no replicates).

Based on the clear inhibitory effects of MT at 50 ppm on SV fingerlike extensions development observed in experiments 1 and 2, a larger experiment was designed. The aim of this experiment was to use a lower MT dose and define the shortest period necessary to inhibit the SV fingerlike extensions development, and to minimize possible inhibitory effects on spermatogenesis caused by higher MT doses. Therefore, in experiment 3, a progeny group was divided over three tanks. Two tanks received the MT-diet (duplicate) at 20 ppm, and one tank received the control-diet. Treatment started at 12 dph and ended at 26, 33, 40 and 47 dph, by taking random samples of 100 fish from each of the 3 tanks at each of the end-days. Fish were, then, fed untreated diet.

Fifty fish per treatment from experiment 1, all fish from experiment 2, and 40 fish per treatment from experiment 3 were sampled at 4 mph, i.e. after sex differentiation. Fish from experiment 3 were also sampled at 10 mph to assess whether the effects of MT treatment would be permanent and irreversible after sexual maturation. At this age, all fish MT-treated for 12-26 and 12-33 dph, all females MT-treated for 12-40 and 12-47 dph, and 30 fish from the control group were sampled. All males MT-treated for 12-40 dph (n=31) and 12-47 dph (n=43) were sexed by urogenital papilla morphology, but only 5 males from each group were sampled to observe the SV development. The other males of these MT-treated groups and some of the control group were saved for a semen release response experiment, at 14 mph.

2. Semen release response experiment

To test the hypothesis whether the absence of the SV fingerlike extensions facilitate stripping of semen, at 14 mph, 19 MT-treated at 20 ppm and 15 MT-control males from experiment 3 received two injections of cPS at 8 and 10 mg/kg body weight, given with a 48-h interval, according to our previous results (Chapter 2 in this thesis). The pituitaries were dissolved in NaCl 0.9% (w/v) and injected intra-muscularly. After the first injection, males were individually housed in 120-L tanks (25°C) to avoid aggressive interaction and to maximize care during the experimental period. Fish were sampled 12 h after the second injection. On the same day as the first cPS injection was given to the other group, the remaining MT-treated (n=22) and MT-control (n=15) males were sampled without any treatment, so that the initial gonad condition could be known.

Parameters used to assess the effects of MT treatments

Fish were sacrificed in tricaine methanesulfonate (TMS; Crescent Research Chemicals, Phoenix, Arizona, USA) at 0.8 g per L tap water. Fish with elongated and pointed urogenital papilla were classified as males and those with a rounded papilla

with a longitudinal cleft, as females. Body weight, gonad weight and SV weight were recorded. Gonado- (GSI) and SV-somatic index (SVSI) were calculated and expressed as percentage of gonad or SV weight, respectively, relative to total body weight. Because at 4 mph, SV weight was usually less than 1 mg, SVSI was calculated only for older fish. The SV development was considered complete when both the SV basal portion and the SV fingerlike extensions were present, and incomplete when only the basal portion was present. At 4 mph, juvenile fish were sexed according to macroscopic observation of the gonads, as: (a) "female", when gonads were reddish, smooth and pear shaped; (b) "male", when white-grey and long; (c) intersex, when a red female-like part was attached to a male-like gonad and; (d) sterile, when gonads were filiforme. At 10 mph, mature fish were sexed according to papilla morphology.

One gonad of each fish from experiment 3 sampled at 4 mph, was fixed in Bouin fluid, dehydrated through a series of alcohol and embedded in paraffin. Five-µm cross sections were stained with Crossman for histological confirmation of the gonad sex, initially scored under macroscopic observation. Gonads were scored as: (a) ovary, when oocytes and ovarian cavity were distinguished; (b) testis, when spermatogenic cysts were evident; (c) intersex, when both oocytes and spermatogenic cysts were present and; (d) sterile, when strands of connective tissue and degenerated seminiferous tubules were observed.

Parameters used to assess semen release response and semen quality

Males were sacrificed in TMS at 0.8 g per L tap water for 40 minutes and weighed. Genital area was dried and hand pressure was applied to the fish abdomen, midway between pectoral and pelvic fins, moving posteriorly right down to the urogenital papilla (van der Waal, 1985). This process was repeated 10-15 times for each male. The stripped fluid, when obtained, was collected in a test tube and volume was measured. Then, males were dissected and testes and SV were carefully removed and weighed. GSI and SVSI were calculated. Testes were slit, IT semen was squeezed out and volume was measured. Stripped fluid and IT semen were kept in crushed ice during all analyses which never took place later than 5 h.

All qualitative and quantitative analyses were carried out in duplicate for each IT and stripped samples. Spermatocrit determination was performed in capillary tubes in a micro-hematocrit IEC MB centrifuge equipped with a capillary tube rotor at 12,800 rpm for 3 minutes, at room temperature. The white packed semen volume was calculated as percentage of total volume. For microscopic sperm cell counting and spectrophotometric measurement, IT semen and stripped fluid were diluted 1000 times with NaCl 0.9% (w/v). A double Burker-Türk counting chamber (W. Schreck, Hofheim TS.) was filled with diluted semen and the number of spermatozoa counted under 400x magnification. Sperm concentration was calculated as the number of spermatozoa per ml of semen. Absorbance was measured with a Beckman DU-64 spectrophotometer at a wavelength of 505 nm in polystyrene disposable cuvettes, according to Ciereszko and Dabrowsky (1993). A solution of NaCl 0.9% (w/v) served as blank. Sperm cell production per fish was calculated based on semen volume and sperm concentration, both using IT samples. A motility test was carried out only in stripped samples to check whether there was any sperm cell alive in this fluid. Fifteen µl of stripped fluid was

mixed with 30 µl of tap water and directly observed under a normal light microscope at 200x magnification. Motility was subjectively classified according to the percentage of moving cells, as: 0, when no movement was observed; 1, when up to 25% cells were moving; 2, when up to 50% cells were moving; 3, when up to 75% cells were moving and; 4, when more than 75% cells were moving.

Immediately after measuring volume, both IT and stripped samples from MT-treated fish, after cPS stimulation, were tested for fertility. One female catfish was treated with cPS at 4 mg/kg body weight and stripped of eggs 12 h later (25°C). Portions of 0.2 g eggs were fertilized with 100 µl of either IT semen diluted 200 times with NaCl 0.9% (w/v) or undiluted stripped fluid. Each semen sample from each male was used to fertilize two portions of eggs (duplicates). The percentage of hatched larvae was calculated 24 h after incubation at 30°C.

Statistical analyses

In each MT-experiment, treatments were compared to the control group of the same progeny. In experiment 1, there was no difference between duplicates, so data were pooled. In experiment 2, there was no duplicate tank, so results represent mean of fish from the same tank. In experiment 3, means and frequencies were compared among treatments and between treatments and control. Because no difference was observed among control fish from different tanks or between MT-treated duplicate tanks, data were pooled. At 10 mph, only 5 males MT-treated for 12-40 and 12-47 dph were sampled. Therefore, GSI and SVSI data from these groups were not tested for significant differences.

When males were treated with cPS, some of them were strippable, and some were not. To detect any difference in IT semen quality caused by removing part of the seminal fluid, data from strippable and non-strippable males were analyzed separated. As no difference was observed between MT-treated duplicate tanks, between males MT-treated for 12-40 and 12-47 dph, or between MT-control groups, data were pooled.

All statistical analyses were carried out using the SAS 6.11 package (SAS Institute Inc., 1990). Frequency distribution of sex ratio, presence of ovary, testis, intersex or sterile gonads scored under histology, and SV development were analyzed by chi-square test of contingency tables. Means ± SD were calculated for GSI, SVSI, hatching rate and for semen analyses data, and were tested for significant differences by ANOVA using the parametric General Linear Model procedure, followed by Duncan's multiple range test for multiple comparisons. For GSI, SVSI, spermatocrit values and hatching rates, the residues of each model were tested for normality using the univariate procedure. Data on spermatocrit and optical density were regressed on sperm concentration per ml to obtain a linear equation. Correlation was expressed as Pearson correlation coefficient (r). P-values <0.05 were regarded significant.

Results

1. *MT-treatments at 50 ppm (experiments 1 and 2)*

In both MT-treated and control groups in both experiments, sex ratio, based on gonad macroscopic observation, was not different from the expected 1:1. Fish scored as "female" had reddish gonads with smooth surface. Fish scored as "male" had grey-white long gonads, but the typical crenated testes normally observed in catfish males were present only in the control group. All MT-treated "males" had abnormal testes, with a little crenated or smooth surface. Fish with intersex gonads were identified only in the group MT-treated for 12-40 dph (n=3). Sterile fish were not observed macroscopically. GSI was not affected ($P>0.05$) by MT treatment, in both experiments (Table 2).

Control "males" had small SV extensions in each lateral side of the sperm ducts. MT-treated "males", however, had only the SV basal portion. The development of the SV fingerlike extensions was inhibited in all MT-treated "males".

Control fish showed a perfect correlation between elongated and pointed urogenital male papilla and presence of a "male" gonad upon dissection. In MT-treated groups, however, the urogenital papilla was of a similar form in both males and females.

2. *MT-treatments at 20 ppm (experiment 3)*

Fish sampled at 4 mph

Sex ratio, based on gonad macroscopic observation, was not influenced ($P>0.05$) by any MT treatment duration.

Fish scored as "female" had reddish gonads with smooth surface, similarly to those observed in experiments 1 and 2. Histological analyses revealed the presence of pre-vitellogenic and vitellogenic oocytes and ovarian cavity in all samples, confirming the previous female score and indicating normal gonad development and normal oogenesis, irrespective of the MT-treatment duration. GSI was higher ($P<0.05$) in females MT-treated for 12-47 dph (0.08%) and lower when treated for 12-33 dph (0.03%), compared to the control group (0.05%; Table 3).

Table 2. Number of African catfish scored as "female", "male", intersex and sterile after macroscopic observation, number of "males" with incomplete seminal vesicle development, body weight and gonadosomatic index, after treatment with 17α-methyltestosterone at 50 ppm and sampled at 4 months post hatch (ph).

Period of treatment		"Female"			"Male"				I	S
	(days ph)	(n)	BW (g)	GSI (%)	(n)	BW (g)	GSI (%)	I-SV (n)	(n)	(n)
Exp. 1	Control	28	132.3 ± 55.4	0.49 ± 0.40[a]	22	124.9 ± 49.5	0.28 ± 0.08[a]	0[b]	0	0
	12-40	24	99.1 ± 37.8	0.36 ± 0.30[a]	23	95.4 ± 41.5	0.13 ± 0.09[a]	23[a]	3	0
Exp. 2	Control	7	70.6 ± 26.6	0.18 ± 0.10[a]	13	60.1 ± 17.1	0.07 ± 0.05[a]	0[b]	0	0
	12-33	9	127.9 ± 61.2	0.12 ± 0.07[a]	7	118.7 ± 19.8	0.05 ± 0.03[a]	7[a]	0	0

BW – body weight
GSI – gonadosomatic index
I-SV – incomplete seminal vesicle development
I – intersex fish
S – sterile fish
[a,b] Means within the same column and experiment followed by the same superscript are not significantly different, according to Duncan's multiple range test, for GSI and to Chi-square analysis for SV development distribution ($P<0.05$).

Table 3. Body weight, gonadosomatic index, number of fish with incomplete seminal vesicle development and histological results of gonads macroscopically scored as "male", "female", intersex and sterile, of African catfish treated with 17α-methyltestosterone at 20 ppm and sampled at 4 months post hatch (ph; experiment 3).

Period of treatment (days ph)	"Female"				"Male"							I	S
	(n)	BW (g)	GSI (%)	O (n)	(n)	BW (g)	GSI (%)	I-SV (n)	T (n)	I (n)	PS (n)	(n)	(n)
control	21	182,3 ± 51.8	0.05 ± 0.02^{b}	21^{a}	17	197,6 ± 91.3	0.03 ± 0.06^{a}	0^{c}	14^{a}	0^{c}	3^{a}	0^{a}	2^{a}
12-26	16	204,8 ± 66.8	0.07 ± 0.02^{ab}	16^{a}	24	182,3 ± 55.4	0.01 ± 0.01^{b}	7^{b}	24^{a}	0^{c}	0^{b}	0^{a}	0^{a}
12-33	16	189,3 ± 80.7	0.03 ± 0.02^{c}	16^{a}	23	147,9 ± 64.1	0.01 ± 0.01^{b}	10^{b}	19^{a}	4^{b}	0^{b}	0^{a}	0^{a}
12-40	14	149,7 ± 117.6	0.07 ± 0.04^{ab}	14^{a}	25	138,5 ± 44.6	0.02 ± 0.02^{ab}	25^{a}	16^{b}	4^{b}	5^{a}	0^{a}	1^{a}
12-47	17	152,5 ± 37.5	0.08 ± 0.03^{a}	17^{a}	23	144,7 ± 48.9	0.02 ± 0.01^{ab}	23^{a}	7^{c}	11^{a}	5^{a}	0^{a}	0^{a}

BW – body weight

GSI – gonadosomatic index

Histological analysis confirmed the macroscopic score of all "female" (presence of ovary – O), sterile (S) and intersex (I) gonads.

"Male" gonads were histologically classified as testis (T), intersex (I) or testis partially sterile (PS).

I-SV – incomplete seminal vesicle development

$^{a-c}$ Means within the same column followed by the same superscript are not significantly different, according to Duncan's multiple range test for GSI and to Chi-square analysis for SV development, ovary, testis, intersex, sterile and partially sterile gonads distribution ($P<0.05$).

Fish scored as "male" had grey-white long gonads, but the typical crenated testis was observed only in control fish, in fish MT-treated for 12-26 dph and in a few fish MT-treated for 12-33 dph. All "males" MT-treated for 12-40 and 12-47 dph had abnormal testes with a little crenated or smooth shape. Histological analyses of these "male" gonads revealed three categories: testis, intersex and partially sterile gonads (Table 3). Intersex gonads, with few oocytes among testicular tissue (Figure 1), were observed in 4 "males" treated for 12-33 dph, in 4 "males" treated for 12-40 dph and in 11 "males" treated for 12-47 dph. Partially sterile gonads, with areas of vacuolized connective tissue and areas of normal seminiferous tubules, were observed in 5 "males" treated for 12-40 dph, in 3 "males" treated for 12-47 dph and in 3 "males" from the control group. Progression of spermatogenesis, however, was observed along the length of the seminiferous tubules, in all testis, intersex and partially sterile gonads. Each tubule was surrounded by connective tissue with blood vessels and contained irregular cysts of germ cells in different stages of development, including spermatozoa in most of the samples. GSI was lower ($P<0.05$) in "males" fed MT for 12-26 and for 12-33 dph, than in control (Table 3).

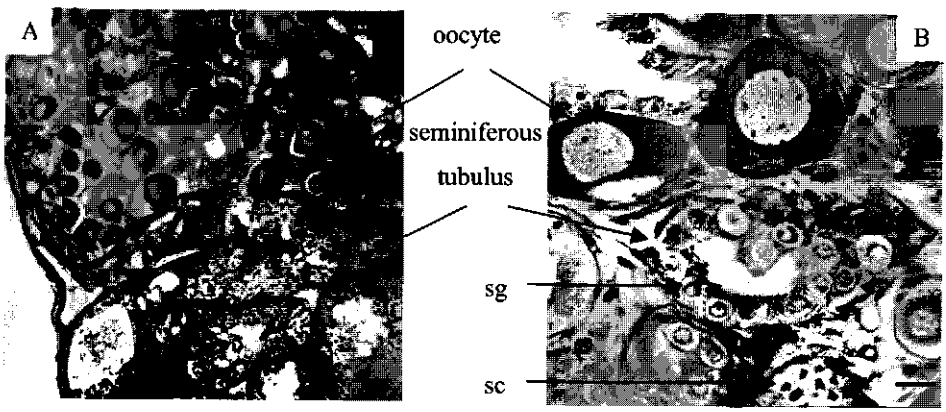

Figure 1 – Intersex gonad identified in an African catfish, Clarias gariepinus, "male" fed 17α-methyltestosterone for 12-40 days post hatch and sampled at 4 months post hatch. A. 100x magnification; horizontal bar = 100 μm. B. 400x magnification; horizontal bar = 25 μm. sg = spermatogonium; sc = spermatocyte

Table 4. Number of females, males with unilateral sterile gonads or with incomplete seminal vesicle development, intersex and sterile fish, scored under gonad macroscopic observation, and gonad- and seminal vesicle-somatic index, after treatment with 17α-methyltestosterone at 20 ppm and sampled at 10 months post hatch (ph; experiment 3).

Period of treatment (days ph)	Female		Male					I	S
	(n)	GSI (%)	(n)	GSI (%)	SVSI (%)	Unilateral sterile (n)	I-SV (n)	(n)	(n)
Control	14	9.4 ± 3.3^{a}	16	0.68 ± 0.18^{a}	0.29 ± 0.06^{a}	0^{a}	0^{a}	0	0
12-26	38	8.1 ± 2.6^{ab}	34	0.57 ± 0.19^{a}	0.18 ± 0.07^{b}	2^{a}	0^{a}	0	0
12-33	28	6.8 ± 3.5^{b}	36	0.66 ± 0.16^{a}	0.14 ± 0.11^{b}	3^{a}	4^{a}	0	0
12-40	31	6.9 ± 3.5^{b}	5	0.87 ± 0.32^{N}	0.10 ± 0.01^{N}	0^{N}	5^{N}	0	0
12-47	31	8.3 ± 3.5^{ab}	5	1.24 ± 0.57^{N}	0.08 ± 0.02^{N}	0^{N}	5^{N}	0	0

GSI – gonadosomatic index
SVSI - seminal vesicle-somatic index
Unilateral sterile – males with one normal testis and one sterile gonad
I-SV – incomplete seminal vesicle development.
I – intersex fish
S – sterile fish
N – Because only 5 males were tested at this age, statistical analysis were not carried out with this data.
[a,b] Means within the same column followed by the same superscript are not significantly different, according to Duncan's multiple range test for GSI and SVSI, and to Chi-square analysis for unilateral sterile gonads and SV development distribution ($P<0.05$).

Intersex fish were not macroscopically observed. One sterile fish was identified in the group MT-treated for 12-40 dph and 2 in the control group, and these gonads were not considered for GSI calculation. Histological analyses confirmed the presence of vacuolized connective tissue and the absence of germ cells in all gonads macroscopically scored as sterile (Table 3).

Control "males" had small SV extensions in each lateral side of the sperm ducts. All "males" MT-treated for 12-40 and 12-47 dph, however, had only the SV basal portion. The development of the SV fingerlike extensions was inhibited in these groups of males.

Control fish showed again a perfect correlation between elongated and pointed urogenital male papilla and presence of "male" gonad upon dissection. In MT-treated groups, however, the urogenital papilla was of a similar form in both males and females.

Fish sampled at 10 mph

Sex ratio, based on papilla form and including males to be sampled at 14 mph, was not significantly different from 1:1. Ovaries had a normal morphology with post-vitellogenic oocytes, in both MT-treated and control females. GSI was significantly reduced in females MT-treated for 12-33 and 12-40 dph, compared to control females. All males had white, crenated testes and semen. The abnormal testis shape, observed during sampling at 4 mph, was not observed. Males showing one normal testis and one sterile gonad (unilateral sterile) were present in groups MT-treated for 12-26 dph (n=2) and for 12-33 dph (n=3). Male GSI was not affected by any MT treatment. Intersex gonads were not macroscopically identified (Table 4).

Control males had a well-developed SV with long fingerlike extensions in each lateral side of the sperm ducts. However, 4 out of 36 males MT-treated for 12-33 dph and all males MT-treated for 12-40 and 12-47 dph, had only the SV basal portion. The development of the SV fingerlike extensions was inhibited in these males. Consequently, SVSI was significantly reduced ($P<0.05$) in all MT-treated males, compared to the control (Table 4).

Urogenital papilla was typically pointed and elongated in males and rounded in females, in both MT-treated and control groups. Males treated for 12-40 and 12-47 dph, however, had a little bit smaller papilla compared to the control males.

3. Semen release response experiment

Testis morphology was normal and similar to that observed at 10 mph. Unilateral intersex gonads were macroscopically observed in 2 MT-treated males. Unilateral sterile gonads were observed in one male from the control group and in 6 MT-treated males. Testes weight, GSI and IT semen volume, however, were not affected in these unilateral sterile and intersex fish, compared to normal males.

Control males had a well-developed SV with long fingerlike extensions in each lateral side of the sperm ducts. However, in all MT-treated males, the development of the SV fingerlike extensions was suppressed (Figure 2), as indicated by the significantly reduced SVSI, compared to the control group (Table 5). Urogenital papilla morphology was similar to that observed at 10 mph.

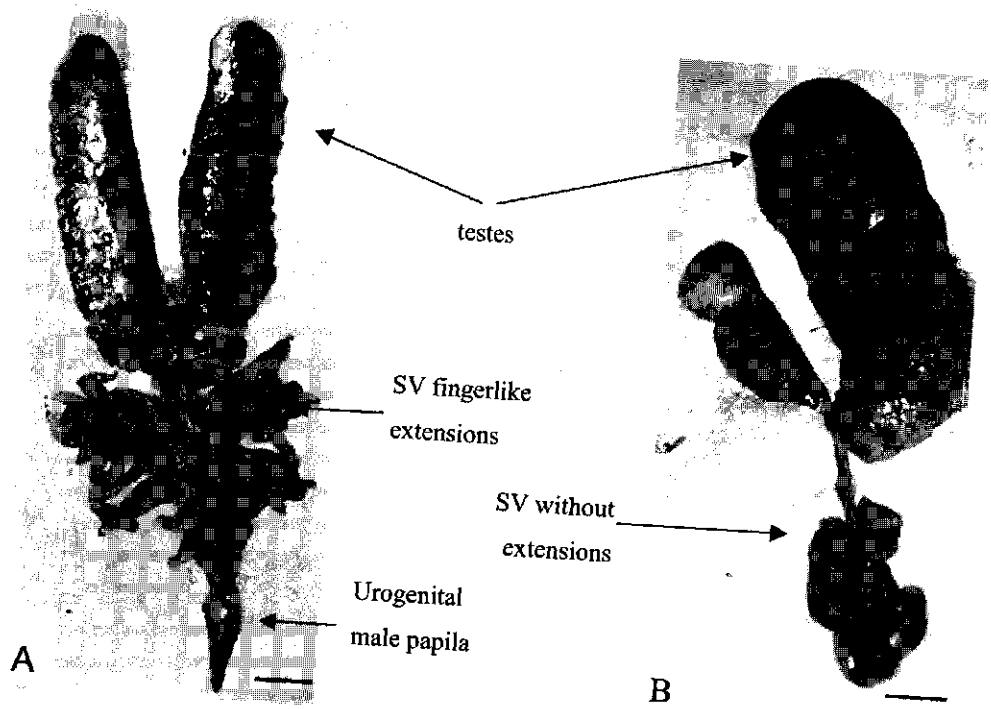

Fig. 2. Reproductive organs of African catfish, Clarias gariepinus, male at 14 months post hatch. A – normal male. B – male fed 17α-methyltestosterone at 20 ppm for 12-47 days post hatch; observe the absence of seminal vesicle (SV) fingerlike extensions;horizontal bar = 1.5 cm.

In MT-control fish, stripping of semen was possible in 4 out of 15 normal males, after cPS stimulation. None of the cPS-control fish was strippable. The amount of stripped fluid was small (range 0.3-0.5 ml). Mean spermatocrit values (2.4%), sperm concentration (0.5 x 10^9 cells/ml) and sperm motility (score: 0.8) were low (Table 5). In IT semen samples, optical density was significantly higher in non-strippable males, compared to strippable males, after cPS treatment. There were no differences in the other parameters analyzed between strippable and non-strippable males, although GSI for non-strippable males was higher ($P<0.05$) than control, but for strippable males it

was not different. Taking together data from strippable and non-strippable males, GSI and IT semen volumes were significantly higher in cPS-treated males than in cPS-control males (Table 6). A larger IT volume was accompanied by a lower concentration ($P<0.05$) in cPS-treated fish, so that sperm production was not affected.

Table 5. Stripped fluid quality of 14 months old African catfish treated with two cPS injections at 8 and 10 mg/kg given with a 48-h interval and sampled 12 h after the second injection (stripping experiment).

	MT – treated[1]		MT – control[2]	
	cPS–treated	cPS–control	cPS–treated	cPS–control
Treated fish (n)	19	22	15	15
Strippable fish (n)	10	0	4	0
Stripped volume (ml)	0.7 ± 1.2		0.5 ± 0.1	
Spermatocrit (%)	2.8 ± 2.9		2.4 ± 2.8	
Cells/ml (x 10^9)	0.9 ± 0.8		0.5 ± 0.3	
Sperm motility[3]	1.3 ± 1.1		0.8 ± 0.5	

[1]Males with incomplete seminal vesicles, due to 17α-methyltestosterone treatment during larval stages

[2] Males with normal seminal vesicles, fed control-diet during larval stages

[3] Motility scored as: 0 (no moving cells), 1 (1-25%), 2 (25-50%), 3 (50-75%) and 4 (more than 75% moving cells)

In MT-treated fish, stripping of semen was possible in 10 out of 19 males with incomplete SV, after cPS stimulation. None of the cPS-control fish was strippable. The amount of stripped fluid was small (range 0.2-4.0 ml). Spermatocrit values (2.8%) and sperm concentration (0.9 x 10^9 cells/ml) were low, but sperm motility (mean score: 1.3) was reasonable (Table 5). There was no difference in IT semen data among strippable and non-strippable males, after cPS treatment. However, SVSI and sperm production for strippable males was higher ($P<0.05$) than the control, while for non-strippable males these values were not different. Taking together data from strippable and non-strippable males, GSI, SVSI and IT semen volume were significantly higher in cPS-treated males compared to cPS-control males (Table 6). A larger IT semen volume was accompanied by a lower spermatocrit and sperm concentration ($P<0.05$) in cPS-treated fish, so that sperm production was not affected.

MT-treatments did not affect the sperm fertilizing capacity, after cPS stimulation. Hatching rates produced with stripped fluid (58.9%) were not different ($P<0.05$) from those produced with IT semen from the same males (56.5%) nor from those produced with IT semen from non-strippable males (61.8%).

A low relationship between spermatocrit and sperm concentration ($r=0.594$) and a high relationship between optical density at 505 nm and sperm concentration ($r=0.831$) was observed when data from all 71 fish was analyzed together.

Table 6. Intra-testicular semen quality of 14 months old African catfish treated with two cPS injections at 8 and 10 mg/kg given with a 48-h interval and sampled 12 h after the second injection (stripping experiment).

	MT - treated[1]			MT – control[2]		
	cPS - treated		cPS - control	cPS - treated		cPS - control
	strippable	non-strippable	non-strippable	strippable	non-strippable	non-strippable
Fish (n)	10	9	22	4	11	15
GSI (%)	1.92 ± 0.78 [a]	2.11 ± 1.31 [a]	1.15 ± 0.22 [b]	1.10 ± 0.36 [ab]	1.63 ± 0.48 [a]	1.01 ± 0.22 [b]
SVSI (%)	0.14 ± 0.04 [a]	0.10 ± 0.03 [ab]	0.08 ± 0.03 [b]	0.58 ± 0.19 [a]	0.62 ± 0.20 [a]	0.49 ± 0.27 [a]
Volume (ml)	27.7 ± 16.3 [a]	29.6 ± 28.6 [a]	10.3 ± 5.3 [b]	16.1 ± 10.5 [a]	17.9 ± 9.8 [a]	11.0 ± 5.5 [b]
Spermatocrit (%)	10.7 ± 3.8 [b]	8.3 ± 3.5 [b]	13.3 ± 7.3 [a]	12.5 ± 5.9 [b]	12.9 ± 4.8 [b]	23.9 ± 11.1 [a]
Cells/ml (x 10^9)	4.7 ± 2.2 [b]	3.9 ± 1.9 [b]	7.2 ± 1.8 [a]	4.9 ± 2.3 [b]	6.8 ± 2.7 [b]	9.2 ± 4.3 [a]
Cells/kg (x 10^9)	48.4 ± 17.4 [a]	40.3 ± 26.4 [a]	40.8 ± 12.6 [a]	29.0 ± 12.3 [b]	55.8 ± 11.6 [a]	40.1 ± 22.5 [ab]
Cells/fish (x 10^9)	107.1 ± 38.8[a]	86.1 ± 55.7 [ab]	69.3 ± 29.2 [b]	69.2 ± 39.2 [a]	107.5 ± 45.2 [a]	102.0 ± 68.8 [a]

[1] Males with incomplete seminal vesicles, due to 17α-methyltestosterone treatment during larval stages

[2] Males with normal seminal vesicles, fed control-diet during larval stages

[a,b] Means within the same MT group (treated or control) and in the same row followed by the same superscript are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

Discussion

The presence of an Y chromosome in mammals and in some amphibians results in the development of a testis. In the absence of an Y chromosome, an ovary will develop. From this point onwards, further sexual differentiation is mediated by hormones produced in the gonads (mainly sex steroids). Thus, secondary sexual characteristics are hormonally mediated and normally confined to one or other sex, while gonadal sex determination is controlled by genes on a sex chromosome (Short, 1982). However, in gonochoristic fish, including catfish species, sex is predominantly determined genetically, but the process can be overruled by environmental factors (mainly temperature) and exogenous steroid hormones bringing about a complete, functional gonad sex reversal (Baroiller *et al.*, 1999). However, it should be emphasized that gonadal sex phenotype in gonochoristic fish can only be manipulated around the phenocritical period of gonad commitment (Davis *et al.*, 1992; Baroiller *et al.*, 1999). African catfish larvae kept under 25ºC show the first signs of histological sex differentiation at 28 dph, with the presence of ovarian cavity and germ cells with nuclei in meiotic prophase in half of the animals of a mixed population, while the other half remains undifferentiated (presumably, the male half). The presence of spermatogonia and Leydig cells are visible at 42 dph (van den Hurk *et al.*, 1989), and the first signs of SV development is observed later on, when fish is of approximately 70 mm total length (Fishelson *et al.*, 1994). Thus, any attempts to manipulate sex differentiation in African catfish should start before 28 dph. In the present study, we treated catfish larvae with 17α-methyltestosterone – a synthetic steroid that has both masculinizing (Liu and Yao, 1995) and feminizing effects (Davis *et al.*, 1990), and kept fish at 27ºC – a temperature known to produce a normal sex ratio in catfish (Patiño *et al.*, 1996). We tested low doses and short treatments aiming to affect only the SV development in males, and not to sex reverse their gonads.

Sex ratio, assessed by gonad morphology, was never influenced by any MT dose or period of treatment tested, although all treatments started before histological sex differentiation. On the other hand, all "males" MT-treated at 20 ppm for 12-40 and 12-47 dph, and all "males" MT-treated at 50 ppm, showed a shift in gonad shape from a male-like crenated shape, to a female-like smooth shape. Besides, the histological results from MT-treated groups in experiment 3, showed that some of the gonads morphologically scored as "male" were, in fact, intersex. At the longest treatment (12-47 dph), for instance, 11 intersex were identified among 23 "males". Because a feminizing effect of MT treatment has been described in African catfish (van den Hurk *et al.*, 1989; Eding *et al.*, 1999), we can predict that these intersex were genetically males. Longer treatment and/or higher MT dose would probably complete the testis feminization and result in a normal and functional ovary (XY females). The feminizing effect of androgens was first reported in Channel catfish (Goudie *et al.*, 1983), where 99% of larvae fed 17α-ethynyltestosterone at 6 mg/kg, for 21 days after yolk-sac absorption, developed in females. These sex reversed females were stable for at least 2.5 years and showed normal ovary development.

The ontogenesis of the SV begins as simple protrusions from the vas efferent, becoming more complex with age. During this process, the SV base form up to 50 fingerlike extensions, each 3-4 cm long, anchoring on each side of the sperm ducts. In adult fish, the basal portion is flattened and wide, and contains the fingerlike extensions (Fishelson *et al.*, 1994). MT treatments inhibited the development of the SV fingerlike extensions in all males fed MT-diet at 20 ppm for both 12-40 and 12-47 dph and at 50 ppm for both 12-33 and 12-40 dph. The inhibition of the SV development was stable for at least 14 months and did not affect testis development or GSI, despite some cases of unilateral intersex or unilateral sterile gonads.

The primary function of the SV is the production of a fluid containing various active compounds including steroid glucuronides. These glucuronides act as sex pheromones that promote ovarian growth and development in pubertal females (van Weerd, 1990), and activate female responsiveness and induce ovulation, thereby improving fertilization in adults (Resink, 1988). Males with incomplete SV, however, were able to sexually stimulate females kept in the same tank, as the control males were. At 10 mph, females fed MT-diet at 20 ppm for 12-47 dph, had a GSI of 8.3%, not significantly different from control females (9.4%). In the same group, males had the lowest SVSI (0.08%; data of only 5 males) compared to control males (0.29%). If males with incomplete SV were not able to stimulate normal ovarian development, we would expect a decrease in female GSI as MT-treatment duration was increasing. In fact, it did happen for 12-33 and 12-40 dph periods, but not for 12-47 dph. Therefore, we speculate that GSI variance in females was caused by MT treatment rather than lack of male stimuli. It seems that MT affected only the SV development, but not SV functionality. However, as all fish received water supply from the same recirculating system, perhaps pheromones produced by control males were diluted in the water system, and may have stimulated females in other tanks.

MT treatments at both 20 and 50 ppm also delayed urogenital papilla differentiation. At 4 mph, while control fish could be sexed by papilla morphology (pointed and elongated in males and round with a longitudinal cleft in females), MT-treated fish had, in general, a papilla with similar morphology in males and females. After sexual maturation (10 mph), all MT-treated fish showed a differentiated male and female papilla, corresponding to a male and female gonad. Males from long treatment groups, however, had a shorter papilla compared to control males. Previous study with catfish fed MT-diet at 50 ppm for 13-40 dph similarly showed no correlation between papilla sex and gonad sex at young age. At 40 dph, 100% of these fish had a male-like papilla, while, upon dissection, 90% of them had female gonad (Eding *et al.*, 1999).

Different from observed earlier in MT-treated fish (Eding *et al.*, 1999), stripping of semen was possible only after cPS treatment, but the absence of the SV fingerlike extensions seems to facilitate this process. Ten out of 19 cPS-treated males with incomplete SV (MT-treated) were strippable, compared to only 4 out of 15 cPS-treated males with normal SV. The sperm concentration in the stripped fluid from MT-treated fish was slightly higher and these sperm cells were generally more active (motility), compared to those in the stripped fluid from MT-control fish. Sperm auto-activation, however, was always present. It is possible that, during stripping, semen was contaminated with urine that activates sperm cells, as it normally occurs during semen

collection in European catfish, Silurus glanis (Linhart and Billard, 1994). Hatching rates produced with the stripped fluid (58.9%) were not different from that produced with IT semen from the same MT-treated males (56.5%). The mean sperm:egg ratio for stripped fluid was 210 x 10^3 and for IT semen the ratio was 21.0 x 10^3 sperm cells/egg. Because the effective insemination ratio for fresh semen, in this species has been suggested as 15 x 10^3 sperm cells/egg (Rurangwa *et al.*, 1998), we probably overestimated the sperm quality of the stripped fluid. High hatching rates were also produced with stripped fluid in the same fish species, after treatment with Clarias pituitary suspension (van der Waal, 1985), but these males were captured from the wild, during their natural breeding season.

Spermatogenesis, according to the parameters analyzed in this study, was not affected by MT treatment, compared to MT-control group. CPS treatment increased GSI and IT semen volume and decreased spermatocrit and sperm concentration, in all cPS-treated fish, compared to cPS-control fish. Sperm cell production per fish, on the other hand, was not increased. Testicular hydration with increased semen volume and decreased spermatocrit, after pituitary suspension treatments, was described for African catfish (Hecht *et al.*, 1982), European catfish (Linhart and Billard, 1994) and South American catfish Rhamdia sapo (Espinach Ros *et al.*, 1984).

The minimum effective MT dose to inhibit the SV development in African catfish males was 20 ppm, for 12-40 dph. Males developed normal testes with occasional oocytes that tended to disappear before sexual maturation. Applying pressure directly on testes was always very difficult, as, in this species, testes are located deep at the posterior end of body cavity and are covered by abdominal organs. The absence of the SV fingerlike extensions facilitated stripping when compared to normal fish, as observed by Eding *et al.* (1999). However, stripping was possible only when fish were treated with a maturational hormone, in this case, cPS. MT treatments have not affected semen quality or quantity.

Acknowledgements

The authors express their gratitude to Bernardo Vargas for statistic support and to Yonas Fessehaye, Mohamed Yagoub, Erik-Jan Lock and Menno ter Veld for technical assistance during sampling. This study is part of the 1st author Ph.D. project and was supported by Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

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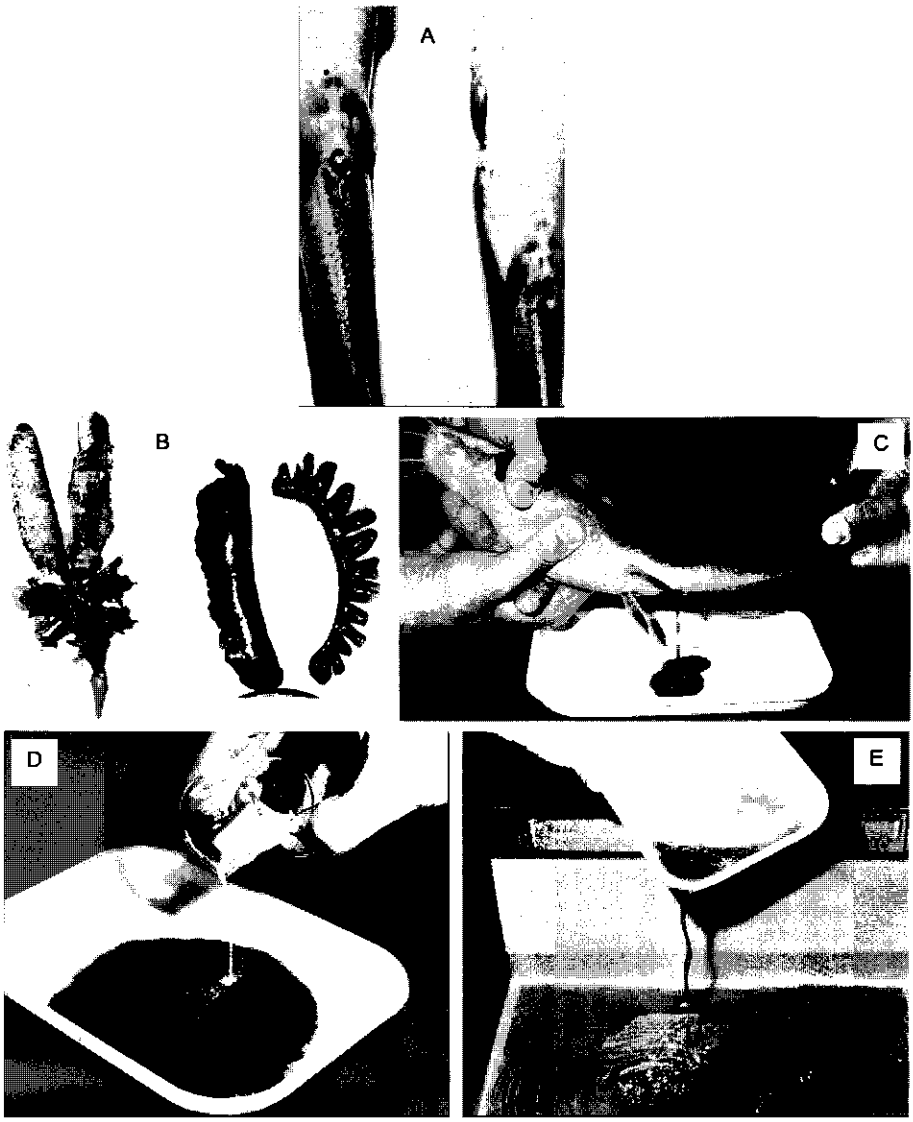

Picture 2. Artificial fertilization in the African catfish. Male (top left) and female (top right) are sexually distinguished by urogenital papilla (A). To collect semen, males are sacrificed, testes are removed (B) and intratesticular semen is squeezed out. Females are stripped of eggs (C). Eggs and intratesticular semen are mixed (D). Water is added to start fertilization. Fertilized eggs are incubated in thermoregulated water at 27-30°C (E).

Chapter 4

Hand-stripping of semen after treatment with oxytocin, in African catfish, Clarias gariepinus, males with undeveloped seminal vesicles

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To be submitted

Abstract

The aim of this study was to investigate the *in vitro* and *in vivo* effects of muscle contractors on semen release in African catfish, Clarias gariepinus. For *in vitro* experiments, testis slices were incubated with oxytocin, vasopressin, epinephrine and prostaglandin F2α as well as LH and Clarias pituitary extract, for 15 and 30 min. Oxytocin increased the optical density and sperm concentration of the medium. The other drugs did not affect optical density or the sperm concentration of the medium. For *in vivo* experiments, oxytocin was tested in combination with carp pituitary suspension (cPS) using two groups of fish: normal males, and males that had been treated with 17α-methyltestosterone during larval stages to inhibit the seminal vesicle development (MT males). Two treatments were tested: 1) two injections of cPS at 8 and 10 mg/kg, respectively, given with a 48-h interval; fish were sampled 12 h later; and 2) the same cPS treatment as before, followed by one injection with oxytocin at 5 IU/kg, 12 h later; fish were sampled 30 min after the oxytocin injection. While there was no effect of oxytocin on stripping, the MT-induced absence of the seminal vesicle, in combination with cPS treatment, facilitated stripping of semen. Taking together cPS and cPS-oxytocin treated fish, 87% MT males and 60% normal males were strippable. The stripped semen volume was low in both groups but MT males produced significantly higher ($P<0.001$) hatching rates (63.1%) than normal males (2.1%). A significant increase in gonadosomatic index and intratesticular semen volume and a decreased intratesticular sperm concentration was observed as a result of the cPS treatment, in both normal and MT males. Intratesticular spermatocrit values after cPS-oxytocin treatment were similar to control, while after cPS treatment these values were lower than control, in both groups of male. There was a consistent but not significant tendency of higher hatching rates with either stripped or intratesticular semen after cPS-oxytocin treatment in both MT and normal males. An adjustment of oxytocin dose and/or latency time is needed to further investigate the role of oxytocin in semen release and sperm quality in African catfish.

Introduction

As the majority of the fish species reared in captivity, the African catfish, Clarias gariepinus exhibits reproduction dysfunction. The absence of the natural conditions of spawning grounds and reproductive cues in catfish farms, results in a limited and continuous secretion of the gonadotropin (luteinizing hormone; LH) by the fish pituitary. This is sufficient for a sustained gametogenesis and gonadal steroid production, but not for spontaneous semen release and male reproductive behavior (van Oordt *et al.*, 1987). The failure to release a large amount of LH is not caused by insufficient storage of the hormone in the gonadotrophes (de Leeuw, 1985). It is more likely that the gonadotropin releasing hormone (GnRH) is not released or is prevented from eliciting its effects (Goos and Richter, 1996). In a previous study (Chapter 2 in this thesis), the effects of treatments that increase plasma LH levels, such as injections of pituitary extracts and hypothalamic hormones were evaluated in hatchery-bred African

catfish males. All treatments with hypothalamic hormones in combination or not with a dopamine antagonist produced a significant LH surge 2 h after injection. Stripping of semen was possible only after ovaprim (salmon GnRH analogue plus a dopamine antagonist) treatments, but the few sperm cells present in the fluid were not motile. Single injections of pituitary extracts not only produced increased plasma LH levels, but also caused a slight testicular hydration. However, stripping of semen was not possible. Only when two injections of carp pituitary suspensions (cPS) were used, a significant testicular hydration was noted, with increased intratesticular (IT) semen volume and decreased sperm concentration. Stripping of semen was possible in 4 out of 5 treated males, although volume and sperm concentration were very low, compared to IT semen from the same males.

Crude pituitary extracts contain several other hormones, factors, peptides, etc. besides LH, that may have facilitated stripping of semen after cPS treatment, as observed in the previous study described above. For instance, high levels of isotocin (the fish oxytocin-like peptide) are present in catfish brain and pituitary (Goos *et al.*, 1997). In mammals, various muscle contractors stimulate sperm transport in the reproductive tracts and increase the number of sperm cells in the ejaculate. In rats, prostaglandins, mainly F2α, modulate the contractility of seminiferous tubules and mediate the sperm transport from the testis (Farr and Ellis, 1980). In sheep (Assinder *et al.*, 2000) and in bulls (Berndtson and Igboeli, 1988), oxytocin has a stimulatory effect on the contractility of the seminiferous tubules during ejaculation, increasing semen volume and concentration in the ejaculate. The related peptide vasopressin also increases epididymal contractions in rams (Knight, 1974) and rabbits (Kihlstrom and Agmo, 1974), although higher doses of vasopressin are required to produce an effect similar to oxytocin. Incubation with epinephrine induced follicle contraction with ovulation in brook trout, Salvelinus fontinalis (Goetz and Bradley, 1994). To the best of our knowledge, these hormones have never been evaluated on their ability to induce a semen release response in catfish species.

In this study, the effects of oxytocin, vasopressin, epinephrine and prostaglandin F2α as well as LH and Clarias pituitary extract on semen release in African catfish, were evaluated *in vitro*, with testicular incubations. The hormone that increased sperm concentration of the incubation medium, was then tested *in vivo* using two groups of fish: normal males and males that were expected to have undeveloped seminal vesicles (SV) as a result of dietary 17α-methyltestosterone (MT) treatment. It has been speculated that the SV fingerlike extensions around the sperm ducts may retain sperm flow during hand-stripping (Richter, 1976). Recently, we demonstrated that MT treatment during larval stages was effective in inhibiting the development of SV extensions and that the semen release response of these males following two consecutive injections of cPS was improved when compared to normal males (Viveiros *et al.*, 2001).

Material and Methods

This experiment was approved by the Ethical Committee judging Animal Experiments (DEC) of Wageningen University.

Animals

The brood stock of African catfish, C. gariepinus, originally came from the Republic of Central Africa and Cameroon, and has been bred for several generations in the hatchery of Fish Culture and Fisheries Group at Wageningen University, The Netherlands. Fish from this stock (2-3 kg and approximately 18 months of age) were used as testes donor for *in vitro* experiments and as normal males for *in vivo* experiments. MT males were produced at Fleuren's catfish farm (Someren, The Netherlands), according to the protocol described previously (Viveiros *et al.*, 2001). Briefly, dietary 17α-methyltestosterone (MT; Sigma Chemical CO. St. Louis. USA) was prepared using the alcohol evaporation method (Guerrero, 1975), by dissolving MT 20 mg in 300 mL ethanol 99.6% and carefully mixing with 1 kg dry feed (20 ppm). MT-diet was dried at room temperature in an airflow cabinet for 2 days and stored at 4°C. Larvae were fed MT-diet from day 12 to 40 post hatch. At 3 months post hatch, MT-treated fish were transferred to the hatchery of Wageningen University, and kept until the beginning of experiments, after sexual maturation (2-3 kg and 12 months of age). All fish were maintained at a constant temperature (26 ± 0.5°C) and photoperiod (12 h of light per day), in tanks with well-aerated water connected to a recirculating system equipped with a biofiltration unit. The flow rate was 8 to 12 L/min and the water quality was monitored daily. NH_4^+ and NO_2 were kept below 1 ppm and pH was kept at 6.5. Fish were fed trout pellets (Trouvit, The Netherlands) *ad libitum*.

Hormones

For *in vitro* experiments, oxytocin, [Arg^8]-vasopressin, epinephrine bitartrate and prostaglandin F2α (all from Sigma Chemical CO, St. Louis, USA) were diluted in 2 mL of Goldfish Ringer (NaCl 124.91 mM; KCl 2.35 mM; MgCl 1.92 mM; $MgSO_4$ 0.58 mM; $CaCl_2$ 3.15 mM; hepes 3.99 mM; glucose 5.55 mM; pH 7.6) and stored at -20°C. Partly purified LH containing 20% LH and Clarias pituitary extract containing LH 1171 μg/mL PBS, a gift from Dr. R.W. Schulz, Utrecht University, were stored at -20°C. Prior to each incubation trial, hormones were thawed and further diluted with Goldfish Ringer to achieve the tested doses per well. Each well contained a total volume of 3 mL of Goldfish Ringer with the addition or not (control wells) of hormones.

For the semen release experiment, cPS (Crescent Research Chemicals, Phoenix, Arizona, USA) was dissolved in NaCl 0.9% (w/v) to a final concentration of 16 and 20 mg/mL and injected intramuscularly always at the same volume of 0.5 mL/kg body weight (bw). Oxytocin (stored in a stock solution with Goldfish Ringer) was further diluted in NaCl 0.9% (w/v) to a final concentration of 10 IU/mL and injected intravenously also at 0.5 mL/kg bw.

In vitro experiments

In vitro experiments were carried out using a static incubation system with testicular slices. Mature catfish males were anaesthetized with tricaine methanesulfonate (TMS; Crescent Research Chemicals, Phoenix, Arizona, USA) at 0.8 g/L, and sacrificed by spinal transection. In each experiment, 2 testes were used. Each testis was cut in 9 slices of *ca.* 3 mm each and placed in a Petri dish with 25 mL of Goldfish Ringer for 1 min. The medium was refreshed once, so that the excess of sperm could be washed out. Slices from 2 testes were divided over three 6-well Coastar plates. Hormone at 2 different doses (oxytocin 1 and 10 IU - *ca.* 2 and 20 μg, respectively; prostaglandin F2α 1 and 10 μg; vasopressin 0.2 and 2 μg; or epinephrine 1 and 10 μg) was previously added to two groups of 6 wells each. The third group of 6 wells served as control and contained medium only (Figure 1A). Each hormone was tested in the same set of 3 plates as a control group, thus comparison was possible only within each set. The testis slices were removed either 15 or 30 min after incubation. The sperm concentration in the wells was assessed by measuring the optical density of the incubation medium (see below).

In one experiment, the efficacy of oxytocin on testis contraction was compared with crude pituitary extract (that contains, among other substances, LH and isotocin) and partly purified LH. Twenty four slices from 2 testes were randomly divided over 24 wells. Each group of 6 wells contained one of the following substances: partly purified LH 1500 ng (containing LH 300 ng), Clarias pituitary extract containing LH 300 ng, oxytocin 10 IU or Goldfish Ringer only (Figure 1B). The testis slices were removed 30 min after incubation and the optical density of the medium was measured. All trials with oxytocin were repeated with a different male to check for repeatability of the results.

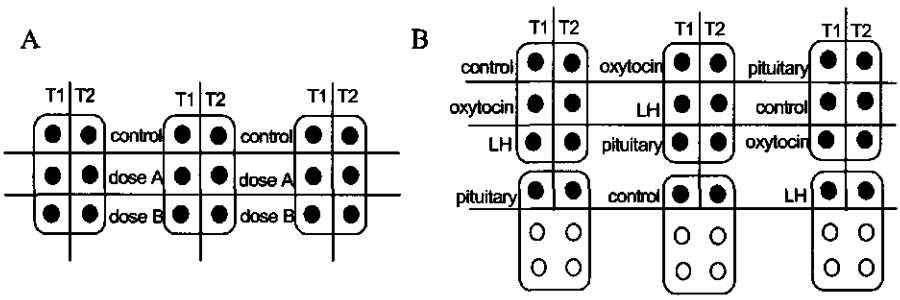

Figure 1. Schematic representation of testicular incubation with the same hormone at 2 doses (A) or combination of 3 substances (B), used during *in vitro* experiments with African catfish. Each incubation trial was carried out with 2 different testes (T1 and T2) and each well contained 3 mL of Goldfish Ringer in combination or not with hormones.

Semen release response experiment

During *in vitro* experiments, oxytocin was the only drug that increased sperm concentration of the medium after 15 and 30 min of incubation, compared to the

control. To test the hypothesis that oxytocin plays a role in sperm transport *in vivo*, 57 MT males and 45 normal males were randomly divided over 3 treatments (control, cPS and cPS-OT) with 19 MT and 15 normal males in each group. All males selected had a large and pointed urogenital papilla. The number of MT fish was larger, because the presence of intersex and sterile fish was expected as a consequence of the dietary steroid treatment. The control group of both MT and normal males were sampled without hormone injection, so that the initial gonad condition could be known. Fish of the other 2 treatments received two injections of cPS at respectively 8 and 10 mg/kg bw, given with a 48-h interval. After the first injection, males were individually housed in 120-L tanks (25°C) to avoid aggressive interaction and to maximize care during the experimental period. Twelve h after the second cPS injection, half of the MT and normal males was sampled (treatment cPS), while the other half received an intravenous injection of oxytocin (5 IU/kg bw; Hecht *et al.*, 1982) and was sampled 30 min later (treatment cPS-OT). Due to the large number of fish to handle and the limited capacity of the hatchery to house fish individually under the same conditions, fish were sampled on 3 different days. All MT and normal fish from the control group were sampled on Day 0. Nineteen MT and 15 normal fish from both cPS and cPS-OT treatments received the 1st cPS injection on Day 0 and were sampled on Day 3. The remaining fish received the 1st injection on Day 3 and were sampled on Day 6.

Parameters used to assess the semen release response

Males were anaesthetized in TMS 0.5 g/L of water, for 3 min. Then, the genital area was dried and hand pressure was applied to the fish abdomen, midway between pectoral and pelvic fins, moving posteriorly right down to the urogenital papilla (van der Waal, 1985). This process was repeated 10-15 times for each male. The stripped fluid, when obtained, was collected in a test tube containing 2 mL of the Saad immobilizing medium (NaCl 200 mM; Tris 30 mM; pH 7.0), used to prevent sperm activation caused by urine or blood contamination (Linhart and Billard, 1994). Volume was immediately measured. After stripping, males were placed back on TMS solution but at a higher concentration (0.8 g/L of water) for 40 min to be sacrificed by spinal transection.

Males were dried with a towel and weighed to the nearest 0.001 kg. Then, males were dissected and testes and SV were carefully removed and weighed to the nearest 0.01 g. Gonado- (GSI) and SV-somatic index (SVSI) were calculated and expressed as percentage of gonad or SV weight, respectively, relative to total body weight. Testes were slit, IT semen was squeezed out in a Beaker and volume was measured. Stripped fluid and IT semen were kept on crushed ice during all analyses.

Parameters used to assess the semen quality

All qualitative analyses were carried out in duplicate. Spermatocrit was determined in capillary tubes in a micro-hematocrit IEC MB centrifuge, equipped with a capillary tube rotor, at 10.000 G for 4 min, at room temperature. The white packed cell volume was calculated as percentage of total volume.

The optical density was measured with a Beckman DU-64 spectrophotometer at a wavelength of 505 nm (Ciereszko and Dabrowsky, 1993) in polystyrene disposable

cuvettes, in both *in vitro* and semen release response experiments, with few differences as follows. To assess the optical density of the testis incubation medium, Goldfish Ringer served as blank. IT semen was previously diluted 100 times in NaCl 0.9% (w/w) so this solution served as blank. To calculate the IT sperm concentration based on optical density, sperm cells of 4 random samples per treatment (n=24 samples) were counted in a double Burker-Türk chamber (W. Schreck, Hofheim TS). Then, a linear equation was obtained when the optical density values were regressed on sperm counts, and used to calculate the concentration of all remaining IT samples. The stripped samples were mostly contaminated with blood that disturbed the optical density reading. Thus, the optical density reading was disregarded and the sperm concentration was assessed on a double Burker-Türk counting chamber for all stripped samples.

The number of sperm cells collected per kg body weight (cell/kg) and per fish (cells/fish) were calculated based on IT semen volume and IT sperm concentration.

Sperm motility was assessed in 15 µL of semen mixed with 30 µL of tap water and directly observed under a microscope at 200x magnification. Motility was subjectively classified according to the percentage of moving cells, as: 0, when no movement was observed; 1, when up to 25% cells were moving; 2, when up to 50% cells were moving; 3, when up to 75% cells were moving and; 4, when more than 75% cells were moving.

When stripping was possible, stripped and IT samples from the same male were tested for fertility. Two females were treated with cPS at 4 mg/kg body weight and stripped of eggs 12 h later (25°C). Portions of 0.25 g containing *ca.* 150 eggs were mixed with 100 µL of either IT semen diluted 200 times with NaCl 0.9% (w/v) or stripped semen diluted in 2 mL of the Saad immobilizing medium. Fertilization was initiated by adding 1.5 mL of copper-free tap water and mixing for 40 seconds. Each semen sample was used to fertilize two portions of eggs (duplicate fertilization). The percentage of hatched larvae of the total eggs was calculated 24 h after incubation at 30°C.

Statistic analyses

When males were treated with cPS, some of them were strippable, and some were not. To detect any difference in IT semen quality caused by removing part of the seminal fluid, data from strippable and non-strippable males were first checked for significant differences. Because no difference was found, data were pooled. Data from 45 MT males were considered for statistical analyses; the other 12 MT males possessed either sterile or intersex gonads. Most of the stripped semen samples were contaminated with blood, thereby hampering accurate spermatocrit and sperm counting. Thus, spermatocrit is referred to as packed cell volume and, except for hatching rates, statistical analyses were not carried out for stripped semen quality.

All statistical analyses were carried out using the SAS 6.11 package (SAS Institute Inc., 1990). Means ± SD were calculated and tested for significant differences by ANOVA using the parametric General Linear Model procedure, followed by Duncan's multiple range test for multiple comparisons. For GSI, SVSI, spermatocrit values and hatching rates, the residues of each model were tested for normality using the univariate

procedure. Data on optical density of IT semen (semen release experiments) were regressed on sperm concentration per mL to obtain a linear equation. Correlation was expressed as Pearson correlation coefficient (r). P-values <0.05 were regarded significant.

Results

In vitro experiments

Table 1. Optical density at 505 nm (mean ± SD; n=6) measured in the medium after incubation of testis slices with different hormones, in the African catfish.

Replicate	Hormones (dose/well [1])	Incubation (min)	Optical density (505 nm)
1	Control	15	0.49 ± 0.23 [b]
	Oxytocin 1 IU		0.82 ± 0.27 [ab]
	Oxytocin 10 IU		0.95 ± 0.36 [a]
1	Control	30	0.60 ± 0.22 [b]
	Oxytocin 1 IU		1.22 ± 0.44 [a]
	Oxytocin 10 IU		1.62 ± 0.15 [a]
2	Control	30	0.22 ± 0.11 [b]
	Oxytocin 1 IU		0.90 ± 0.26 [a]
	Oxytocin 10 IU		0.88 ± 0.27 [a]
1	Control	30	0.61 ± 0.44 [b]
	LH 300 ng		0.59 ± 0.24 [b]
	Clarias pituitary 300 ng LH [2]		0.70 ± 0.51 [b]
	Oxytocin 10 IU		1.42 ± 0.37 [a]
2	Control	30	0.46 ± 0.27 [b]
	LH 300 ng		0.64 ± 0.40 [ab]
	Clarias pituitary 300 ng LH [2]		0.64 ± 0.37 [ab]
	Oxytocin 10 IU		0.95 ± 0.29 [a]

[1] Testis slices were incubated in 3 mL of Goldfish Ringer plus hormones or Goldfish Ringer only (control).
[2] The Clarias pituitary dose was set according to its LH content.
[a,b] Means within the same replicate and in the same column followed by the same superscript are not significantly different (Duncan; P<0.05).

Increased ($P<0.05$) optical densities of the medium were observed after both 15 and 30 min of incubation with oxytocin at 10 IU/well, compared to control. The lower oxytocin dose (1 IU/well) also increased the optical density of the medium, but only after 30 min of incubation (Table 1). Incubation of testis slices with vasopressin, epinephrine or prostaglandin F2α did not affect the optical density of the medium (results not shown), nor did incubation with LH or Clarias pituitary extract.

Semen release response experiment

A very high correlation was found when optical density values of IT samples were regressed on sperm counting ($r=0.92$; $n=24$). The formula used to estimate the sperm concentration of the remaining samples was $y = 145.7x - 17.137$; where x=optical density value.

Normal males showed well-developed SV with long fingerlike extensions at each lateral side of the sperm ducts. Stripping of semen was possible in 18 out of 30 normal males after cPS treatment. Fish that did not receive the cPS treatment combined or not with oxytocin, were not strippable. The amount of stripped fluid was small, with relatively low sperm concentration and sperm motility. Oxytocin treatment did not affect the number of strippable males, nor did it affect the quality of the stripped fluid, compared to cPS treatment (Table 2). When tested for fertility, stripped fluid of only one fish produced hatching rates above 20%; the other 17 samples produced extremely low values (Table 4).

In normal males, the cPS treatment significantly increased GSI, SVSI and IT semen volume, while IT spermatocrit, optical density and IT sperm concentration decreased, compared to the control (Table 3). Sperm motility and the number of sperm cells per kg and per fish were not affected. The combined treatment of oxytocin and cPS resulted in IT spermatocrit values similar ($P>0.05$) to the control. There were no significant differences between cPS-treated and cPS-OT-treated fish for any of the parameters recorded for IT semen.

In all MT males, the development of the SV fingerlike extensions was suppressed, as indicated by the significantly reduced SVSI, compared to normal fish (Table 3). From the 57 MT males, 10 were sterile and 2 were intersex. These fish were removed from the analyses and the following data refer to 45 MT males. Stripping of semen was possible in 26 out of 30 MT males, after cPS stimulation. Fish that did not receive the cPS treatment were not strippable. The amount of stripped fluid was small, with low packed cell volume and sperm motility. Oxytocin treatment did not affect the number of strippable males, nor did it affect the quality of the stripped fluid, compared to cPS treatment (Table 2). When stripped samples were tested for fertility, 15 samples produced hatching rates similar ($P>0.05$) to the IT semen from the same MT male. Hatching rates below 20% were produced with 5 samples only (Table 4).

Table 2. Stripped fluid quality of African catfish treated with two injections of carp pituitary suspension (cPS) at 8 and 10 mg/kg given with a 48-h interval. Twelve h later, half of the fish received oxytocin (OT) at 5 IU/kg. All fish were sampled 30 min later.

	Normal males			MT males [1]		
	control	cPS	cPS-OT	control	cPS	cPS-OT
Treated fish (n)	15	15	15	15	17	13
Strippable fish (n)	0	8	10	0	15	11
Volume (mL)		1.0 ± 0.8	0.8 ± 0.8		0.8 ± 0.6	0.8 ± 0.6
Packed cell volume (%)		3.7 ± 3.5	7.4 ± 1.7		2.0 ± 2.0	2.9 ± 3.1
Cells/mL (x 10^9)		0.2 ± 0.2	0.4 ± 0.4		1.1 ± 0.8	4.6 ± 3.4
Sperm motility [3]		0.3 ± 0.5	0.2 ± 0.3		0.9 ± 0.1	0.6 ± 0.5
Hatching rates (%)		0.7 ± 1.4 [b]	3.0 ± 7.0 [b]		61.8 ± 35.4 [a]	65.0 ± 34.9 [a]

[1] Males treated with dietary 17α-methyltestosterone during larval stages, in order to inhibit the development of the seminal vesicle fingerlike extensions.
[2] Absorbance 505 nm; stripped semen was diluted in 2 mL of the Saad immobilizing medium.
[3] Motility scored as: 0 (no moving cells), 1 (1-25%), 2 (25-50%), 3 (50-75%) and 4 (more than 75% moving cells)
[a,b] Means in the same row followed by the same superscript do not differ significantly (Duncan; $P<0.05$).

Table 3. Intratesticular semen quality of African catfish treated with two injections of carp pituitary suspension (cPS) at 8 and 10 mg/kg given with a 48-h interval. Twelve h later, half of the fish received oxytocin (OT) at 5 IU/kg. All fish were sampled 30 min later.

	Normal males			MT males [1]		
	control	cPS	cPS-OT	control	cPS	cPS-OT
Treated fish (n)	15	15	15	15	17	13
GSI (%)	1.19 ± 0.32^{b}	2.03 ± 0.63^{a}	2.00 ± 0.42^{a}	0.99 ± 0.40^{b}	1.43 ± 0.52^{a}	1.15 ± 0.38^{ab}
SVSI (%)	0.60 ± 0.18^{b}	1.04 ± 0.43^{a}	0.89 ± 0.22^{a}	0.08 ± 0.03^{b}	0.11 ± 0.05^{ab}	0.12 ± 0.06^{a}
Volume (mL)	17.0 ± 9.2^{b}	33.6 ± 13.4^{a}	29.2 ± 10.8^{a}	14.3 ± 9.4^{b}	21.1 ± 11.3^{a}	15.8 ± 6.5^{ab}
Spermatocrit (%)	25.1 ± 20.5^{a}	13.5 ± 5.2^{b}	15.5 ± 7.6^{ab}	11.2 ± 6.1^{a}	6.6 ± 2.2^{b}	8.9 ± 2.8^{ab}
Optical density [2]	0.92 ± 0.36^{a}	0.67 ± 0.27^{b}	0.72 ± 0.23^{b}	0.46 ± 0.22^{a}	0.31 ± 0.11^{b}	0.34 ± 0.09^{b}
Cells/mL (x 10^9)	12.1 ± 4.9^{a}	8.0 ± 4.3^{b}	8.8 ± 3.3^{b}	4.8 ± 3.1^{a}	2.8 ± 1.4^{b}	2.9 ± 1.1^{b}
Cells/kg (x 10^9)	81.7 ± 38.9^{a}	108.9 ± 47.5^{a}	109.8 ± 32.8^{a}	29.3 ± 15.7^{a}	26.9 ± 13.9^{a}	19.8 ± 10.0^{a}
Cells/fish (x 10^9)	177.5 ± 75.1^{a}	231.6 ± 97.9^{a}	237.2 ± 85.0^{a}	60.3 ± 39.5^{a}	53.7 ± 29.4^{a}	43.2 ± 27.8^{a}
Sperm motility [3]	3.7 ± 0.5^{a}	3.7 ± 0.5^{a}	3.7 ± 0.8^{a}	3.9 ± 0.4^{a}	3.9 ± 0.2^{a}	3.8 ± 0.4^{a}
Hatching rates (%) [4]		85.5 ± 4.5^{a}	89.1 ± 2.5^{a}		80.4 ± 11.8^{a}	82.0 ± 8.6^{a}

[1] Males treated with dietary 17α-methyltestosterone during larval stages, in order to inhibit the development of the seminal vesicle fingerlike extensions.
[2] Absorbance 505 nm; semen was diluted 100 times in NaCl 0.9%.
[3] Motility scored as: 0 (no moving cells), 1 (1-25%), 2 (25-50%), 3 (50-75%) and 4 (more than 75% moving cells).
[4] Only intratesticular samples from strippable males were tested for fertility.
[a,b] Means within the same group of fish (normal or MT) and in the same row followed by the same superscript are not significantly different (Duncan; $P<0.05$).

Table 4 . Comparison between hatching rates produced with stripped and intratesticular semen from the same individual African catfish male. All fish were treated with two consecutive injections of carp pituitary suspension with a 48-h interval. Twelve 12 h after, some males received an additional injection of oxytocin (^{OT}). All males were sampled 30 min later.

Normal fish			MT fish [1]		
Fish	Hatching rates (%)		Fish	Hatching rates (%)	
code	Stripped	Intratesticular	code	Stripped	Intratesticular
60	0.0*	90.5	45	93.4	92.5
61	0.0*	78.4	46	89.3	82.8
66	1.0*	80.4	47	94.1	73.7
85	0.0*	83.9	48	70.7	45.2
87	3.9*	88.4	50	40.5*	68.9
88	0.0*	86.3	64	56.7*	89.3
89	0.0*	91.0	67	1.0*	87.9
90	0.5*	84.7	69	88.4	87.8
52^{OT}	0.0*	89.8	70	86.2	85.6
53^{OT}	21.8*	89.1	71	56.6*	78.5
54^{OT}	0.0*	88.1	72	0.5*	89.6
58^{OT}	0.0*	91.5	73	0.0*	76.1
92^{OT}	0.0	[A]	74	73.1*	87.8
93^{OT}	7.7*	88.3	83	80.8	77.4
96^{OT}	0.0*	88.9	84	95.4	83.6
97^{OT}	0.0*	87.0	33^{OT}	75.3	78.0
98^{OT}	1.0*	85.4	36^{OT}	78.2	83.7
99^{OT}	0.0*	93.9	37^{OT}	0.0*	78.1
Mean	2.0 ± 5.3*	87.4 ± 4.0	38^{OT}	92.9	89.1
			39^{OT}	0.0*	88.8
			40^{OT}	81.0	81.3
			75^{OT}	91.5	83.5
			76^{OT}	83.2	89.2
			78^{OT}	92.7	60.1
			79^{OT}	76.4*	90.7
			80^{OT}	43.2*	79.9
			Mean	63.1 ± 34.5*	81.1 ± 10.4

[1] Males treated with dietary 17α-methyltestosterone during larval stages, in order to inhibit the development of the seminal vesicle fingerlike extensions.

[A] Larvae from this incubator escaped before counting, so the hatching rate could not be calculated.

*Means significantly different from the intratesticular semen of the same male (Duncan; $P<0.05$).

In MT males, the cPS treatment significantly increased GSI and IT semen volume, while IT spermatocrit, optical density and IT sperm concentration decreased, compared to the control (Table 3). SVSI, the number of sperm cells collected

per kg and per fish, and sperm motility were not affected. The combined treatment of oxytocin and cPS resulted in IT spermatocrit values similar ($P>0.05$) to the control. There were no significant differences between cPS-treated and cPS-OT-treated fish for any of the parameters recorded for IT semen.

The MT treatment used to inhibit the SV development did not affect the fertilizing capacity of the IT semen after cPS stimulation, as MT males produced hatching rates similar ($P>0.05$) to the normal males (Table 3).

Discussion

Oxytocin belongs to a very conservative family of molecules, with representatives throughout the animal kingdom: from worms and insects to vertebrates. It is inevitable that most emphasis in oxytocin research is on the mammalian female, as it is involved in uterine contractions at birth, and it is the prime agent in mediating the milk let-down reflex in response to suckling (Ivell and Russell, 1996). In recent years, it has been found that oxytocin is also important in males. Oxytocin promotes spermiation and sperm transport, increases the volume of fluid and the number of sperm released from the cauda epididymis and increases volume and sperm concentration in the ejaculate (Assinder *et al.*, 2000). Oxytocin also influences Leydig cells' steroidogenesis (Whittington *et al.*, 2001) and regulates male sexual behavior leading to penile erection and ejaculation (Mckenna, 1999). In some fish species, the involvement of oxytocin in spawning and parturition is established (Venkatesh *et al.*, 1992). However, the role of oxytocin in fish reproduction is not as clearly understood as it is in other vertebrate classes. To the best of our knowledge, this is the first report about oxytocin effects on semen release response in a fish species.

When oxytocin was tested in testis incubations, an increased optical density of the medium was observed after 15 and 30 min. Because optical density is highly correlated with sperm concentration (Chapter 2 in this thesis), these results suggest that oxytocin might have stimulated testis contraction, thereby increasing sperm concentration of the incubation medium. Similar testis incubations were carried out with vasopressin, epinephrine and prostaglandin F2α, but the optical density of the medium was not affected. Vasopressin increases the contractility of the epididymis in rams (Knight, 1974) and in rabbits (Kihlstrom and Agmo, 1974), catecholamines stimulate the contractions of deferent ducts causing emission of semen (Cross and Glover, 1958) and prostaglandin F2α increases volume, sperm concentration and the number of motile spermatozoa per ejaculate in buffalo bulls (Mar, 1988). As only 2 concentrations of each hormone were tested here, it is possible that the doses necessary to stimulate contraction of the testis in African catfish are not in this range. Different hormone concentrations combined with other latency times should be investigated.

It has been described that the African catfish pituitary contains large amounts of isotocin (the fish oxytocin-like peptide; Goos *et al.*, 1997). To check whether the isotocin present in the pituitary extract stimulates testis contraction, the *in vitro* effects of oxytocin and crude pituitary extracts were compared. To separate the effects of LH

present in the pituitary extract from the other components (such as isotocin), the LH content of the pituitary was measured. Then, a treatment with partly purified LH containing exactly the same concentration of LH present in the pituitary extract, was included. Pituitary extract and LH did not affect the optical density of the incubation medium, compared to the control. The optical density of the medium, containing testis slices incubated with oxytocin was increased ($P<0.05$), compared to the control. It is possible that the isotocin content present in the concentration of pituitary extract used here, was too low to stimulate contractility of the seminiferous tubules.

In the semen release response experiment, cPS treatment increased GSI and IT semen volume and decreased spermatocrit and sperm concentration, in both MT and normal males. Similar testis hydration after pituitary suspension treatments has been described for the same fish species (Viveiros *et al.*, 2001; Hecht *et al.*, 1982), Asian green catfish, Mystus nemurus (Christianus *et al.*, 1996), European catfish Silurus glanis (Linhart and Billard, 1994) and South American catfish Rhamdia sapo (Espinach Ros *et al.*, 1984). The number of sperm cells collected per kg or per fish was not affected by cPS treatment in both MT and normal fish, as previously reported (Viveiros *et al.*, 2001). However, in another study (Chapter 2 in this thesis), 3 times more sperm cells per kg were collected from normal males after similar cPS treatment, compared to the control group. The reason for the different response to cPS treatment is not clear, but could be related to differences in testis development due to age or genetic background.

There was no effect of oxytocin treatment on GSI, SVSI or IT semen quality in both groups of male. However, spermatocrit after cPS-OT treatment resulted in values similar to the control, in both normal and MT males. Because testis incubation with oxytocin increased the optical density of the medium compared to the control, in 5 different trials (Table 1), these results together suggest that oxytocin may play a role in the movement of spermatozoa within the catfish testis. However, correspondent increased optical density of the intratesticular semen after cPS-OT treatment was not observed. So far, it is not possible to describe the oxytocin effects on semen output of the African catfish.

Stripping of semen was possible after both cPS and cPS-OT treatments. Taking these treatments together, 26 out of 30 MT males and 18 out of 30 normal males were strippable. In normal males, the stripped semen volume was low, and the packed cell volume was relatively high (cPS-OT treatment: 7.4%). This high value was probably caused by blood contamination during stripping and does not represent the real value. This is consistent with the low hatching rates (3.0%) obtained when this fluid was tested for fertility. Similar poor hatching rates were obtained with stripped fluid from normal males, after treatment with cPS plus ovaprim (Chapter 2 in this thesis). The only study that reported high hatching rates produced with stripped fluid of normal African catfish treated with Clarias pituitary suspension, used males captured from the wild, during their natural breeding season (van der Waal, 1985).

On the other hand, when the stripped semen of MT males treated with cPS or cPS-OT was tested for fertility, hatching rates as high as 63.1% were produced. Furthermore, these hatching rates were significantly higher ($P<0.001$) than that produced with stripped semen of normal males (2.1%). These results confirm our previous findings

that dietary 17α-methyltestosterone treatment for 12-40 days post hatch inhibits the development of the SV fingerlike extensions in all treated males, and that the absence of the SV extensions facilitates hand-stripping of semen of this fish species (Viveiros *et al.*, 2001).

There was a slight but consistent tendency of higher hatching rates being produced with both IT (Table 3) and stripped semen (Table 2) after oxytocin treatment in both MT and normal males, compared to cPS treatment. This suggests that oxytocin, used at an optimal concentration, may improve the fertilizing capacity of catfish semen. In rabbits with poor fertility, intratesticular injections of oxytocin increased ($P<0.01$) sperm motility, litter size and litter weight at birth, compared to saline-injected rabbits (Abd-El-Kariem *et al.*, 1998).

The use of oxytocin to induce reproduction in African catfish has been reported in only one study (Hecht *et al.*, 1982). Females were treated with oxytocin at the same dose used here, to induce ovarian contraction and to facilitate egg release. About 50% of females spawned after oxytocin treatment, in comparison with almost 100% of the females after treatments with combinations of human chorionic gonadotropin and pituitary extracts. Taking this result and ours together, one can conclude that the reproductive tract of the African catfish is sensible to the effects of oxytocin: contraction of the follicles with ovulation was observed in females and contraction of the testis with increased sperm concentration of the incubation medium was observed in males. Certainly, an adjustment of oxytocin dose and/or latency time for this species is needed. The use of oxytocin to stimulate contractility of the seminiferous tubules and promote sperm transport, thereby increasing sperm concentration in the semen output in African catfish, as has been described in rams (Assinder *et al.*, 2000) and bulls (Berndtson and Igboeli, 1988), should be further investigated

Acknowledgements

The authors express their gratitude to Ir. Willie Fleuren for producing the MT males, to Herzo van der Waals for statistic support and to Menno ter Veld, Ronald Booms, Neil Ruane, Catarina Martins and Rosane Barreto for technical assistance during sampling. This study is part of the 1st author Ph.D. project and was supported by Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

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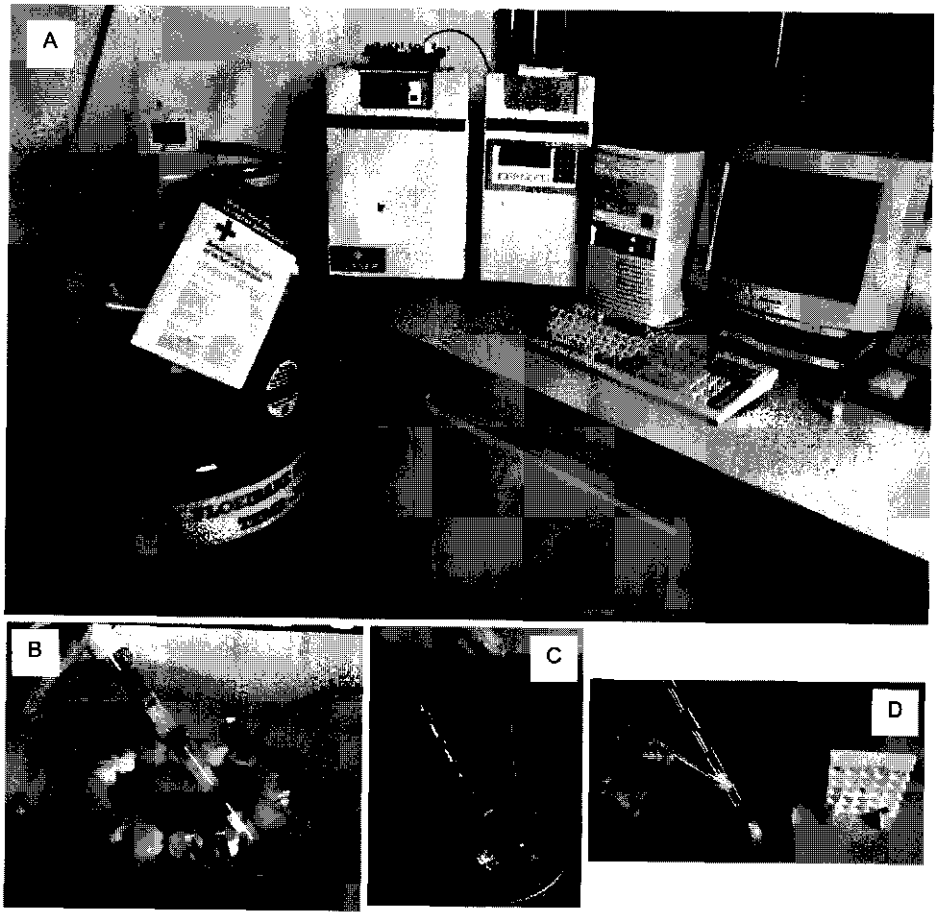

Picture 3. Materials used for semen cryopreservation. The freezer apparatus and the liquid nitrogen container (A); a closer view of the freezer top with some cryovials inserted (B); the liquid nitrogen vessel for semen storage (C) and; rack with cryovials and a polystyrene plate for thawing (D).

Chapter 5

Sperm cryopreservation of African catfish, Clarias gariepinus: cryoprotectants, freezing rates and sperm:egg dilution ratio

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Theriogenology (2000) 54, 1395-1408

Abstract

Methods for cryopreserving spermatozoa and optimizing sperm:egg dilution ratio in African catfish Clarias gariepinus were developed. Five percent to 25% dimethylsulphoxide (DMSO) and methanol were tested as cryoprotectants, by diluting semen in Ginzburg fish ringer and freezing in 1-milliliter cryovials in a programmable freezer. To avoid an excess of spermatozoa per egg, post-thaw semen was diluted 1:20, 1:200 or 1:2000 before fertilization. Highest hatching rates were obtained by spermatozoa frozen in 10% methanol and post-thaw diluted to 1:200. Then, slow freezing rates (-2, -5 or -10°C/min) to various endpoint temperatures (range -25 to -70°C) before plunging into liquid nitrogen (LN_2) were evaluated. Hatching rates equal to control ($P>0.05$) were obtained by spermatozoa frozen at -5°C/min to -45 to -50°C and at -10°C/min to -55°C. In 3-step freezing programs, at -5°C/min, the effect of holding spermatozoa for 0, 2 or 5 min at -30, -35 or -40°C before plunging into LN_2 was analyzed. Hatching rates equal to control ($P>0.05$) were produced by spermatozoa frozen to, and held at, -35°C for 5 min and at -40°C for 2 or 5 min. Finally, frozen spermatozoa (10% methanol, -5°C/min, 5-min hold at -40°C, LN_2, post-thaw diluted to 1:200) were tested in on-farm fertilization conditions. Again, no difference ($P>0.05$) in hatching rate was observed between frozen and fresh spermatozoa. Cryopreservation offers utility as a routine method of sperm storage and management for catfish.

Introduction

Catfishes are an economically important group of fresh and brackish water fish worldwide. Several species have been successfully introduced in aquaculture (Teugels, 1996), and the African catfish, Clarias gariepinus (formerly C. lazera; Fishelson *et al.*, 1994), is perhaps the most important one, not only in Africa but also in S-E Asia (e.g., Thailand) and in Europe (e.g., The Netherlands).

The availability of gametes throughout the year is important to ensure a constant supply of fish. In captivity (25°C; 12 h light per day), C. gariepinus gametogenesis is continuous once sexual maturity is reached (Huisman and Richter, 1987). However, whereas females can be stripped of eggs after treatments with carp pituitary suspension (cPS; Hogendoorn, 1979) or human Chorionic Gonadotropin (hCG; Eding *et al.*, 1982), spermiation and male reproductive behavior do not take place spontaneously (van Oordt *et al.*, 1987), even after hormonal therapy. To obtain spermatozoa it is necessary to kill male brood fish (Steyn *et al.*, 1985) or surgically remove part of their testes (Bart and Dunham, 1990). Storing batches of spermatozoa by cryopreservation would significantly improve the reproductive potential of male catfish.

Cryopreservation of fish spermatozoa has been the subject of many investigations, especially in salmonids (e.g., Stoss, 1983). Successful cryopreservation depends not only on the right choice of cryoprotectant and extender, but also on the freezing protocol used. Cryoprotectant and freezing rate together determine the damage to spermatozoa due to intracellular ice crystallization (Mazur, 1977). Research on sperm

cryopreservation of African catfish and related species is summarized on Table 1. African catfish spermatozoa were first successfully cryopreserved by Steyn *et al.* (1985), who obtained 40% motility 24 h after storage in LN_2. Glucose in combination with glycerol has been the most widely used cryoprotective solution. Recently, glucose in combination with DMSO was also shown to be effective (Urbanyi *et al.*, 1999). Freezing rates can be rapid (e.g., pellet freezing on dry ice or in LN_2 vapor; Linhart *et al.*, 1993; Oteme *et al.*, 1996; Padhi and Mandal, 1995) or slow (e.g., at fixed rates in a programmable freezer; Steyn, 1993; van der Walt *et al.*, 1993). However, in most cases, sperm quality was only evaluated in terms of motility after thawing. When fertilization was included in the evaluation, sperm:egg ratios were not optimized and were often excessive (Steyn and van Vuren, 1987). Using excess spermatozoa for fertilization obviously masks the quality of cryopreserved spermatozoa, making comparison of protocols difficult.

In the present paper we evaluate different combinations of cryoprotectant and freezing protocols using defined sperm:egg fertilization ratios, with the aim of developing a reliable protocol for catfish sperm cryopreservation under both laboratory and on-farm conditions.

Materials and Methods

Husbandry of brood stock

The brood stock of the African catfish, C. gariepinus, originated from the Republic of Central Africa (4.22°N, 18.35°E), has been bred for several generations in the Hatchery of the Fish Culture and Fisheries Group at Wageningen University (52°N, 5.5°E), The Netherlands. Mature males and females were kept together under constant temperature (25°C) and photoperiod (14 h of light per day) in 250-L rectangular tanks connected to a recirculating system. The flow rate was 8 to 12 L/min. Fish were fed trout pellets (Trouvit, The Netherlands) at a maintenance level of 0.8% of body weight daily.

Sperm cryopreservation

Males (1 per experiment) weighing 850 to 2300 g were anesthetized with 8 g tricaine methanesulfonate (TMS; Crescent Research Chemicals, Phoenix, Arizona, USA) dissolved in 10 L tap water and sacrificed by spinal transection. Testes were removed by dissection and perforated with a needle, and semen was gently squeezed out. Motility was determined subjectively by mixing 1 drop of fresh semen with 2 drops of tap water and observed under a microscope at magnification 200x. Only samples with more than 80% motile spermatozoa were frozen.

Table 1: Literature review on semen cryoconservation of African catfish and related species. The data presented shows recommended treatments based on the current literature.

Catfish species	Extender	Cryoprotectant	Container	Freezing rates	Hatching rates (%)	Spermatozoa per egg	Reference
Clarias gariepinus	5% glucose	5% glycerol	straw or bio-freeze vial	-7°C/min to -65°C; LN_2	not measured	not measured	Steyn *et al.*, 1985
	5% glucose	11% glycerol	1-mL cryo tube	-11°C/min to -70°C; LN_2	frozen: 51% control: 51%	245 x 10^3	Steyn & van Vuren, 1987
	4% glucose	9% glycerol	1-mL cryo tube	-5 and -11°C/min to -70°C; LN_2	not measured	not measured	Steyn, 1993
	4% glucose	9% glycerol	1-mL cryo tube	-5°C/min to -70°C; LN_2	not measured	not measured	van der Walt *et al.*, 1993
	333 mmol L^{-1} fructose	10% DMSO	250-µL straw	-11°C/min to -80°C; LN_2	not measured	not measured	Urbanyi *et al.*, 1999
Clarias batrachus	0.6% NaCl	10% glycerol	1.5-mL tube	directly to -70°C; stored at -70°C	75% of control	13.6 x 10^6	Padhi & Mandal, 1995
Heterobranchus longifilis	Mounib solution [a]	5% DMSO + 5% glycerol + 10% egg yolk	5-mL straw	20 min at 3cm above LN_2 level; LN_2	frozen: 79% control: 81%	50 x 10^3	Oteme *et al.*, 1996

[a] Mounib solution: (g/L) reduced glutathione 2.0; $KHCO_3$ 10.0; sucrose 42.0

The mean sperm concentration of 10 randomly chosen catfish males was determined by counting spermatozoa diluted 1:10,000 in a Burker-Turk counting chamber (W. Schreck, Hofheim TS.).

Ginzburg fish ringer (123.2 mM NaCl; 3.75 mM KCl; 3.0 mM $CaCl_2$; 2.65 mM $NaHCO_3$; pH 7.6; 244 mOsm) was used as extender and mixed with methanol or DMSO (Merck, Germany) at various concentrations in a 10-milliliter (mL) tube. Semen was gently added at 1:10 (v/v) final concentration. Aliquots of 500 microliter (μL) of diluted spermatozoa (containing 50 μL semen) were immediately transferred to 1-mL cryovials (Greiner Labortechnik; Düsseldorf, Germany) and frozen. The equilibration time between mixing spermatozoa with cryoprotective solution and freezing was approximately 2 min at 5°C.

For each treatment (i.e., a specific combination of cryoprotective agent, cryoprotectant concentration, freezing rate, endpoint temperature in the first step of freezing and holding time at the endpoint), 6 cryovials (replicates) were frozen in a programmable freezer (Planer Kryo 10 series 3 Controlled Rate Freezer; Middlesex, UK) and stored in LN_2 at -196°C for 1 to 3 weeks before being tested in fertilization trials.

Artificial insemination and hatching of eggs

Female catfish, 1 per experiment, were injected with 4 mg cPS/kg body weight and stripped 12 h later (25°C). Eggs were kept at room temperature (23°C) while being used and for a maximum of 2 h. Three cryovials per treatment were thawed in a water bath at 27°C for 5 min. The remaining 3 cryovials were kept as backup samples. After thawing, spermatozoa from each cryovial (previously diluted 1:10) were diluted again with fresh fish ringer to a final concentration of 1:20 (in Experiment 1A and 1C), 1:200 (in all experiments except 1A) or 1:2000 (in Experiment 1C).

From each sample of post-thaw diluted spermatozoa, 2 aliquots of 100 μL were mixed with 2 portions of 0.2 g of fresh eggs (approximately 150 eggs) in a plastic Petri dish. Fertilization was initiated by adding 1 mL tap water and mixing for 40 sec. The eggs were then transferred to a 10-cm diameter PVC basket with a 0.5-mm mesh bottom and incubated in shallow trays at 30°C, connected to a recirculating system. Every 30 min and at the end of each fertilization trial (that lasted for a maximum of 2 h), control egg batches were inseminated with fresh spermatozoa, diluted in fish ringer to the same ratio as the post-thaw spermatozoa (i.e., 1:20, 1:200 or 1:2000, depending on the experiment) to check for changes in egg quality.

Cryoprotectant agent, concentration and final sperm dilution ratio

In this section, 3 experiments were conducted. In the first experiment (1A), spermatozoa was frozen in 5, 10, 15, 20 or 25% methanol or DMSO, and diluted post-thaw to 1:20 (v/v final concentration). Based on the results of this experiment, the same design, except for the cryoprotectant concentration of 25%, was repeated in Experiment 1B. However, spermatozoa were diluted post-thaw to 1:200. In Experiment 1C,

spermatozoa were frozen only in 10 or 15% methanol or DMSO and diluted post-thaw to 1:20, 1:200 or 1:2000. In all 3 experiments, spermatozoa were frozen at -5°C/min from +5 to -50°C, plunged into LN_2, and tested in fertilization trials.

Freezing rates and endpoint temperatures in 2-step freezing programs

Four 2-step freezing programs were designed to define the best freezing rate and endpoint temperature. After reaching the target endpoint (first step), sperm samples were directly plunged into LN_2 (second step). In Experiment 2A, spermatozoa were frozen at -5°C/min (the same rate used in the first series of experiments) from +5 to -35, -50 or -70°C. Based on these results, in Experiment 2B the same freezing rate was used, but more endpoints were tested: -40, -45, -50, -55, -60 or -65°C. In Experiments 2C and 2D, a slower and a faster rate (-2 and -10°C/min, respectively) were used, and spermatozoa were frozen from +5 to -25, -30, -35, -40, -45, -50, -55 or -60°C. All sperm samples were frozen in 10% methanol and diluted post-thaw to 1:200 (v/v final concentration).

Holding spermatozoa at supraoptimal endpoints in 3-step freezing programs

Based on results of the previous experiment, we have hypothesized that holding sperm samples at endpoint temperatures higher than the optimal ones (supraoptimal temperatures) for a few minutes before fast freezing in LN_2 would increase sperm survival. To Test this hypothesis, spermatozoa was frozen at -5°C/min from +5 to -30, -35 or -40°C and either plunged immediately into LN_2 (0-min hold) or held at the endpoint temperature for 2 or 5 min (second step) before plunging (third step). All sperm samples were frozen in 10% methanol and diluted post-thaw to 1:200 (v/v final concentration).

Cryopreserved spermatozoa in on-farm conditions

Semen was obtained after surgical removal of a part of the testis from 3 adult male catfish (4-5 years old and about 10 kg) from farm brood stock (Ir. Fleuren, Someren, The Netherlands). Spermatozoa were frozen according to one of our best protocols (10% methanol, at -5°C/min from +5 to -40°C and held for 5 min before plunging into LN_2) and diluted post-thaw 1:200 (v/v final concentration). Ten mL diluted post-thaw spermatozoa (50 µL semen, 1 cryovial) were mixed with 20 g eggs (3 cryovials per male). After fertilization, 2 aliquots of 0.2 g eggs from each sample of 20 g were incubated separately in 2 mesh-bottom baskets suspended in an aquarium to determine the hatching rate. The remaining 19.6 g were incubated in another tank, but in the same recirculating system as the other samples, at 30°C.

Statistical analysis

The number of hatched larvae, as a percentage of total eggs exposed to spermatozoa, was calculated 24 h after fertilization. For cryopreserved spermatozoa, data from 6 replicates per treatment (2 samples of 0.2 g egg per vial and 3 vials per treatment) were pooled to calculate mean and standard deviation. For fresh spermatozoa, hatching rate

data from fertilization every 30 min and at the end of each trial were pooled to calculate mean and standard deviation. All statistical analyses were done using the SAS 6.11 package (SAS Institute Inc., 1990). Hatching rates from fresh and cryopreserved spermatozoa per fertilization trial were tested for significant differences by ANOVA using the parametric General Linear Model procedure, followed by Duncan's Multiple Range Test. The residues from the different ANOVA models were tested for normal distribution using the univariate procedure. P-values <0.05 were considered to be significant.

Results

The mean sperm concentration, counted in 10 randomly chosen males, was 3.4×10^9 (SD 2.0×10^9; range 1.8 to 7.2×10^9) spermatozoa per mL. From this value, sperm concentration per vial was estimated as 17×10^7 spermatozoa in 0.5 mL solution and the insemination ratio, as 113×10^3 at 1:20, 11.3×10^3 at 1:200, and 1.13×10^3 spermatozoa per egg at 1:2000 final sperm dilution.

Hatching rates of control groups, in the laboratory conditions, ranged from 55.7 to 94.3%. The hatching rate in on-farm conditions was 49.6%.

Cryoprotectant agent, concentration and final sperm dilution ratio

Table 2: Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen in DMSO or methanol at different concentrations and diluted post-thaw to 1:20 (Experiment 1A) or 1:200 v/v final dilution (Experiment 1B).

Cryoprotectant		Final sperm dilution ratio (v/v)	
Concentration (%)		1:20	1:200
Methanol	5	81.5 ± 6.1 [a]	40.8 ± 14.6 [bc]
	10	82.3 ± 6.2 [a]	77.8 ± 10.5 [a]
	15	81.3 ± 5.1 [a]	38.9 ± 19.8 [c]
	20	75.0 ± 8.0 [a]	6.2 ± 7.1 [e]
	25	0 [b]	
DMSO	5	82.1 ± 3.7 [a]	55.5 ± 22.1 [b]
	10	73.9 ± 3.9 [a]	38.1 ± 28.4 [c]
	15	85.7 ± 5.1 [a]	24.1 ± 14.0 [d]
	20	81.6 ± 8.7 [a]	28.6 ± 21.6 [cd]
	25	81.4 ± 6.8 [a]	
Control		77.4 ± 3.2 [a]	85.2 ± 12.6 [a]

[a-d] Means within the same column with different superscripts are significantly different (P<0.05).

In Experiment 1A, all samples, except those frozen in 25% methanol, protected by either methanol or DMSO and diluted post-thaw to 1:20 v/v final dilution, produced hatching rates not significantly different ($P>0.05$) from the control. However, when spermatozoa was diluted post-thaw to 1:200 v/v final dilution (Experiment 1B), only samples frozen in 10% methanol produced hatching rates (77.8%) not significantly different from control (85.2%). A significant ($P<0.05$) decrease in hatching rate was observed in samples frozen in 5, 15 or 20% methanol and in 5 to 20% DMSO (Table 2).

When spermatozoa were diluted post-thaw to 1:20 v/v final dilution in Experiment 1C, different results from Experiment 1A were obtained. Only samples frozen in 10% methanol produced hatching rates (60.0%) similar to control spermatozoa (65.9%). Samples frozen in 15% methanol and 10 and 15% DMSO produced lower hatching rates ($P<0.05$) compared with control (Table 3). At 1:200 post-thaw sperm dilution ratio, 10% methanol was again the most effective in protecting spermatozoa against freezing, maintaining the same ($P>0.05$) hatching rate (58.3%) as control (55.7%). At 1:2000 v/v final dilution, none of the samples tested produced satisfactory results (Table 3).

Table 3: Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen in 10 or 15% methanol or DMSO and diluted post-thaw to 1:20, 1:200 or 1:2000 (final dilution; Experiment 1C).

Cryoprotectant		Final sperm dilution ratio (v/v)		
Concentration (%)		1:20	1:200	1:2000
Methanol	10	60.0 ± 8.8^{ab}	58.3 ± 6.6^{a}	29.3 ± 22.4^{b}
	15	52.0 ± 2.5^{bc}	43.6 ± 12.9^{b}	17.5 ± 9.0^{b}
DMSO	10	48.3 ± 6.0^{c}	9.5 ± 5.1^{c}	1.5 ± 1.1^{c}
	15	38.1 ± 5.3^{d}	15.8 ± 3.7^{c}	0.3 ± 0.4^{c}
Control		65.9 ± 14.7^{a}	55.7 ± 12.8^{a}	58.1 ± 16.3^{a}

[a-d] Means within the same column with different superscripts are significantly different ($P<0.05$).

Freezing rates and endpoint temperatures in 2-step freezing programs

In Experiment 2A, when a freezing rate of -5°C/min was used, spermatozoa frozen to -35°C (first step endpoint) then plunged into LN_2 produced a hatching rate of 0%. However, when samples were frozen to -50°C, the hatching rates (95.6%) were similar ($P>0.05$) to control (94.3%). A deeper endpoint temperature, -70°C, had a negative effect on hatching, producing rates (43%) significantly lower ($P<0.05$) than the control (Table 4).

In Experiment 2B, when more endpoint temperatures were tested with the same freezing rate of -5°C/min, spermatozoa frozen to -40°C and then plunged into LN_2 produced a very low hatching rate of only 3.9% (Table 4). However, samples frozen to either -45 or -50°C produced hatching rates equal ($P>0.05$) to control (77.2 and 66.2%

vs. 79.9% of control). Endpoint temperatures of -55°C or lower had a negative effect on hatching rate.

At a rate of -2°C/min (Experiment 2C), spermatozoa frozen to -25 or -30°C (first step endpoint) and then plunged into LN_2 produced a hatching rate of 0%. When spermatozoa were frozen to -35°C, less than 3% of eggs hatched (Table 4). Maximum hatching rates were observed when samples were frozen to -40°C (40.7%), although this was significantly ($P<0.05$) lower than control (72.5%). Endpoint temperatures of -45°C or lower had a negative effect on hatching rate.

At a faster freezing rate of -10°C/min (Experiment 2D), spermatozoa frozen to -25, -30, -35 or -40°C and then plunged into LN_2 produced a hatching rate of 0%. When spermatozoa were frozen to -45°C, only 1.5% of eggs hatched (Table 4). Hatching rates equal ($P>0.05$) to control (59.8%) were observed only when spermatozoa were frozen to -55°C (51.8%). Endpoint temperatures of -60°C or lower had a negative effect on hatching rate.

Table 4: Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen at -2, -5 or -10°C/min to different endpoints, then plunged into liquid nitrogen (Experiments 2A to 2D).

First step endpoints (°C)	Freezing rates (°C/min)			
	-2	-5	-5	-10
- 25	0 ^{d}	N	N	0 ^{d}
- 30	0 ^{d}	N	N	0 ^{d}
- 35	2.7 ± 1.1 ^{d}	0 ^{c}	N	0 ^{d}
- 40	40.7 ± 4.8 ^{b}	N	3.9 ± 2.0 ^{d}	0 ^{d}
- 45	20.6 ± 10.3 ^{c}	N	77.2 ± 3.3 ^{a}	1.5 ± 2.2 ^{d}
- 50	11.2 ± 5.3 ^{cd}	95.6 ± 3.9 ^{a}	66.2 ± 12.1 ^{a}	28.7 ± 28.5 ^{c}
- 55	8.3 ± 3.4 ^{cd}	N	41.1 ± 14.4 ^{b}	51.8 ± 9.7 ^{ab}
- 60	5.6 ± 3.5 ^{cd}	N	25.5 ± 18.1 ^{bc}	38.6 ± 16.1 ^{bc}
- 65	N	N	10.3 ± 1.7 ^{c}	N
- 70	N	43.0 ± 18.0 ^{b}	N	N
Control	72.5 ± 7.1 ^{a}	94.3 ± 6.4 ^{a}	79.9 ± 15.5 ^{a}	59.8 ± 10.7 ^{a}

N = endpoint not tested within a given freezing rate.
$^{a-d}$ Means within the same column with different superscripts are significantly different ($P<0.05$).

Holding spermatozoa at supraoptimal endpoints in 3-step freezing programs

No egg hatched when fertilized with spermatozoa frozen at -5°C/min to either -30°C at all holding times tested or to -35°C with 0-min holding time (Table 5). However, hatching rates as high ($P>0.05$) as control (85.9%) were produced with spermatozoa

frozen to, and held at, -35°C for 5 min (85.1%) and -40°C for either 2 min (78.4%) or 5 min (86.8%).

Table 5: Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen at -5°C/min to supraoptimal endpoint temperatures in the first step of freezing, with varying holding times (second step) and plunged into liquid nitrogen (third step; Experiment 3).

First step endpoints	Holding time, in min (second step)		
(°C)	0	2	5
- 30	0 [b]	0 [b]	0 [b]
- 35	0 [b]	4.3 ± 4.3 [b]	85.1 ± 0.9 [a]
- 40	0.8 ± 1.2 [b]	78.4 ± 2.5 [a]	86.8 ± 3.6 [a]
Control	85.9 ± 3.5 [a]		

[a,b] Means with different superscripts are significantly different ($P<0.05$).

Cryopreserved spermatozoa in on-farm conditions

No significant difference in hatching rate was observed when eggs were fertilized with either fresh or post-thaw spermatozoa tested on a commercial scale (Table 6). However, when data from frozen spermatozoa were separated by male, significantly higher ($P<0.05$) hatching rates (57.7%) compared with control (49.6%) were produced by male # 1.

Table 6: Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen in 10% methanol at -5°C/min to, and held at, -40°C for 5 min, plunged into liquid nitrogen and tested in on-farm fertilization conditions (Experiment 4).

Spermatozoa		Hatching rates (%)
Frozen	Male # 1	57.7 ± 2.9 [a]
	Male # 2	53.9 ± 5.0 [ab]
	Male # 3	51.8 ± 3.1 [ab]
	Average	54.6 ± 4.3 [ab]
Control		49.6 ± 1.6 [b]

[a,b] Means with different superscripts are significantly different ($P<0.05$).

Discussion

Sperm dilution ratio

To avoid such high sperm:egg ratios that even post-thaw spermatozoa with low numbers of live cells could yield adequate hatching rates, the maximum sperm dilution ratio to achieve hatching rates similar to control was investigated (Experiment 1C). Spermatozoa could be frozen and diluted as much as 200 times without losing fertilization ability. However, at 1:2000 the hatching rates produced with frozen spermatozoa were lower than the control (Table 3). These dilution ratios are higher than those reported in the literature. African catfish, Heterobranchus longifilis, spermatozoa were diluted 1:3 before freezing and 1:10 after thawing, and had the same fertilization ability (78.9%) as the control (81.1%; Oteme *et al.*, 1996). In Cyprinus carpio, however, no spermatozoa survived when diluted higher than 1:5 before or after freezing (Lubzens *et al.*, 1997).

Sperm:egg ratio

As sperm collection in African catfish, as well as in channel and Asian catfish, involves testis destruction or male death, it is important to maximize the use of a single male by optimization of sperm:egg insemination ratio. For fresh spermatozoa, the effective insemination ratio was estimated as 15×10^3 spermatozoa per egg in C. gariepinus (Rurangwa *et al.*, 1998) and as 4 to 8×10^3 spermatozoa per egg in C. macrocephalus (Tambasen Cheong *et al.*, 1995). Because a percentage of spermatozoa die during freezing and thawing processes, the effective insemination ratio for frozen spermatozoa should be higher. In channel catfish, 50×10^6 post-thaw spermatozoa per 0.5-mL straw enabled fertilization of 250 eggs (200×10^3 spermatozoa per egg; Tiersch *et al.*, 1994). In blue catfish I. furcatus, a minimum of $13{,}000 \times 10^3$ post-thaw spermatozoa per egg were needed to achieve 54% of control fertilization (Bart *et al.*, 1995). In C. gariepinus (Steyn and van Vuren, 1987), 49×10^3 live post-thaw spermatozoa per egg achieved a hatching rate (51.2%) equal to the control (51%). In our experiments, mean fresh sperm concentration was 3.4×10^9 per mL, while only a small aliquot of 100 µL of post-thaw spermatozoa diluted to 1:200 ($1{,}700 \times 10^3$ spermatozoa) enabled fertilization of about 150 eggs. In our field trial, 10 mL of post-thaw diluted (1:200) spermatozoa (50 µL semen) were able to fertilize 20 g (15,000) eggs. The insemination ratio in both situations was within the range 6 to 24×10^3 spermatozoa per egg, which is comparable to the ratio tested by Steyn and van Vuren (1987). Based on the present data, 5 mL of semen from a single male, frozen according to our protocol, should be enough to fertilize 1,500,000 eggs (2 kg) and produce 750,000 larvae.

Cryoprotectants

At 1:20 final sperm dilution (Experiment 1A; Table 2), it was possible to determine neither the best cryoprotectant nor the most effective cryoprotectant concentration to protecting spermatozoa against freezing. All treatments, except 25% methanol, reached

hatching rates comparable with control. However, when this dilution ratio was used again in Experiment 1C, only spermatozoa frozen in 10% methanol produced high hatching rates (Table 3). It is possible that the explanation for these contrasting results reflects differences in sperm quality (better in Experiment 1A than in Experiment 1C) and not in egg quality, because fertilization with fresh spermatozoa produced high hatching rates in both experiments.

Using 1:200 as the final sperm dilution, 10% methanol was the most effective cryoprotectant for C. gariepinus spermatozoa compared with methanol at other concentrations and DMSO at all concentrations tested, in both Experiment 1B and 1C. Methanol also proved effective for freezing zebra fish spermatozoa (Harvey *et al.*, 1982) and was a better cryoprotectant for channel catfish, Ictalurus punctactus, spermatozoa than DMSO, glycerol, sucrose, polyvinylpyrrolidone (Tiersch *et al.*, 1994) and n,n-dimethyl acetamide (Christensen and Tiersch, 1997). In contrast, it was less effective (18.9% hatching rate) than glycerol (51.2%) and DMSO (40.5%) for protecting C. gariepinus spermatozoa against freezing (Steyn and van Vuren, 1987). Glycerol has been the most widely used cryoprotectant for African catfish spermatozoa (Table 1). Ten percent glycerol also proved effective for freezing Asian catfish, Heteropneustes fossilis and C. batrachus, spermatozoa, yielding 69 to 84% of control hatching rates (Padhi and Mandal, 1995). However, glycerol was toxic to salmonid spermatozoa, whereas DMSO could be used for cryopreservation (Stoss and Holtz, 1981).

According to Tiersch *et al.* (1994), a longer time of equilibration before freezing can enhance the effectiveness of cryoprotectants that act more slowly than methanol. This could explain why DMSO was less effective than methanol as the equilibration time used in the present experiments was only 2 min.

Freezing rates and endpoints

According to Mazur (1970), when any kind of cell is subjected to subzero temperatures, it initially supercools while ice forms in the external medium. The manner in which cells regain equilibrium with medium depends chiefly on the rate at which they are cooled and on their permeability to water. If cells are frozen slowly or if their permeability to water is high, cells will equilibrate by transferring intracellular water to external ice (dehydration and shrinkage). On the other hand, if cells are cooled rapidly, or if their permeability to water is low, or even if cells are plunged into LN_2 before nucleation is completed, cells will equilibrate, at least in part, by intracellular freezing.

In our experiments, sperm survival was variable among different freezing rates, even within the same endpoint. For instance, when the endpoint was -50°C, hatching rates similar ($P>0.05$) to control were produced only by spermatozoa frozen at -5°C/min (Experiment 2A and 2B; Table 4); at -2 (Experiment 2C) and -10°C/min (Experiment 2D), hatching rates were lower. However, it is easy to distinguish, for each freezing rate, an optimal temperature endpoint to which spermatozoa must be frozen slowly, before fast freezing in LN_2 where highest sperm survival are obtained. At -2°C/min, this optimal endpoint was -40°C; at -5°C/min, both -45 and -50°C and at -10°C/min, -55°C. When sperm samples were plunged into LN_2 before these optimal endpoints and used to

fertilize eggs, the hatching rates produced were, in most cases, very low (Table 4). Based on Mazur's conclusion, we assume that dehydration was not completed and intracellular ice was formed in those samples. The ice formed inside the cells as a result of rapid freezing is likely to grow by recrystallization during warming, especially if warming is slow, as in our experiments. However, at -5°C/min, the optimal endpoint could be increased to supraoptimal temperatures like either -35 or -40°C when a 5-min holding time was included before fast freezing (Experiment 3; Table 5). Perhaps, during the holding time, sperm cells could dehydrate enough and not form intracellular ice, since hatching rates produced by spermatozoa frozen as described were similar ($P>0.05$) to those of fresh spermatozoa. However, this hypothesis requires further testing.

Regardless of whether cells equilibrate by dehydration or by intracellular freezing, they are subjected to a second class of physical-chemical events associated with the removal of liquid water and its conversion to ice. As temperature decreases, the amount of cell water decreases, extracellular and intracellular solutes concentrate, solutes precipitate as their solubilities are exceeded (thus changing pH), and all solutes precipitate below the eutectic point (the temperature at which the solution starts freezing) of the system. These physical-chemical events are referred to as "solution effects" (Mazur, 1970; Watson, 1995). Using an optimal freezing rate, once intracellular nucleation temperature has been passed, cells can be safely plunged into LN_2 at -196°C. If not, cells will be exposed for too long to the "solution effects." This phenomenon could explain our results in the second series of experiments, when spermatozoa frozen further than the optimal endpoint (-40°C a -2°C/min; -45/50°C at -5°C/min; -55°C at -10°C/min) before fast freezing in LN_2 produced decreasing hatching rates (Table 4). As far as we know, several freezing rates have been tested in African catfish spermatozoa, but no other endpoint in the first step of freezing but -65, -70 and -80°C were evaluated (Table 1). It is possible that the nucleation point had been surpassed and better hatching rates could have been obtained at other temperatures.

Solution effects are responsible for injury when freezing is slower than optimal, and intracellular freezing is responsible for injury when freezing is faster than optimal. The optimal rate, then, is slow enough to prevent production of intracellular ice and yet rapid enough to minimize the length of time cells are exposed to solution effects (Mazur, 1970). The critical rate varies among different types of cells. Yeast, for example, will contain intracellular ice when cooled faster than -10°C/min, whereas red blood cells will contain intracellular ice only when cooled faster than -5000°C/min (Mazur, 1970). In our experiments with catfish spermatozoa, 3 slow rates were tested in the first step of freezing (-2, -5 and -10°C/min), followed by plunging samples into LN_2 (second step of freezing). Because better results were obtained at -5°C/min (Table 4), we suggest it as the best freezing rate for Clarias gariepinus spermatozoa. Our results agree with Steyn (1993), who tested several freezing rates between -2 and -17°C/min and found better motility rates at -5°C/min.

Cryopreservation of spermatozoa should be useful as a routine method of gamete storage and management for catfish. African catfish, C. gariepinus, spermatozoa stored at -196°C retained good fertility for 6 weeks in our experiments (unpublished data) and for 16 months in the experiments of Steyn and van Vuren (1987). Frozen African catfish, H. longifilis, spermatozoa had the same fertilizing capacity as fresh spermatozoa

after 8 months of storage (Oteme *et al.*, 1996) and channel catfish spermatozoa, after 13 months of storage (Tiersch *et al.*, 1994). These data suggest that catfish spermatozoa will remain viable for years of storage and will allow greater flexibility in timing of induced spawning.

Acknowledgments

This research is supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) - Brazil and is part of the first author's Ph.D. project. We thank Mr. and Mrs. Willie Fleuren for providing facilities during on-farm experiment and Menno ter Veld for assistance on experiments.

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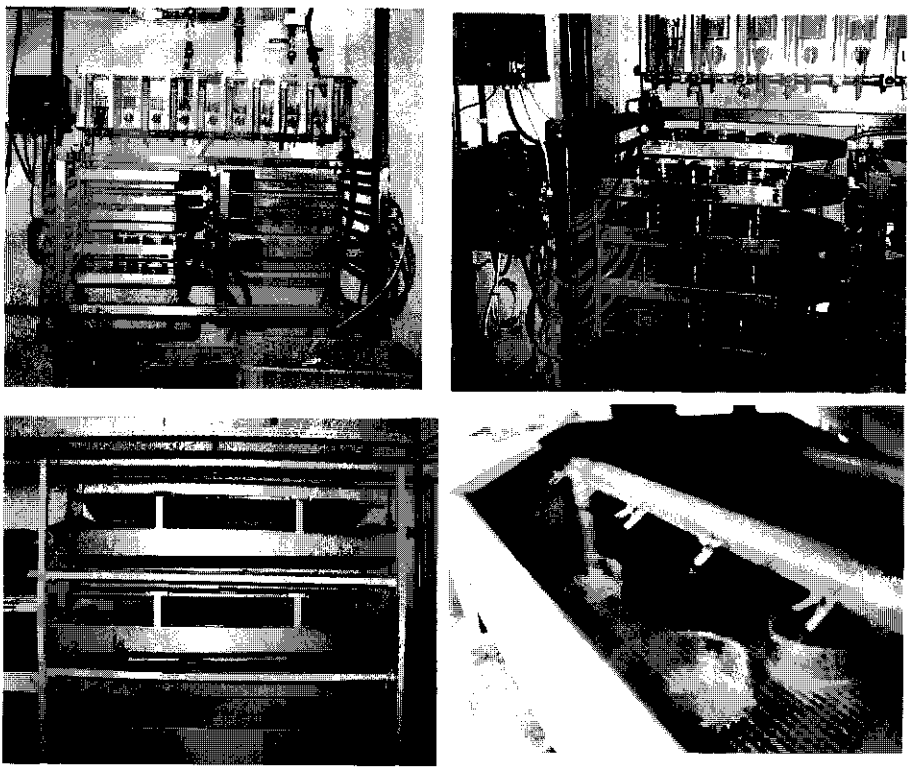

Picture 4. Incubation systems used in this thesis. In the upper system, water flows underneath the incubation units. In the lower system, water flows from the top over each incubation unit. Water is thermoregulated and recirculates in each system separately.

Chapter 6

Influence of cooling rates and plunging temperatures in an interrupted slow freezing procedure for semen of the African catfish, Clarias gariepinus

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Cryobiology (accepted)

Abstract

The objective of the present study was to optimize interrupted slow freezing protocols for African catfish semen. Semen diluted with methanol and extender was frozen in 1-ml vials in a programmable freezer. The temperatures of the freezer ($T_{chamber}$) and of the semen (T_{semen}) were measured simultaneously. We first tested 2-step freezing protocols with different cooling rates (-2, -5 and -10°C/min) and different temperatures at plunging into liquid N_2. The difference between T_{semen} and $T_{chamber}$ increased with faster cooling rates. In all programs, survival of spermatozoa, expressed as hatching rates, increased from near zero when T_{semen} at plunging was higher than -30°C, to values equal to control when T_{semen} at plunging was equal to or lower than -38°C. The inclusion of an isothermal holding period before plunging into liquid N_2 (3-step freezing protocols), resulted in an equilibration between T_{semen} and $T_{chamber}$, and improved semen survival. Semen could be plunged at temperatures as high as -37°C when cooled at -5 or -10°C/min, without compromising post-thaw semen survival. Cooling at -2°C/min in combination with a 5-min holding period reduced post-thaw survival. We conclude that at a cooling rate of -2°C/min, hatching rates can be maximized by plunging as soon as T_{semen} reaches -38°C. The isothermal holding period is beneficial when faster rates are used. A simple and efficient protocol for freezing African catfish semen can be obtained by cooling at a rate of -5-10°C/min combined with a 5-min holding period in the freezer, at -40°C.

Introduction

Cryopreservation of semen has long been seen as a means of benefiting the breeding of animals of agricultural importance, and has been recognized as contributing to the conservation of endangered species and to overcoming aspects of male infertility in humans (Watson, 2000). In aquaculture, semen cryopreservation is applied for synchronization of artificial reproduction and maintenance of the genetic variability of original fish stocks, as the continued development of this industry depends on the availability of the wild (sometimes endangered) fish species (Holt, 1997). Catfishes are an economically important group of fresh and brackish water fish worldwide. Several species have been successfully introduced in aquaculture and the African catfish, Clarias gariepinus, is perhaps the most important one, not only in Africa but also in S-E Asia (e.g., Thailand) and in Europe (e.g., The Netherlands). Stripping of semen in these species, however, is difficult and many times impossible, and for artificial reproduction, males are killed, testes are removed and intra-testicular semen is squeezed out over the eggs. Semen cryopreservation of catfish species significantly increases the reproduction potential of a single male and is a tool in selection and genetic improvement.

A variety of methods for freezing semen of a number of different mammals and fish species have been described. In so-called interrupted slow freezing (ISF) procedures an initial slow cooling period is followed by rapid cooling, as samples are plunged into liquid N_2 (LN_2) for final storage. During the first step, samples are cooled either in vapor of LN_2 for a certain time, or in a programmable freezer. Spontaneous or induced

ice nucleation occurs at a temperature just below the solution freezing point. The ice formation results in an increase of the extracellular solute concentration in the unfrozen fraction, which in turn leads to dehydration of the cells. As the ISF procedure continues, the slow cooling step is terminated at an intermediate temperature (T_p) at which plunging into LN_2 occurs (second step; Mazur, 1990). As plunging into LN_2 implies a phase of rapid cooling, cell survival is only possible if dehydration has reached the level necessary to preclude intracellular ice formation (IIF) at that fast cooling rate. If plunging happens too early, cells will equilibrate with the unfrozen fraction, at least in part, by IIF. On the other hand, if slow cooling is continued longer than necessary to reach the required level of dehydration, cell injury could arise from too long exposure to high concentrations of electrolytes (the so-called solution effects; Mazur, 1977), and/or from a continued osmotic shrinkage of the cells. Thus, the optimal T_p may be an important key factor for a successful post-thaw semen survival. As the optimal T_p relates to the level of dehydration of the cells, it is likely to depend on the initial concentration of the permeating cryoptotective agent present in the solution and the slow cooling rate (Liu *et al.*, 2000).

Most of the experimental work on cryopreservation for African catfish semen has focused on trials to identify the optimal extender for conservation (Urbanyi *et al.*, 1999; Rurangwa *et al.*, 2001), the cryoprotective agent (Steyn and van Vuren, 1987; Horvath and Urbanyi, 2000; Viveiros *et al.*, 2000) and the cooling rates (Steyn, 1993; Viveiros *et al.*, 2000). However, the effects of T_p other than -65 to -80°C, have been evaluated in only one study (Viveiros *et al.*, 2000). The results obtained in the latter study showed decreasing hatching rates when semen was plunged in LN_2 from freezer chamber temperatures ($T_{chamber}$) below -40°C (at -2°C/min), -50°C (at -5°C/min) or -55°C (at -10°C/min). It suggests that the negative effects of prolonged slow cooling reduce semen survival at T_p below these values.

In this study, different combinations of T_p and cooling rates for African catfish semen were tested, aiming to identify a range of optimal T_p where injuries caused by IIF and solution effects could be minimized. First, the effects of 2-step freezing protocols were analyzed. Then, the effects of an isothermal holding period before plunging into LN_2 (3-step freezing protocols) on semen temperature (T_{semen}) and semen survival were evaluated. Methanol was chosen as the permeable cryoprotective agent, based on previous results (Viveiros *et al.*, 2000).

Materials and methods

Animals

The brood stock of the African catfish, C. gariepinus, originated from the Republic of Central Africa, has been bred for several generations in the Hatchery of the Fish Culture and Fisheries Group at Wageningen University, The Netherlands. Mature males and females were kept together under constant water temperature (25°C) and photoperiod (12 h of light per day) in 250-l rectangular tanks connected to a recirculating system equipped with a biofiltration unit. The flow rate was 8 to 12 l/min

and the water quality was monitored daily. NH_4^+ and NO_2 were kept below 1 ppm and pH was kept at 6.5. Fish were fed trout pellets (Trouvit, The Netherlands) at a maintenance level of 0.8% of body weight daily.

Semen

Males were anesthetized in tricaine methanesulfonate (Crescent Research Chemicals, Phoenix, Arizona, USA) and sacrificed by spinal transection. Males were dissected, testes were removed, and intra-testicular semen was gently squeezed out. Sperm cells of each male were counted in a double Burker-Türk chamber (W. Schreck, Hofheim TS) to estimate sperm concentration.

Cryopreservation

Based on previous results (Viveiros *et al.*, 2000), Ginzburg fish Ringer (123.2 mM NaCl; 3.75 mM KCl; 3.0 mM $CaCl_2$; 2.65 mM $NaHCO_3$; pH 7.6; 244 mOsm) and methanol 2.47 M were used as cryosolution. Semen was added at 10% (v/v) to the cryosolution. Aliquots of 500 µl of diluted semen were transferred to 1-ml vials and immediately placed in the freezer.

For each treatment, 3 vials (triplicates) were cooled in a programmable freezer (Planer Kryo 10 series III). This freezer has a platinum resistance thermometer that measures the chamber temperature ($T_{chamber}$) and controls the programmed cooling rate. To follow the cooling process of the cryosolution, T_{semen} was measured with a thermocouple (Omega T Thermocouple D5331, USA) placed inside one vial, containing the same solution as the others, and cooled simultaneously. All freezing programs started when $T_{chamber}$ was at 5°C and T_{semen} was at 7°C. Thus, the equilibration time between mixing semen with cryosolution and the start of the freezing program was about 4-6 min. At the end of each program, semen was quickly plunged into LN_2 at -196°C and stored until next morning, except in experiment 3, in which samples were stored for only 40 min (see experimental design). Semen was thawed in a water bath at 27°C for 3 min. A sample of fresh semen was kept undiluted at 4°C, and used as unfrozen control.

Fertilization trial

Female catfish, one per experiment, were injected with carp pituitary extract at 4 mg/kg body weight and stripped of eggs 15 h later (23.5°C). Eggs were collected in a dry plastic basket and kept at room temperature (23°C) while being used and for a maximum of 2 h. According to the sperm concentration, post-thaw semen from each vial was diluted again (about 1000 times) with fresh Ginzburg fish Ringer to a constant concentration of 17×10^6 spermatozoa/ml. One hundred µl of this solution was mixed with 0.25 g of fresh eggs (approximately 150 eggs), in duplicate. Fertilization was initiated by adding 1.5 ml of copper-free tap water and mixing for 40 seconds. Based on previous results (Viveiros *et al.*, 2000), the fertilization ratio was set at approximately 11,300 spermatozoa/egg. Fertilization was initiated by adding 1.5 ml of copper-free tap water and mixing for 40 seconds. The eggs were then transferred to a 10-cm diameter

PVC basket with a 0.5-mm mesh bottom and incubated in shallow trays at 29.5 ± 0.5°C, connected to a recirculating system. In the beginning and at the end of each experiment, control egg batches were inseminated with unfrozen semen from the same male, diluted in fish Ringer to the same ratio as the post-thaw semen. The fertilization yield was evaluated as hatching rates, measured 24 h after incubation.

Experimental design

All experiments were conducted in duplicate, with a different pair of male and female catfish each time.

In the first series of experiments, 2-step freezing protocols were used to cool semen at rates of -2, -5 and -10°C/min (Figure 1A). A set of vials (n=3) was plunged into NL_2 at every 5°C (see Table 1 for temperature range), according to the $T_{chamber}$.

In a second series of experiments, 3-step freezing protocols were used to cool semen at rates of -2, -5 and -10°C/min (Figure 1B). At the end of each program, half of the samples were immediately plunged into LN_2, while the other half was held at the end temperature for 5 min longer. In the case that the end temperature was -40°C, an additional set of vials (n=3) were held in the freezer for as much time as necessary until the T_{semen} had reached -38°C, and then plunged into LN_2.

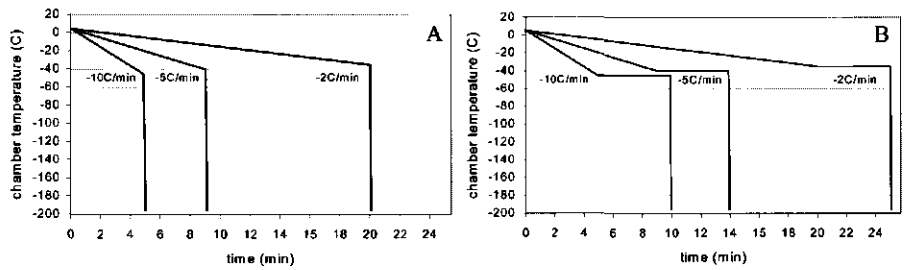

Figure 1. Schematic representation of 2-step (A) and 3-step (B) freezing protocols at cooling rates of -2, -5 and -10°C/min used to freeze African catfish semen.

In a third series of experiments, 2-step freezing protocols were used to cool semen at a rate of -5°C/min. Samples (n=6) were removed from the freezer every 5°C over the range of -30 to -55°C, according to $T_{chamber}$. Then, samples were either immediately rewarmed from those temperatures (not plunged in LN_2), or first plunged in LN_2 for 40 min, and then thawed.

Statistical analyses

The number of hatched larvae, as a percentage of total eggs exposed to semen, was determined 24 h after fertilization. For cryopreserved semen, data from 6 replicates per

treatment (2 samples of 0.25 g egg per vial and 3 vials per treatment) were pooled to calculate mean and standard deviation. For fresh semen, hatching rate data from fertilization in the beginning and at the end of each trial were pooled to calculate mean and standard deviation, after checking for differences. All statistical analyses were done using the SAS 6.11 package (SAS Institute Inc., 1990). Hatching rates from fresh and cryopreserved semen per fertilization trial were tested for significant differences by ANOVA using the parametric General Linear Model procedure, followed by Duncan's multiple range test. The residues from the different ANOVA models were tested for normal distribution using the univariate procedure. P-values <0.05 were considered to be significant.

Results

Hatching rates produced with control semen samples (unfrozen) ranged from 73.7 to 87.0%, indicating good gamete quality.

Among all experiments, T_{semen} did not correspond to $T_{chamber}$, as can be observed in Tables 1, 2 and 3 and Figure 2. The largest differences were (a) during the crystallization process when the latent heat warmed up the semen solution to around -5°C, while $T_{chamber}$ continued to drop, governed by the cooling rate used; and (b) at a faster rate of -10°C/min.

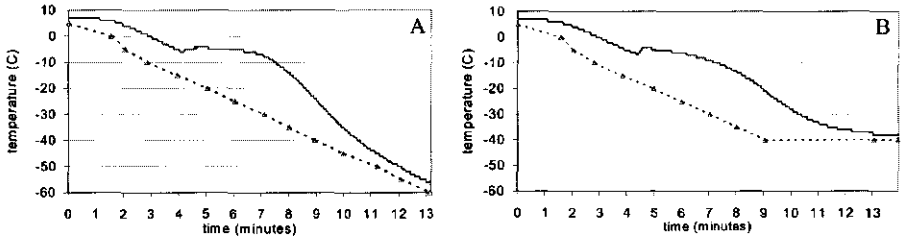

Figure 2. Examples of semen temperature (full line) variation in comparison with chamber temperature (dotted line) during a 2-step (A) and a 3-step (B) freezing protocols, at a cooling rate of -5°C/min.

In the range of the cooling rates studied here, the results produced with semen frozen in 2-step protocols, were all in the same pattern. Hatching rates were always very low when eggs were fertilized with semen plunged in LN_2 at high $T_{chamber}$ (above -35°C at -2°C/min; above -40°C at -5°C/min; or above -55°C at -10°C/min). When the semen was plunged at lower values of $T_{chamber}$, hatching rates increased drastically to values similar (P>0.05) to control. However, with even lower $T_{chamber}$ at plunging, post-thaw semen produced again lower hatching rates, which is seen most clearly at the slowest cooling rate (Table 1).

Table 1. Hatching rates (mean ± SD, n=6 fertilizations) produced with African catfish semen frozen in 2-step freezing protocols and plunged in liquid nitrogen at different temperatures. Semen temperature just prior to plunging is also shown.

Cooling rates (°C/min)	Temperature of plunging (°C)		Hatching rates (%)
	Chamber	Semen	
-2	- 35	-32	0.2 ± 0.4^{d}
-2	- 40	-38	74.3 ± 6.0^{ab}
-2	- 45	-44	74.8 ± 4.6^{ab}
-2	- 50	-49	66.6 ± 4.2^{bc}
-2	- 55	-53	52.4 ± 24.1^{c}
-5	- 40	-24	4.3 ± 6.0^{d}
-5	- 45	-35	48.5 ± 13.5^{c}
-5	- 50	-45	77.4 ± 3.4^{ab}
-5	- 55	-50	81.7 ± 5.0^{a}
-5	- 60	-56	67.3 ± 9.6^{bc}
-10	- 45	-9	2.2 ± 2.6^{d}
-10	- 50	-18	1.8 ± 1.3^{d}
-10	- 55	-31	3.7 ± 4.6^{d}
-10	- 60	-41	50.7 ± 36.9^{c}
-10	- 65	-50	63.4 ± 10.0^{bc}
	Control		83.1 ± 6.0^{a}

[a-d] Means followed by different superscript are significantly different (Duncan, $P<0.05$).

In 3-step freezing protocols, a 5-min holding period was added at the end of the freezing programs, before plunging into LN_2. At the cooling rate of -2°C/min, the holding period at -30°C was ineffective in increasing semen survival. Semen plunged after the holding period at -35°C, produced hatching rates higher ($P<0.05$) than samples cooled without hold, but still lower than control semen. And semen plunged after a 5-min holding period at -40°C, produced lower ($P<0.05$) hatching rates than samples cooled without hold. On the other hand, at faster cooling rates, the additional holding period was always beneficial and T_{semen} could equilibrate with $T_{chamber}$ (Figure 2B). All samples cooled at a rate of either -5 or -10°C/min and held at the end temperature for 5 min or until T_{semen} dropped to -38°C, produced hatching rates ($P<0.05$) higher than those samples cooled to the same end temperatures, but without hold (Table 2).

Table 2. Hatching rates (mean ± SD, n=6 fertilizations) produced with African catfish semen frozen in 3-step freezing protocols.

a) Samples were plunged in liquid nitrogen immediately or after 5-min hold at the plunging temperature. Semen temperature in the beginning and at the end of the hold, just prior to plunging is also shown.

Cooling rates	Temperature at plunging			Hatching
(°C/min)	Chamber (°C)	Hold (min)	Semen (°C)	(%)
-2	- 30	0:00	-20	0.0 ± 0.0[d]
-2	- 30	5:00	-28	0.0 ± 0.0[d]
-2	- 35	0:00	-30	5.1 ± 16.9[d]
-2	- 35	5:00	-33	25.9 ± 6.4[c]
-2	- 40	0:00	-36	60.9 ± 9.3[ab]
-2	- 40	5:00	-39	26.7 ± 10.0[c]
-5	- 35	0:00	-13	0.3 ± 0.8[d]
-5	- 35	5:00	-33	52.4 ± 6.4[b]
-5	- 40	0:00	-21	1.2 ± 1.9[d]
-5	- 40	5:00	-38	80.9 ± 4.1[a]
-10	- 40	0:00	-5	1.6 ± 3.9[d]
-10	- 40	5:00	-37	82.9 ± 3.4[a]
-10	- 45	0:00	-8	2.8 ± 2.6[d]
-10	- 45	5:00	-43	80.5 ± 6.9[a]
-10	- 50	0:00	-11	5.2 ± 4.8[d]
-10	- 50	5:00	-48	33.8 ± 8.8[c]
	Control			73.7 ± 1.5[a]

b) After reaching a chamber temperature of -40°C, samples were held at -40°C until the semen temperature reached -38°C and were then plunged in liquid nitrogen.

Cooling rates (°C/min)	Hold (min)	Hatching (%)
-2	0:56	56.7 ± 18.2[b]
-5	4:20	84.8 ± 3.8[a]
-10	5:57	86.2 ± 5.7[a]
Control		73.7 ± 1.5[a]

[a-d] Means followed by different superscript are significantly different (Duncan, $P<0.05$).

Semen cooled at a rate of -5°C/min and rewarmed from the end temperature produced hatching rates similar (P>0.05) to control over the $T_{chamber}$ range of -30 to -50°C. Samples rewarmed from a $T_{chamber}$ of -55°C produced lower hatching rates than control. Samples thawed from LN_2, produced very low hatching rates when cooled to the $T_{chamber}$ range of -30 to -45°C. Only samples plunged into LN_2 from the $T_{chamber}$ of -50°C produced hatching rates similar (P>0.05) to control (Table 3).

Table 3. Hatching rates (mean ± SD, n=6 fertilizations) produced with African catfish semen frozen at the rate of -5°C/min, to different temperatures. Samples were either immediately rewarmed from the end temperature (not plunged), or first plunged into liquid nitrogen (LN_2), and then thawed. Semen temperature at the end temperatures is also shown.

Temperature at the end of cooling		Not plunged	Plunged
Chamber (°C)	Semen (°C)	into LN_2	into LN_2
- 30	-7	86.4 ± 9.3[a]	0.0 ± 0.0[c]
- 35	-13	84.3 ± 6.9[a]	0.2 ± 0.4[c]
- 40	-20	88.0 ± 7.2[a]	0.2 ± 0.4[c]
- 45	-30	88.1 ± 2.8[a]	39.5 ± 35.9[b]
- 50	-40	77.8 ± 14.2[a]	78.1 ± 6.9[a]
- 55	-47	54.9 ± 26.2[b]	37.7 ± 28.0[b]
Control		84.6 ± 15.8[a]	

[a-c] Means followed by different superscript are significantly different (Duncan, P<0.05).

To have an overview of the results obtained in this study and allow comparison between different males, all hatching rates were transformed as percentage of control and plotted in Figure 3 as a function of the T_{semen} at plunging.

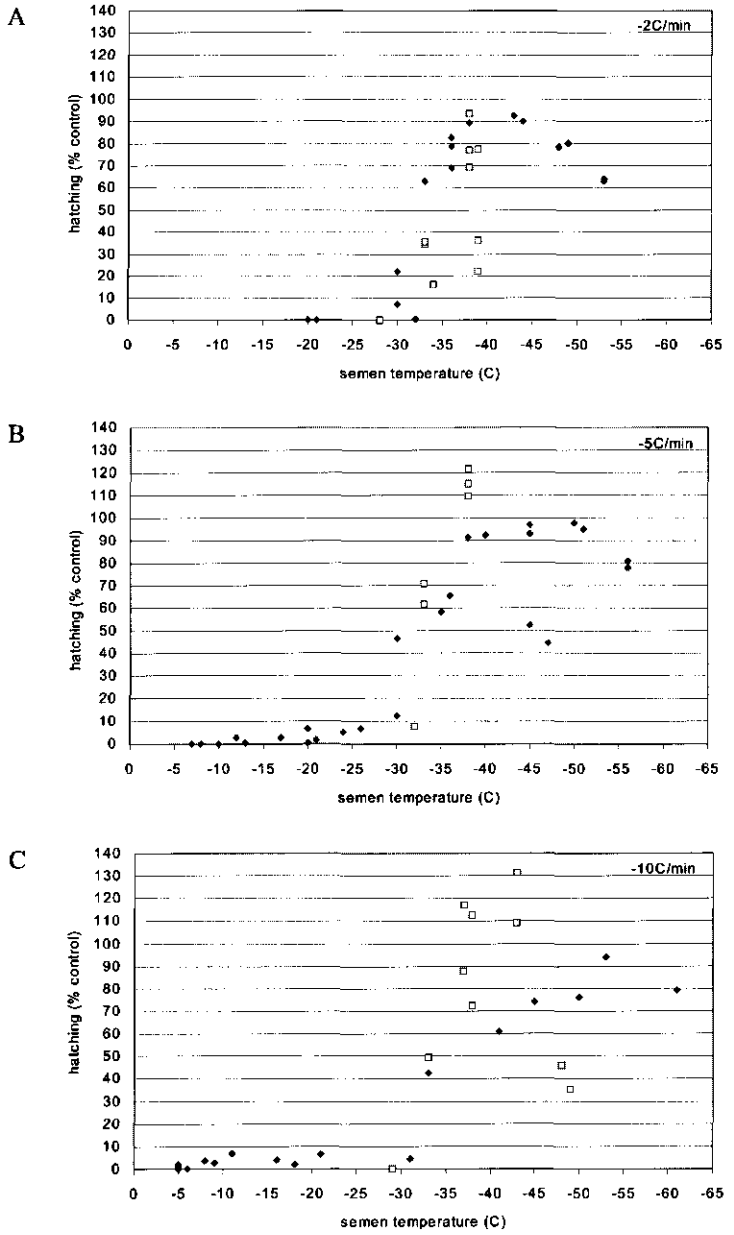

Figure 3. Hatching rates as percentage of control, of African catfish semen frozen at -2 (A), -5 (B) or -10°C/min (C), in 2- (closed symbols) or 3-step freezing protocols (open symbols). Each symbol represents mean of 6 fertilizations and includes data from different experiments.

Discussion

To develop an optimal freezing protocol with the highest survival of spermatozoa (measured in terms of hatching rates), one should use a low spermatozoa:egg ratio. Using too many spermatozoa would mask the decrease of the fertilizing capacity of post-thaw spermatozoa and produce good hatching rates even when using sub-optimal freezing protocols. According to our previous study (Viveiros *et al.*, 2000), a ratio of 11,300 spermatozoa:egg was used in all experiments described here. With the same catfish species, Rurangwa *et al.* (Rurangwa *et al.*, 2001) used a ratio of 101,400 spermatozoa:egg. In other catfish species, to produce hatching rates similar to control with post-thaw semen, 200,000 spermatozoa:egg were needed for channel catfish, Ictalurus punctatus (Tiersch *et al.*, 1994) or as many as 13000,000 spermatozoa:egg for blue catfish, I. furcatus (Bart *et al.*, 1995).

During the process of cooling and freezing, spermatozoa are subjected to drastic changes in their physical and chemical environment. One of these changes takes place when liquid water is converted into ice. Spontaneous ice nucleation will usually occur after the solution is supercooled to a temperature between -5 and -15°C. Ice crystals will grow rapidly in all directions. The latent heat warms up the sample (Figure 2) until the remaining unfrozen fraction of the solution reaches the freezing temperature. At this point, ice formation stops or proceeds at a rate governed by the rate at which the sample is further cooled. The solute concentration and, consequently, the osmotic pressure of the unfrozen fraction increase rapidly, causing an efflux of water from the cells. Therefore, cells dehydrate and shrink. When cells are cooled slowly, dehydration can proceed to the point of osmotic equilibrium between intra- and extracellular space. Cellular dehydration and, consequently, osmotic shrinkage of the cells will be maximal, and cells will be exposed for too long to a continued increase of solute concentration in the unfrozen fraction, which may cause the so-called solution effects. Raising the cooling rate will lead to less excessive cellular dehydration, intracellular solute concentrations and shrinkage. Moreover, cells will remain vulnerable to the unfavorable conditions for a shorter period of time. But if the cooling rate is increased too much, dehydration may not be fast enough to prevent the occurrence of IIF, which is lethal (Mazur, 1977). A clear line between damages caused by IIF and solution effects cannot be drawn, mainly because solution effects happen throughout the entire freezing process.

The results observed in this study can be explained by the mechanisms described above. As can be observed in Figure 3, when semen was plunged into LN_2 at T_{semen} higher than -30°C, the hatching rates were extremely low. Also, virtually all sperm cells were immotile (data not shown). We assume that the loss of motility and fertility of the semen is caused by IIF when plunged into LN_2 from these relatively high T_{semen}. Semen plunged from temperatures between -30 and -35°C produced increased hatching rates, but maximal hatching rates were produced with semen plunged from -36°C or lower, suggesting that the optimal range of T_p starts at this point.

Liu *et al.* (2000) estimated theoretically for rat embryos in medium containing DMSO that plunging into LN_2 would be possible from temperatures in the range of -26

and -35°C, provided that cooling rate was slow enough to allow sufficient efflux of water. Their calculations were based on the assumption that the zygotes need to attain a critical intracellular solute concentration in the range known to enable vitrification of a ternary (water/DMSO/NaCl) system, using vitrification data from Ren *et al.* (1994). In our study we used methanol as cryoprotectant. However, whereas different solutes will have different solute concentrations at one temperature, the free energy of free (unfrozen) water, or the osmolality of the extracellular unfrozen fraction will only depend on the temperature, and not on the type of cryoprotectant, nor on the initial concentrations of cryoprotectant and other solutes. During slow cooling, the osmolality of the unfrozen fraction of aqueous media increases from 14.0 Osm/kg of water at -26°C to 25.3 Osm/kg of water at -47°C (as $\Delta T = 1.86 \times$ Osmol/kg; Weast, 1984). For the freezing medium used in our study, the latter value results in molar concentrations of 11.9 M methanol and 0.64 M extender (see material and methods for extender formula). We found that vials containing 500 µl of 11.9 M methanol plus 0.64 M extender remained glassy during cooling and during warming in a water bath. However, 10.7 M methanol plus 0.58 M extender remained glassy when plunged in LN_2 but became opaque during slow warming. This indicates that the remaining unfrozen fraction reaches an osmolality (or a solute concentration) that enables vitrification of bulk liquid ultimately at a temperature of -47°C. Hence, it can be expected that IIF does not occur when semen is plunged into LN_2, at a temperature of or below -47°C, provided that cells get ample time to reach or approach osmotic equilibrium with the extracellular unfrozen fraction. The chance of ice nucleation in the extremely small intracellular aqueous volume of spermatozoa is much smaller than that in bulk liquid. Hence, the upper limit of temperatures at which the semen can be safely plunged in LN_2 could be higher than -47°C, which indeed we found.

Our results show that T_{semen} at plunging should not be higher than -36°C. With the cooling rates used in the present study, the apparent optimal T_p ranged from -38 to -50°C when 2-step protocols were used, and from -36 to -43°C when 3-step protocols were used. Furthermore, the optimal T_p appeared to be lower at faster cooling rates in 2-step protocols (Table 1). This finding indicates that at faster cooling rates, cell dehydration lags behind and osmotic equilibrium is not reached or approached, whereas it appears that a holding period could provide extra time needed for the efflux of water and to eliminate spatial temperature gradients within the semen sample.

Possibly, T_p could even be higher than -36°C at even slower cooling rates, or longer holding periods. We have no data on the water permeability for fish semen to calculate the necessary slow cooling rate or holding time to reach full osmotic equilibrium, as Liu *et al.* (2000) did for rat embryos. We did not consider slower cooling rates or longer holding times for practical reasons and because prolonged exposure of the spermatozoa to high solute concentrations are expected to be deleterious for their survival. The deleterious effects of continued slow cooling become apparent, as T_{semen} goes down in 2-step freezing protocols. Post-thaw semen produced decreasing hatching rates when cooled at the rate of -2°C/min below -45°C; at -5°C/min, below -55°C; and at -10°C/min, below -60°C (Figure 3). It is evident, however, the difference in semen quality between males, with the good freezers yielding still high results (78-81% of hatching) at temperatures as low as -55°C, and the bad freezers yielding lower results (45-52% of hatching) at temperatures as high as -45°C (Figure 3B). The negative effects

of prolonged slow cooling were also observed at higher temperatures, when a combination of slow rate and holding period was used (3-step freezing protocol). At -2°C/min, the 5-minute holding period included at $T_{chamber}$ of -40°C had a negative effect on hatching rate, probably because cells remained at low temperatures (-36 to -39°C) for too long (Figure 3A). Cell dehydration was already enough for plunging at the beginning of the holding period, as indicated by the high hatching rates produced with samples frozen to a $T_{chamber}$ of -40°C, without hold (Table 2). These results indicate that when cells are held for longer than necessary, or when slow cooling is continued for longer than necessary, spermatozoa become damaged by prolonged exposure to high solute concentrations (solution effects), and other phenomena associated with excessive dehydration, such as mechanical stress of cell shrinkage.

We conclude that, in the range of the cooling rates and the cryoprotective agent used in this study, injuries caused by IIF and solution effects are minimized when T_{semen} at plunging is in the range of -36 to -50°C and the introduction of an isothermal holding period is beneficial when relatively fast cooling rates are used. A simple and efficient protocol for freezing African catfish semen can be obtained by cooling at a rate of -5-10°C/min combined with a 5-min holding at a $T_{chamber}$ of -40°C.

Acknowlegments

This research was financed by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Brazil. We thank Menno ter Veld, Rui Rocha and Tania Fernandes for assistance on experiments.

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Chapter 7

General discussion

Reproduction of the African catfish male kept in captivity

The reproductive process in all vertebrates is mainly regulated by the brain-pituitary-gonadal axis. The brain is the integrator of most information coming from the outside (such as photoperiod, temperature, and cues from conspecifics) and from the inside (such as gonad development), and is actively involved in all steps of the sexual cycle (Kah *et al.*, 1993). According to the kind of information (positive or negative stimuli), the brain hormone secretion is changed. The main brain hormones controlling reproduction are gonadotropin releasing hormone (GnRH) and dopamine. GnRH stimulates the release of pituitary gonadotropins (follicle stimulating hormone – FSH, and luteinizing hormone – LH), while dopamine inhibits this release (Nagahama, 1994).

In African catfish, two GnRH forms have been described: the chicken GnRH II (cGnRH-II; that seems to be present in all vertebrates, except in highly evolved placental mammals) and catfish GnRH (cfGnRH; described only in African and Thai catfish until now; Bogerd *et al.*, 1992). During the annual reproduction cycle under natural conditions, plasma LH levels are fairly low, possibly because cGnRH-II partially inhibits the cfGnRH-stimulated LH secretion. At the time of spawning, high plasma LH levels are required, which may be generated by increased cGnRH-II amounts in the pituitary. These, together with cfGnRH or alone, and possibly in combination with a reduced dopaminergic inhibition (de Leeuw *et al.*, 1985), result in the spawning-associated LH surge (Schulz *et al.*, 1993). Under husbandry conditions, cGnRH-II may be one of the factors that keep circulating LH levels relatively low throughout the year (Bosma, 1995).

Despite the low circulating LH levels, spermatogenesis in the African catfish kept in captivity occurs normally and males remain in a condition comparable to the pre-spawning phase in the natural environment. LH stimulates Leydig cells to produce and secrete androgens (steroidogenesis). During this process, the main testicular steroidogenic product is 11β-hydroxyandrostenedione, which is converted into 11-keto-testosterone (11-KT) at extra-testicular sites, probably hepatic tissue (Cavaco, 1998). 11-KT seems to be the biologically active compound and exerts direct effects on the Sertoli cells, stimulating premitotic spermatogonia and the complete process of spermatogenesis in the fish. At all seasons, catfish testis contains spermatogenic cysts at different stages from spermatogonia to spermatozoa, and several ripe sperm cells inside the seminiferous tubules lumen. The quality of these gametes is good and fertilization using intratesticular semen results in high hatching rates. These gametes also survive the freezing and thawing processes, thereby enabling semen cryopreservation of this species (Chapters 5 and 6). However, spontaneous semen release does not occur and hand-stripping of semen is practically impossible. For artificial reproduction, either males are operated or sacrificed, testes are removed and macerated over the eggs, or brood fish are placed in freshly filled ponds that had been dry for some time to spawn naturally, but then predation on and cannibalism between the offspring are heavy.

The aim of this thesis was to improve the reproductive efficiency of the African catfish males kept under husbandry conditions, using a two-pronged approach. Firstly, different methods to facilitate hand-stripping of semen were tested; these results are

discussed in the 1st paragraph of this Chapter. Then, an effective protocol for catfish semen cryopreservation was developed; the important findings are reported and discussed in the 2nd paragraph. Several concluding remarks are made in the last paragraph of this Chapter.

Hand-stripping of semen

It is normally accepted that catfish males in captivity do not release semen spontaneously because their pituitary fails to release LH in large amounts, either because GnRH is not released or because it is prevented from eliciting its effects (Goos and Richter, 1996). Results presented in Chapter 2 show an LH surge induced 2 h after treatments with mammalian LH-releasing hormone analogue (LHRHa), LHRHa plus a dopamine antagonist or salmon GnRH analogue plus a dopamine antagonist (ovaprim). Hand-stripping of semen, however, was possible only in a few ovaprim-treated fish. Although it would have been better to test the stripped fluid quality on a fertilization trial, the lack of motility observed after water addition suggests that either sperm cells were stripped in an activated form (possibly due to urine or blood contamination) and thereby lost their motility soon after that, or the collected fluid was deprived of sperm cells. To avoid sperm activation during stripping, it is recommended to add an immobilizing solution to the sampling recipient prior to stripping, as described in Chapter 4. Similar disappointing results with hypothalamic hormone treatments were observed in Blue catfish, Ictalurus furcatus. LHRHa treatment induced, after 24 h, stripping of 2-30 mL of a clear liquid containing a few viable sperm. This fluid gave a fertilization rate of 50% when directly stripped onto eggs. However, when this fluid was first diluted in extender and then used for fertilization of 2,000 eggs 5 min later, only 3 embryos hatched (Dunham, 1993).

It is possible that a sustained LH surge rather than a brief LH peak would be necessary to enable collection of viable semen and of a larger volume. The effects of either weekly injections of the hypothalamic hormones or treatment with a sustained-release GnRH delivery system on semen collection of catfish species should be investigated. The use of such a GnRH delivery system results in high LH levels for at least 8 weeks and induces both spermatogenesis and spermiation in salmonids (Weil and Crim, 1983), white bass, Morone chrysops (Mylonas *et al.*, 1997), European seabass Dicentrarchus labrax (Sorbera *et al.*, 1996) and other species.

The effect of exogenous LH, present in crude pituitary extracts, on semen collection was also evaluated in this thesis. The carp pituitary suspension (cPS)-induced plasma LH level, measured 2 h after injection, was lower than the plasma LH levels observed after LHRHa and ovaprim treatments, but significantly higher than the control. Single injections of cPS induced a slight testis hydration, but hand-stripping of semen remained impossible. Only when 2 consecutive injections of cPS were used, hand-stripping of semen was possible in 80% (Chapter 2), 27% (Chapter 3) and 53% (Chapter 4) of the treated males. The reason for this different response to cPS treatment is not clear. Perhaps it is due to differences in maturational stages, progeny groups or genetic

background. The sperm cells present in the stripped fluid could be activated with water, both when the fluid was stripped into an immobilizing solution (Chapter 4) and when not (Chapters 2 and 3). However, the fertilizing capacity of the stripped fluid was very low as only 0.7% of the eggs hatched (Chapter 4). Furthermore, a strong testicular hydration with increased testes and seminal vesicles (SV) weights, increased intratesticular semen volume and decreased intratesticular sperm concentration, was observed after 2 consecutive cPS injections. It has been described that the seminal fluid production, possibly as a result of testicular hydration, is the earliest effect of a brief elevation in plasma LH (Mylonas *et al.*, 1997). Because both hypothalamic hormones and cPS treatments elevated plasma LH levels, but testis hydration was observed only when cPS or homologous pituitary suspension (Clarias-PS) was used (Chapter 2), this hydration could not have been caused by a brief elevation in plasma LH. Crude pituitary extracts contain all hormones, factors, etc. produced or stored in the pituitary. It is possible that the substantial cPS-induced testicular hydration was related to another compound of the cPS (such as FSH) that is able to activate fluid production by testicular Sertoli cells. Similar testicular hydration in response to pituitary extract treatments has been reported for the same species (Hecht *et al.*, 1982), Asian green catfish, Mystus nemurus (Christianus *et al.*, 1996), European catfish, Silurus glanis (Linhart and Billard, 1994) and South American catfish, Rhamdia sapo (Espinach Ros *et al.*, 1984).

Similarly, cPS treatments but not hypothalamic hormone treatments, facilitated stripping of sperm cells that could be activated with water, although with low fertilizing capacity. It is possible that one or more of the pituitary compounds, such as oxytocin-like or vasopressin-like peptides, may have acted on testicular functionality and facilitated the stripping of fluid. It has been demonstrated that high levels of isotocin (the oxytocin-like peptide) are present in catfish brain and pituitary (Goos *et al.*, 1997). Furthermore, the paraventricular nucleus of the hypothalamus, which uses oxytocin as a neuro-transmitter, plays a key role in sexual functions in mammals (McKenna, 1999). Oxytocin promotes spermiation and sperm transport, increases the volume of fluid and the number of sperm released from the cauda epididymis and increases volume and sperm concentration in the ejaculate (Assinder *et al.*, 2000). Oxytocin also regulates male sexual behavior leading to penile erection and ejaculation (McKenna, 1999). However, the role of oxytocin in the reproductive behavior and semen release in male fish is yet not clear. In Chapter 4, the results of 5 different trials showed increased sperm concentration of the medium after incubation of testis slices with oxytocin. However, when tested *in vivo*, oxytocin treatment did not significantly improve the effects of 2 consecutive injections of cPS on stripping of semen. Stripping was possible in 67% of the cPS-oxytocin treated fish and in 53% of the cPS treated males. The hatching rates obtained with stripped semen after cPS-oxytocin treatment were low (3.0%) as were those obtained after cPS treatment (0.7%). The only "positive" effect was that the intratesticular spermatocrit values observed after cPS-oxytocin treatment were similar to the control, while after cPS treatment, these values were lower than the control. This suggests that, in a modest way, oxytocin may have stimulated contractions of the seminiferous tubules and increased the number of sperm cells collected from the testes. However, correspondent increased optical density was not observed. To elucidate this point, this experiment should be repeated with a larger number of animals and using a higher oxytocin dose.

The use of oxytocin to induce reproduction in African catfish has been reported in only one study (Hecht *et al.*, 1982), besides this thesis. Females were treated with oxytocin at the same dose used here, to induce ovarian contraction and to facilitate egg release. About 50% of females spawned after oxytocin treatment, in comparison with 0% of the control and almost 100% of the females treated with Clarias-PS or cPS in combination or not with human chorionic gonadotropin. Taking these results and ours together, we can conclude that the reproductive tract of both male and female African catfish responds to oxytocin treatment. The use of oxytocin to stimulate contractility of the seminiferous tubules and promote sperm transport, thereby increasing sperm concentration in the semen output in African catfish, as has been described in rams (Assinder *et al.*, 2000) and bulls (Berndtson and Igboeli, 1988), should be further investigated. Research should focus on dose and latency time, as well as on other combinations of cPS and oxytocin treatments.

Since we could not facilitate hand-stripping of fertile sperm cells in catfish males after treatments that induced LH surge, testis hydration or contractions of the reproductive tract, another source of failure/blockage might be present. Already in 1976, Richter stressed that the fingerlike extensions of the SV surrounding the sperm ducts in the African catfish, may retain semen flow when pressure is applied on the male abdomen. Later on, this hypothesis was confirmed. In our laboratory, during studies on sex differentiation in catfish, we found that about 19% of the males treated with dietary 17α-methyltestosterone (MT) were strippable. Under dissection, all MT-treated males had undeveloped SV (Eding *et al.*, 1999). MT is a synthetic aromatizable androgen. Aromatization is thought to decrease the masculinization effectivity of some androgens producing the observed paradoxical feminizing effects. However, whether these feminizing effects are due to a decrease in androgen concentration or an increase in estrogen concentration is unknown (Baroiller *et al.*, 1999). In Chapter 3, we tested different dietary MT treatments and developed an efficient protocol to completely suppress the development of the SV extensions without affecting testicular functionality. Such a diet should contain 20 ppm MT and be fed to larvae during days 12-40 after hatching. This period coincides with the phenocritical period of gonad commitment, *i.e.*, before sexual differentiation, when gonadal sex phenotype can still be manipulated by external factors, thereby overruling the genetic sex determination (Baroiller *et al.*, 1999). It has been demonstrated that the hypothalamic-pituitary axis is potentially active around the time of sex differentiation and that steroids can have a feedback effect on this axis in the same way as they do in adults (Kah *et al.*, 1993). After sexual maturation, all MT-treated fish with a female urogenital papilla possessed normal ovary development. All MT-treated fish with a male urogenital papilla had undeveloped SV extensions, but 21% of them had either sterile or intersex gonads (Chapter 4).

Fertile sperm cells could be stripped in 53% (Chapter 3) and in 88% of the MT-treated males (Chapter 4) after 2 consecutive cPS injections, and in 85% of the MT-treated males after cPS-oxytocin treatment (Chapter 4). Differences in genetic background or maturational stages may explain the different response to cPS treatment observed between animals from these two Chapters. Despite the low volume (from a few drops to 4 mL), hatching rates obtained with the stripped fluid were similar to those

obtained with intratesticular semen from the same MT-treated males, in most of the samples. These results confirm the negative effects of the SV extensions on hand-stripping of semen, as previously speculated by Richter (1976). These findings open a new field of research on semen collection of catfish males. However, species-specific responses to dietary MT treatment may exist, and generalization of this method for other catfish species should be avoided.

The primary function of the SV is the production of a fluid containing various active compounds including steroid glucuronides. These glucuronides act as sex pheromones that promote ovarian growth and development in pubertal females (van Weerd, 1990), and activate female responsiveness and induce ovulation, thereby improving fertilization in adults (Resink, 1988). In Chapter 3, we observed that males with incomplete SV were able to sexually stimulate females kept in the same tank. After long MT treatments, females showed normal ovarian growth, while males from the same treatments and present in the same tanks possessed reduced SV development. However, as all fish received water supply from the same recirculating system, perhaps pheromones produced by control males were diluted in the water system, and may have stimulated females in other tanks. Under natural conditions, however, it is possible that MT-treated males would be less attractive to females than normal males.

Semen cryopreservation

Semen of the African catfish maintained good fertilizing capacity after freezing and thawing (Chapters 5 and 6), although the percentage of post-thaw live/intact cells was not assessed. Methanol proved a better cryoprotectant than dimethyl sulphoxide (DMSO; Chapter 5). In our preliminary experiments (unpublished), fresh sperm was incubated with methanol, DMSO or glycerol at a concentration of 0 to 25% and for a period of 0 to 60 min prior to fertilization. Incubations with methanol at concentrations up to 20% for a period of 60 min, did not affect the sperm fertilizing capacity. Only when methanol was used at 25%, the sperm fertilizing capacity decreased. DMSO could be used at a maximum of 15% for 5 min equilibration and glycerol at 5% and for 5 min equilibration. Longer equilibration periods or higher DMSO or glycerol concentrations affected sperm viability and significantly decreased the hatching rates. Similar catfish sperm sensitivity to glycerol was observed by Horvath and Urbanyi (2000) who found that 5% glycerol in combination with only 2 min equilibration significantly reduced the sperm viability. However, the same authors found very low post-thaw sperm motility when methanol was used and reported DMSO and dimethyl acetamide (DMA) as the most successful cryoprotectants for semen of African catfish. Contrarily to our results, glycerol has shown good freezing protection to sperm of the same fish species, at a concentration of 5-11% and an equilibration period of 10 to as long as 60 min (Steyn *et al.*, 1985; Steyn and van Vuren, 1987; Steyn, 1993). The difference in catfish sperm sensitivity to cryoprotectants observed in the literature, may be due to different protocols used to incubate semen (such as cryoprotectant:extender:semen ratio or temperature during equilibration) or different methods to assess sperm motility or fertility (such as spermatozoa:egg ratio). Furthermore, it is possible that differences on

seminal plasma composition exist between wild and domesticated catfish, and among domesticated catfish reared under different conditions and that these differences may interfere on sperm sensitivity to cryoprotectors.

Methanol has the smallest molecule size and thus the fastest cellular permeation when compared to other permeating compounds such as DMSO, DMA and glycerol. However, whether to be protective, these compounds have to permeate a cell, is still unclear. Experiments using different ways of blocking cryoprotector permeation (such as addition of copper ions in the medium, a very short equilibration time of 30 sec, or equilibration at temperatures that a cell is not permeable to the cryoprotector anymore) have yet demonstrated good post-thaw cell survival. Perhaps, the ability of a cell to survive freezing depends more on protection of the cell surface than on protection of the cell interior (Mazur, 1970).

When any kind of cells are subjected to subzero temperatures, they initially supercool. The manner in which cells regain equilibrium with the extracellular medium depends chiefly on the rate at which they are cooled and on their permeability to water. If cells are cooled slowly or if their permeability to water is high, they will equilibrate by the transfer of water to the external ice (dehydration). But if cells are cooled rapidly or if their permeability to water is low, cells will equilibrate, at least in part, by intracelular freezing. But, regardless whether cells equilibrate by dehydration or by intracellular freezing, cells will be exposed to loss of liquid water, increased concentration of intra- and extracellular solutes (the so-called solution effects), dehydration and shrinkage. The optimal cooling rate, thus, is fast enough to prevent solution effects and excessive shrinkage, and yet slow enough to prevent intracellular freezing. Because different cells have different volume:surface area ratio and permeability to water, the optimal cooling rate varies over a wide range, from -1.6°C/min for marrow stem cells to about -3000°C/min for the human erythrocyte (Mazur, 1970). The optimal cooling rate for sperm cells varies from -1-10°C/min for human semen (Henry *et al.*, 1993) to -100°C/min for bull semen (Woelders, 1997). For African catfish semen, -5°C/min has been described as the optimal cooling rate (Steyn, 1993).

All cooling rates tested in this thesis, -2, -5 and -10°C/min, were effective to freeze African catfish semen as long as the right program was used. A freezing program combined cooling rate, program type (2- or 3-step) and temperature at which plunging into liquid nitrogen (LN_2) occurred.

The cooling rate should neither be too fast nor too slow, as described above. The type of program depends on the cooling rate. At slower rates, the use of 2-step program is beneficial. In general, this program consists of an initial slow cooling period followed by rapid cooling as samples are plunged into LN_2 for final storage. In 2-step programs, cells cooled at a rate of -2-5°C/min survived plunging at a semen temperature (T_{semen}) of -38°C, while cells cooled at -10°C/min did not. At -10°C/min, high post-thaw sperm survival was obtained only when plunging occurred at a lower T_{semen} of -51°C (Chapter 6). It appears that, at a cooling rate of -10°C/min, and certainly at faster rates, cell dehydration lags behind and osmotic equilibration is not reached or approached. At

faster rates, the use of 3-step program is more appropriated. This program consists of an isothermal holding period added to the end of the slow cooling period and before plunging. During the holding period, while the freezer temperature ($T_{chamber}$) remains constant, T_{semen} continues decreasing until equilibrium with $T_{chamber}$ is achieved or until plunging occurs. Thereby, extra time is provided for water efflux and to eliminate spatial temperature gradients within the semen sample. At a cooling rate of -10°C/min combined with a 3-step program, semen could be plunged into LN_2 at a T_{semen} of -36°C instead of -51°C (2-step program) to produce high hatching rates. For the sake of clarity, the use of a holding period is not always beneficial. At the slowest cooling rate tested, -2°C/min, the addition of holding periods was negative for sperm viability. It seems that at such a rate, sperm cells were already in osmotic equilibrium with the surrounding medium. Remaining at a constant low temperature would expose the cells for too long to a highly concentrated solute and may force excessive dehydration and shrinkage.

To define the correct temperature at which plunging should occur, we strongly recommend measuring the T_{semen} rather than the $T_{chamber}$. On Chapter 6, we found that T_{semen} did not correspond to the $T_{chamber}$ over the whole freezing program. During the crystallization process, while T_{semen} was warmed from -10-15°C up to -5°C, $T_{chamber}$ continued to drop governed by the programmed cooling rate. When the crystallization process was finished, while T_{semen} was -5-6°C, $T_{chamber}$ was already -15°C (at -2°C/min) or as low as -40°C (at -10°C/min).

The high temperature at which plunging into LN_2 should occur, as observed in our research, is one point that merits special attention. While most of the research on semen cryopreservation focuses on freezing injuries caused by fast freezing (the intracellular ice formation), the other source of injuries caused by slow freezing has received less importance. In fact, the formation and/or dissolution of intracellular ice is lethal and 0% hatching is obtained when semen is frozen too fast or is plunged into LN_2 before dehydration has reached the level necessary to preclude intracellular freezing. That's why most of the protocols use a very low plunging temperature for semen, not only for African catfish (-65°C; Steyn *et al.*, 1985; -80°C; Rurangwa *et al.*, 2001), but also for channel catfish (-80°C; Christensen and Tiersch, 1997), carp (-80°C; Linhart *et al.*, 2000), the marine ocean pout (-95°C; Yao *et al.*, 2000) and also for bulls (-140°C; de Leeuw *et al.*, 1993). However, in our studies (Chapter 6) decreasing hatching rates were obtained with semen plunged in LN_2 at T_{semen} below -50°C. We speculate that, below -50°C, sperm cells were damaged by too long exposure to high concentrations of electrolytes and/or continued osmotic shrinkage. According to our results, catfish semen should be cooled at a rate of -2, -5 or -10°C/min to -40°C and plunged into LN_2 as soon as the T_{semen} reaches -38°C. Similar research on optimal plunging temperature should be carried out with semen of other species.

Concluding remarks

Although we have increased plasma LH levels, induced a testicular hydration and used muscle-contractors, stripping of viable and fertile spermatozoa proved possible

only when the SV development was suppressed. Furthermore, stripping by applying pressure directly on the testes proves very difficult, as, in this species, testes are located deeply at the posterior end of the body cavity and are covered by abdominal organs. As a result of this pressure, most of the samples were contaminated with blood, but the quality of the stripped fluid was good as high hatching rates could be obtained.

Although the reproductive biology on male African catfish in captivity remains quite a puzzle, and no conclusive treatment was found, substantial progress was made to understand the difficulties of semen release and to facilitate hand-stripping of semen in catfish species. More research especially in the area of muscle-contractors such as oxytocin and prostaglandin F2α is recommended to further explore this subject. The practical application of the methods described in this thesis should be evaluated.

As we observed in our hatchery over the past few years, one mature African catfish male can produce at least 7 mL of intratesticular semen. For immediate use, 1 mL of fresh semen can be diluted 2000 times in NaCl 0.9%. This 1 mL of semen is enough to effectively fertilize 5 kg of eggs (from about 10 females). The other 6 mL then, can be diluted 10 times and cryopreserved in 120 vials, according to the methods described in this thesis. Vials should be thawed according to the necessity, and the content of each vial is enough to fertilize 25 g of eggs (*ca.* 15,000 eggs). Thus, semen from one individual male can fertilize a total of 8 kg of eggs (*ca.* 4,800,000 eggs) and produce at least 2,400,000 larvae throughout the years.

The importance of preserving genetic resources for the future is widely recognized, and the conservation of semen would be a major contribution with great potential application in agriculture, biotechnology, species conservation and clinical medicine (see Introduction Chapter). As males are killed for artificial reproduction in catfish culture, semen cryopreservation offers a unique tool for genetic preservation. Conservation of genetic variability of the wild catfish population is also of importance, as domestication could lead to the formation of genetically altered strains with reduced genetic variability in a very short period of time. Van der Walt *et al.* (1993) reported that the use of limited numbers of brood stock without adding genes from unrelated fish to successive generations, is responsible for the occurrence of genetic drift and the founder effect in most domesticated African catfish populations.

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Summary

Stock improvement (or germplasm enhancement) using quantitative and molecular genetics is an essential part of nowadays production of farm animals and fish. To achieve this in aquaculture, germplasm of both parental sexes should be obtained in a life-saving manner. In captivity, male African catfish, Clarias gariepinus, do not release semen under normal hatchery procedures, *viz.* abdominal massage and have to be sacrificed to obtain sperm from the macerated testes. Of course, this is regarded as a major constrains by the catfish farming sector. Against this background, the research of the present thesis had a two-pronged approach and aimed (a) to induce semen release and facilitate stripping of semen under abdominal massage, and (b) to optimize protocols for cryopreserving semen of the African catfish.

Catfish males kept under husbandry conditions are subjected to a constant and favorable environment throughout the year, resulting in the absence of a pre-spawning gonadotropin (luteinizing hormone – LH) surge. To confirm whether LH surge is the missing part of the reproduction process of males in captivity, in **Chapter 2** the effect of drugs that increase plasma LH levels were tested. LH-releasing hormone analogue (LHRHa), LHRHa plus a dopamine antagonist (LHRHa-PIM), salmon gonadotropin releasing hormone analogue plus a dopamine antagonist (ovaprim), carp pituitary suspension (cPS), Clarias pituitary suspension and combinations of cPS and ovaprim were injected. Stripped fluid, when present, was compared to intratesticular semen, 12 or 24 h after injection (latency time). Plasma LH levels increased ($P<0.05$) 2 h after injection in all hormone treatments, compared to the control fish. Stripping of a few drops of fluid and few viable spermatozoa was possible in 4 of 5 males treated with two consecutive injections of cPS, sampled 12 h later, and in 13 of 24 males treated with cPS-ovaprim combinations. Volume, spermatocrit and sperm concentration of the stripped fluid, however, were very low compared to those obtained with intratesticular semen, from the same males. When tested for fertility, stripped fluid of only one of 13 stripped males treated with cPS-ovaprim combinations could fertilize eggs, but produced low hatching (13%). The number of sperm cells collected per kg body weight increased only in the group of fish treated with two cPS injections and a 12-h latency time. Treatments using single injections of pituitary suspensions, LHRHa, LHRHa-PIM or ovaprim, did not facilitate hand-stripping of viable sperm cells, nor did they increase the number of sperm cells collected per kg. Based on these results, it is unlikely that catfish males in captivity are not strippable because of a lack of the gonadotropin surge.

Several anatomical blockages have been regarded as a cause of the impossibility to collect semen of catfish species. One of these blocks may be the presence of the seminal vesicle (SV) fingerlike extensions around the sperm ducts that may retain the sperm flow during abdominal massage. In **Chapter 3**, the effects of dietary 17α-methyltestosterone (MT) on SV development were evaluated. Treatment of larvae with MT-diet at 50 ppm for days 12-33 or 12-40 after hatching, or at 20 ppm for days 12-26, 12-33, 12-40 or 12-47 after hatching, inhibited the development of the SV fingerlike extensions in males, but did not affect the sex ratio. The minimum effective dose and the most effective period to inhibit the SV development in all treated males were 20 ppm MT and for days 12-40 after hatching. Males from this treatment developed normal

testes that, in some cases, contained few oocytes that tended to disappear before sexual maturation. After sexual maturation, the semen release response in males with undeveloped SV was evaluated. Fluid with viable spermatozoa was obtained following two consecutive injections of cPS (the best treatment tested in Chapter 2) from 10 of 19 males previously fed MT-diet at 20 ppm for days 12-40 or 12-47 after hatching, but from only 4 of 15 control males. When tested for fertility, stripped and intratesticular semen of MT-treated males produced similar hatching rates as the control, indicating good quality of both samples, after MT and cPS treatments. These results demonstrate that the MT-induced absence of SV combined with cPS treatment facilitate the collection of fertile semen by hand-stripping from this species.

In Chapters 2 and 3, hand-stripping of sperm cells was possible only after treatments with pituitary. As crude pituitary extracts contain all hormones, factors, etc. produced or stored in the pituitary, perhaps one of these hormones, besides LH, may have acted on testicular functionality and facilitated the hand-stripping of the fluid described in the previous chapters. High levels of isotocin (the fish oxytocin-like peptide) have been found in the catfish brain and in the pituitary. Furthermore, the paraventricular nucleus of the hypothalamus, which uses oxytocin as a neuro-transmitter, has a key role in sexual function in mammals. In male fish, however, the role of oxytocin in the reproductive behavior and semen release is yet not clear. Therefore, in **Chapter 4**, the *in vitro* and *in vivo* effects of muscle contractors on semen release were investigated. For *in vitro* experiments, testis slices were incubated with oxytocin, vasopressin, epinephrine and prostaglandin F2α as well as LH and Clarias pituitary extract, for 15 and 30 min. Oxytocin was the only drug that increased the optical density and the sperm concentration of the medium, compared to the control. For *in vivo* experiments, oxytocin was tested in two groups of fish: normal males, and males that had been treated with MT-diet to inhibit the SV development, as described in Chapter 3. Each group of males received 2 treatments: a) two injections of cPS, followed by 12 h of latency time, as in Chapter 2; and b) two injections of cPS, followed by one injection of oxytocin, 12 h later; fish were sampled 30 min after the oxytocin injection. While there was no effect of oxytocin on stripping, the MT-induced absence of the SV in combination with cPS treatment, facilitated stripping of semen. Taking together cPS and cPS-oxytocin treated fish, 87% MT-treated males and 60% normal males were strippable. The stripped semen volume was low in both groups but MT-treated males produced significantly higher ($P<0.001$) hatching rates (63.1%) than normal males (2.1%). The only "positive" effect of the cPS-oxytocin treatment was that the intratesticular spermatocrit values were similar to control, while after cPS treatment these values were lower than control. There was a consistent but not significant tendency of higher hatching rates produced with either stripped or intratesticular semen after oxytocin treatment in both MT-treated and normal males. An adjustment of the oxytocin dose and/or latency time is needed to investigate the role of oxytocin in semen release and sperm quality in African catfish.

Possibly, collection of semen using abdominal massage and the methods developed in Chapters 2, 3 and 4, cannot be easily introduced on catfish farms or may not be economically viable compared to the male sacrifice to obtain semen. Therefore, farmers should be able to maximize the use of each male by freezing the excess of semen when a male is sacrificed for artificial reproduction. Firstly, the catfish semen sensitivity to

cryoprotectors was tested (**Chapter 5**). Semen was diluted in 5 to 25% of dimethyl sulphoxide or methanol, and frozen in a programmable freezer. To avoid an excess of spermatozoa per egg, post-thaw semen was diluted 1:20, 1:200 or 1:2000 before fertilization. Fresh semen could be diluted 2000 times without loosing fertilization capacity. Post-thaw semen, however, could be maximally diluted 200 times to produce hatching rates similar to the control, as long as 10% methanol had been used as cryoprotectant agent. Then, 2-step freezing programs with slow cooling rates (-2, -5 or -10°C/min) were evaluated in combination with various temperatures (range of -25 to -70°C) at which plunging into liquid nitrogen (LN_2) occurred. Hatching rates equal to the control ($P>0.05$) were obtained with semen cooled at -5°C/min to -45 and -50°C and at -10°C/min to -55°C. In 3-step freezing programs, at -5°C/min, the effect of holding semen in the freezer for 0, 2 or 5 min at the end of the programs (-30, -35 or -40°C) before plunging, was analyzed. Hatching rates equal to the control ($P>0.05$) were obtained with semen cooled to, and held at, -35°C for 5 min and at -40°C for 2 or 5 min. Finally, semen cooled with one of our best protocols (10% methanol, at a rate of -5°C/min, to and held at -40°C for 5 min, plunged into LN_2 and post-thaw diluted to 1:200) was tested under large-scale on-farm conditions. Again, no difference ($P>0.05$) in hatching rate was observed between post-thaw and fresh semen.

After developing effective protocols for freezing catfish semen, we tried to understand why the addition of a holding period to the freezing program was beneficial. We speculated that, during the holding period, cells had more time to reach the level of dehydration necessary to avoid intracellular freezing. The rate of cell dehydration is determined by water permeability which is temperature dependent. Therefore, in **Chapter 6**, semen temperature (T_{semen}) was followed throughout the freezing programs. Semen diluted in 10% methanol (based on Chapter 5) was frozen in a programmable freezer. During freezing, the temperatures of both freezer ($T_{chamber}$; which regulates the cooling rates) and semen solution inside the vial (T_{semen}) were measured simultaneously. We first tested 2-step freezing programs with different cooling rates (-2, -5 and -10°C/min) in combination with different temperatures at which plunging into LN_2 occurred. The difference between T_{semen} and $T_{chamber}$ increased with faster cooling rates. In all experiments, regardless the cooling rate or the $T_{chamber}$, survival of spermatozoa, expressed as hatching rates, increased from near zero when T_{semen} at plunging was higher than -30°C, to values equal ($P>0.05$) to the control when T_{semen} at plunging was equal to or lower than -38°C. The inclusion of an isothermal holding period before plunging into LN_2 (3-step freezing program), was beneficial when faster cooling rates were used. During the holding period, $T_{chamber}$ remained constant, while T_{semen} cooled down and equilibrated with $T_{chamber}$. Semen cooled at -5 or -10°C/min, could be plunged into LN_2 from a T_{semen} as high as -36°C without compromising post-thaw semen survival. However, cooling at -2°C/min in combination with a 5-min holding period reduced post-thaw semen survival. We concluded that, in the range of the cooling rates tested, sperm cells can reach the level of dehydration necessary to survive the freezing and thawing processes when the temperature in the semen is -36-45°C. With cooling rates of -2 to -10°C/min, hatching rates can be maximized by plunging into LN_2 as soon as T_{semen} reaches -38°C.

Based on the results presented in this thesis, it is unlikely that catfish males kept under husbandry conditions are not strippable because of a lack of gonadotropin surge.

Instead, the promising results obtained with MT-treated males point toward anatomical blockage of the sperm flow. Oxytocin may play a role in sperm transport in catfish, but more research is needed to optimize dose and latency time. The use of cryopreserved semen of African catfish to fertilize eggs is viable as high hatching rates can be obtained. Cryopreservation of semen is a valuable tool for selection and conservation of genetic diversity in catfish species.

Samenvatting

Genetische verbetering met gebruikmaking van quantitatieve en moleculair genetische technieken is tegenwoordig een essentieel onderdeel van de productie van landbouwhuisdieren.

Om dit ook bij vissen te kunnen doen is het allereerst noodzakelijk om ten alle tijden over gameten van mannen en vrouwelijke individuen te kunnen beschikken. Bij de mannelijke Afrikaanse meerval, Clarias gariepinus, is het echter niet mogelijk sperma af te strijken of anderzins te bekomen, zonder dat het dier hierbij geopereerd of gedood wordt. Dit wordt als een belangrijke belemmering voor de ontwikkeling van de meerval teelt sector gezien. Met dit gegeven werd het onderzoek beschreven in dit proefschrift opgestart, waarbij gewerkt werd in twee richtingen, t.w.:

(a) het hormonaal stimuleren van de sperma productie
(b) het ontwikkelen van een protocol voor het invriezen van sperma van de meerval.

Mannelijke afrikaanse meeval, worden in gevangschap onder constante optimale houderij condities gehouden. Dit heeft o.a. tot gevolg dat de z.g. piek in de afgifte van LH, typerend voor de periode voorafgaand aan de voortplanting, niet optreedt. Om na te gaan of deze LH piek noodzakelijk is voor het normale voortplantingsgedrag van mannetjes werden in **Hoofdstuk 2** een aantal hormonen, welke de LH afgifte in het bloed stimuleren, getest.

In een eerste experiment werden vissen geinjecteerd met resp. LHRHa (analoog), LHRHa in combinatie met de dopamine antagonist Pimozide (LHRHa-PIM), Ovaprim, een preparaat bestaande uit zalm gonadotropine stimulerend hormoon en een dopamine antagonist, karper hypophyse suspensie (cPS), Clarias hypophyse suspensie en combinaties van cPS en Ovaprim. De afgestreken vloeistof werd vergeleken met intra-testiculair sperma op resp. 12 en 24 uur na injectie (latencie tijd). Plasma LH spiegels waren in alle gevallen 2 uur na injectie significant verhoogd ($P<0.05$) in vergelijking met controle vissen. Een paar druppels vloeistof met daarin een paar levende spermatozoa, kon worden afgestreken in 4 van de 5 mannetjes, behandeld met 2 opeenvolgende injecties van cPS en 12 later afgestreken, en in 13 van de 24 mannetjes behandeld met een combinatie van cPS en Ovaprim. De spermatocrit en de sperma concentratie van de afgestreken vloeistof waren echter zeer laag in vergelijking met intra-testiculair semen van dezelfde mannetjes. Slechts 1 van de 13 afgestreken mannetjes, behandeld met cPS-Ovaprim, produceerde een vloeistof waarmee eieren bevrucht konden worden, maar het bevruchtings percentage was zeer laag (13%). Het aantal spermacellen per kg lichaamsgewicht nam alleen toe in de groep behandeld met twee cPS injecties en een latencie tijd van 12 uur. Behandelingen met eenmalige injecties van hypophyse suspensie, LHRHa, LHRHa-PIM of Ovaprim, hadden geen invloed op het afstrijken van sperma; evenmin stimuleerden zij de sperma productie, uitgedrukt als aantallen spermacellen per kg vis. Op basis van deze resultaten werd geconcludeerd dat meerval mannetjes gehouden in gevangschap wel goed sperma

produceren maar niet afstrijkbaar zijn, en dat dit niet komt door de afwezigheid van een pre-ejaculatoire LH-piek.

Een van de mogelijke oorzaken voor het probleem van afstrijkbaarheid bij meerval mannen is de aanwezigheid van een anatomische blokkade. Deze zou gevormd kunnen worden door de aanwezigheid en structuur van de vesicula seminalis (SV). Dit vingervormig orgaan vormt de basis van de spermaduct en zou het sperma kunnen tegenhouden tijdens het masseren van de buik om het sperma vrij te krijgen. In **Hoofdstuk 3** werden de effecten van een behandeling met 17α-methyltestosterone (MT) op de ontwikkeling van de SV geevalueerd. Het voeren van larven met een dieet waarin 50 ppm MT was geincorporeerd, tussen 12 en 33 of 12 en 40 dagen na bevruchting, of een dieet met 20 ppm MT gedurende 12 tot 26, 12 tot 33, 12 tot 40 of 12 tot 47 dagen na bevruchting, remde de ontwikkeling van de SV zonder dat het de geslachtsratio verder beinvloedde. De minimale effectieve dosis in combinatie met de meest effectieve periode van behandeling was 20 ppm MT toegediend van 12 tot 40 dagen na bevruchting. De mannetjes uit deze behandeling ontwikkelden normale testes, waarin incidenteel oocyten werden waargenomen. Deze verdwenen echter op het moment dat de dieren geslachtsrijp werden.

De afstrijkbaarheid van geslachtsrijpe mannetjes met een onderontwikkelde SV werd geevalueerd. Tien van de 19 geslachtsrijpe mannetjes, welke waren behandeld met 20 pm MT tussen 12 en 40, of 12 en 47 dagen na bevruchting, produceerden een vloeistof met levende spermacellen na 2 opeenvolgende injecties met cPS (de beste behandeling uit hoofdstuk 2), in tegenstelling tot slechts 4 van de 15 controle dieren. Er was geen verschil in fertiliteit tussen afgestreken en intra-testicular semen van MT-behandelde mannetjes. Bevruchtings percentages waren vergelijkbaar met die van onbehandelde controle dieren, wat duidde op een goede kwaliteit sperma na MT en cPS behandelingen. Deze resultaten tonen aan dat de MT-geinduceerde afwezigheid van een vesicula seminalis in combinatie met een behandeling met cPS het verzamelen van sperma door middel van afstrijken vergemakkelijkt.

In Hoofdstuk 2 en 3 was het afstrijken van sperma cellen alleen mogelijk na behandeling met hypophyse extract. Aangezien hypophyse extracten naast alle hypophyse hormonen ook andere factoren bevatten is het mogelijk dat wellicht een van deze stoffen, naast LH, een effect op de testis had wat de afstrijkbaarheid vergrootte. Grote hoeveelheden isotocine (het teleost homoloog van oxytocine) zijn aangetoond in de hersenen en hypophyse van de Afrikaanse meerval. Bovendien heeft de paraventriculaire nucleus van de hypothalamus, welke oxytocine gebruikt als neuro-transmitter, een sleutelrol in het sexueel functioneren van zoogdieren. In mannelijke vissen is de rol van isotocine/oxytocine in het voortplantingsgedrag en de ejaculatie verre van duidelijk. Derhalve werden in **hoofdstuk 4** de *in vitro en in vivo* effecten van een aantal spiercontractie stimulerende stoffen getest. In de *in vitro* experimenten werden plakjes testis weefsel geincubeerd met resp. oxytocine, vasopressine, epinephrine en prostaglandine F2α gedurende 15 en 30 min. Daarnaast werd ook het effect van gedeeltelijke gezuiverd LH en van Clarias hyophyse extract getest. Oxytocin was het enigste peptide dat de optische dichtheid van het medium en de sperma concetratie in het medium significant verhoogde. Vervolgens werd in een *in vivo* experiment oxytocine getest in twee groepen vissen: normale mannetjes, en mannetjes welke eerder waren behandeld met MT om de ontwikkeling van de vesicula seminalis te

remmen (zoals beschreven in hoofdstuk 3). Elke groep mannetjes werd verdeeld in twee behandelingsgroepen: groep A kreeg 2 injecties met cPS, gevolgd door een latentie tijd van 12 uur, groep B kreeg 2 injecties met cPS, gevolgd door een injectie met oxytocine, 12 uur later. Alle vissen werden 30 minuten na de oxytocine injectie geanalyseerd.

Er was geen effect van oxytocine op de afstrijkbaarheid, maar de MT geinduceerde afwezigheid van de SV faciliteerde het afstrijken van sperma. Op basis van de gepoolde resultaten van de cPS en cPS-oxytocine behandelde vissen, waren 87% MT-behandelde mannen en 60% normale mannen afstrijkbaar. De hoeveelheid afgestreken vloeistof was klein in beide groepen, maar sperma van MT-behandelde dieren gaf significant hogere bevruchtings percentages ($P<0.001$; 63.1%) in vergelijking met sperma van normale mannetjes (2.1%). Het enigste positieve effect van de cPS-oxytocine behandeling was dat de intratesticulaire spermatocrit waarden gelijk waren aan die van de controle groep, terwijl na cPS behandeling deze waarden lager waren dan de controle. Er was een consistente maar niet significante tendens dat afgestreken, of intratesticulair, semen verkregen na oxytocine behandeling, hogere bevruchtings percentages realiseerden in zowel MT-behandelde als normale mannetjes. Een aanpassing van de oxytocine dosis en/of latencie tijd is nodig om de rol van oxytocine in spermiatie en ejaculatie bij Afrikaanse meerval verder te onderzoeken.

Het is mogelijk dat de methoden, beschreven in hoofdstuk 2, 3 en 4 niet gemakkelijk geintroduceerd kunnen worden in commerciele meerval vermeerderings bedrijven, of dat ze economisch niet rendabel zijn in vergelijking met de gangbare methode waarbij mannetjes geslacht worden om het sperma te winnen. In dat geval zouden kwekers in staat moeten kunnen zijn om maximaal gebruik te kunnen maken van het sperma van elk mannetje, door overtollig sperma in te vriezen.

Allereerst werd de gevoeligheid van meerval sperma voor cryoprotectanten onderzocht (**hoofdstuk 5**). Semen werd verdund in 5 to 25% dimethyl sulphoxide (DMSO) of methanol, en ingevroren in een pogrammeerbare vriezer. Na ontdooien werd het sperma gestandardiseerd verdund tot 1:20, 1:200 of 1:2000 om de hoeveelheid spermacellen per ei te optimaliseren. Vers sperma kon 2000 keer verdund worden zonder dat dit invloed had op het bevruchtings percentage. Na ontdooien kon het sperma echter maximaal 200 keer verdund worden om bevruchtingspercentages te krijgen die niet verschillend waren van de controle. Sperma ingevroren met 10% Methanol bleek onder deze condities de beste bevruchtingsresultaten te geven.

Vervolgens werden zg. 2-staps invries programma's geevalueerd, waarbij sperma eerst langzaam werd afgekoeld (-2, -5 or -10°C/min) tot een bepaalde temperatuur (-25 tot -70°C) waarna het werd ondergedompeld in vloeibare stikstof. Bevruchtingspercentages die niet afweken van de controle ($P>0.05$), werden gevonden met sperma afgekoeld met -5°C/min tot -45 of -50°C, en met -10°C/min tot -55°C. In 3-staps invriesprogramma's werd het effect van het inlassen van een pauze van 2 of 5 minuten aan het eind van het afkoelingstraject, bij -30, -35 of -40°C, onderzocht (koelsnelheid -5°C/min). De beste resultaten, niet verschillend van de controle ($P>0.05$), werden verkregen wanneer sperma werd afgekoeld tot -35°C, gevolgd door een 5 min pauze voordat het sperma in stikstof werd ondergedompeld, of tot -40°C gevolgd door een pauze van 2 of 5 min. Tenslotte werd het beste protocol getest onder commerciele kwekerij condities. Sperma werd ingevroren in 10% methanol, bij -5°C/min tot -40°C,

en na 5 min pauze ondergedompeld in vloeibare stikstof. Na ontdooien werd het sperma 1:200 verdund. Ook nu was er geen verschil (P>0.05) in bevruchtingspercentage tussen ontdooid en vers sperma.

Nu er een efficient protocol voor het invriezen van meerval sperma was ontwikkeld kon worden onderzocht waarom het inlassen van een pauze tijdens het invriezen zoveel betere resultaten gaf. Een hypothese was dat de pauze nodig was om de sperma cellen meer tijd te geven het niveau van dehydratie te bereiken wat nodig was om schade veroorzaakt door intracellulaire ijsvorming te vermijden. Cel dehydratie of membraan permeabiliteit voor water kon niet gemeten worden, maar de theorie voorspelt dat cel dehydratie nauw gerelateerd is aan de omgevings temperatuur. Daarom werd in **Hoofdstuk 6** de sperma (semen) temperatuur (T_{semen}) gedurende het gehele afkoelings/invriestraject gemeten. Semen verdund met 10% methanol (gebaseerd op Hoofdstuk 5) werd ingevroren in een programmeerbare vriezer. Gedurende het invriezen werd de temperatuur van de vrieskamer ($T_{chamber}$) en sperma vloeistof (T_{semen}) simultaan gemeten. Eerst werden 2-staps invries programma's met verschillende afkoelings-snelheden (-2, -5 and -10°C/min) in combinatie met verschillende dompel temperaturen onderzocht. Het verschil tussen T_{semen} en $T_{chamber}$ nam toe met toenemende afkoel-snelheden. In alle experimenten, ongeacht de afkoelsnelheid of de $T_{chamber}$, nam de overleving van spermatozoa, uitgedrukt als bevruchtings percentage, toe van bijna nul, wanneer T_{semen} bij dompelen hoger was dan -30°C, tot waarden gelijk aan de controle (P>0.05) wanneer T_{semen} bij dompelen gelijk of lager was dan -38°C. Het inlassen van een isothermische pauze voorafgaand aan het dompelen in vloeibare stikstof (3-staps invries programma), was alleen voordelig wanneer hogere invries snelheden werden gebruikt. Gedurende de pauze bleef $T_{chamber}$ constant, terwijl T_{semen} verder omlaag ging tot er een evenwicht werd bereikt met $T_{chamber}$. Semen afgekoeld bij -5 of -10°C/min, kon worden ondergedompeld in stikstof bij een T_{semen} van minstens -36°C zonder effect op de overleving na ontdooien. Een afkoelsnelheid van -2°C/min in combinatie met een 5-min pauze verlaagde de overleving na ontdooien. De conclusie is dat binnen het bereik van de invries snelheden die getest zijn, de sperma cellen voldoende kunnen dehydreren om intracellulaire ijsvorming tegen te gaan wanner de temperatuur van het sperma –36 -45°C is. Met afkoelsnelheden van -2 to –10 °C/min kunnen bevruchtingspercentages gemaximaliseerd worden zodra T_{semen} -38°C is geworden.

Samenvattend kan gesteld worden dat, gebaseerd op de resultaten gepresenteerd in dit proefschrift, het onwaarschijnlijk is dat mannelijke meerval gehouden onder optimale houderij omstandigheden, niet afstrijkbaar zijn door een gebrek aan een pre-ejaculatoire LH piek. De veelbelovende resultaten verkegen met 17a methyltestosteron laten zien dat het probleem veeleer gelegen is in een anatomische blokkade van de sperma duct. Oxytocin speelt mogelijk een rol in het sperma transport in mannelijke meerval, maar meer onderzoek is nodig om de dosis en latentie tijd te optimaliseren. Het gebruik van ingevroren sperma van Afrikaanse meerval om eieren te bevruchten is een economisch en maatschappelijk alternatief voor het doden van mannetjes voor sperma. Cryoconservering van sperma is een waardevolle techniek voor selectie en conservering van de genetische diversiteit bij diverse meerval soorten.

Resumo

Atualmente, a melhoria na qualidade dos gametas, através do uso de genética quantitativa e molecular, é essencial na produção animal. Para atingir essa melhoria de qualidade também na produção de peixes, os gametas de ambos os sexos devem ser coletados de reprodutores vivos. Porém, quando mantido em cativeiro, o macho do peixe-gato Africano, Clarias gariepinus, não libera sêmen através dos procedimentos normais, como a extrusão manual do sêmen pela massagem abdominal. Para reproduzir artificialmente essa espécie, o macho precisa ser sacrificado e o sêmen coletado diretamente dos testículos. Com base nessa limitação, as pesquisas conduzidas na presente tese seguiram duas direções: (a) facilitar a extrusão manual de sêmen, e (b) desenvolver métodos que possibilitem a criopreservação do sêmen do peixe-gato Africano.

O peixe-gato Africano criado em cativeiro está sujeito a um ambiente favorável e constante ao longo do ano. Isso resulta na ausência da onda pré-espermiatória de gonadotropina (hormônio luteinizante; LH) que normalmente acontece pelo menos uma vez durante a estação reprodutiva nos peixes selvagens e é seguida por acasalamento. Para confirmar a hipótese de que a falta da onda de LH é responsável pela falha no processo reprodutivo dessa espécie em cativeiro, no **Capítulo 2** vários hormônios que elevam os níveis plasmáticos de LH foram analisados. Machos foram tratados com hormônio liberador de gonadotropinas (LHRHa), LHRHa associado a um antagonista da dopamina (LHRHa-PIM), GnRHa de salmão associado a um antagonista da dopamina (ovaprim), extrato de pituitária de carpa (cPS), extrato de pituitária de Clarias (Clarias-PS), e combinações de cPS e ovaprim. Quando presente, o sêmen extruído manualmente foi comparado com o sêmen intra-testicular dos mesmos machos, 12 ou 24 h após cada tratamento (período de latência). Os níveis plasmáticos de LH aumentaram ($P<0.05$) 2 h após todos os tratamentos, comparado com o grupo controle. Foi possível extruir manualmente algumas gotas de um fluido contendo alguns espermatozóides em 4 dos 5 machos tratados com 2 injeções de cPS, e em 13 dos 24 machos tratados com combinações de cPS e ovaprim. Volume, espermatócrito e concentração espermática do sêmen extruído, entretanto, foram muito reduzidos quando comparados com o sêmen intra-testicular dos mesmos machos. Embora o sêmen extruído dos machos previamente tratados com combinações de cPS e ovaprim, apresentasse motilidade após ativação com água, apenas uma das 13 amostras conseguiu fertilizar ovos, mas com baixa taxa de eclosão (13%). O número de espermatozóides coletados por kg peso vivo aumentou apenas nos peixes tratados com 2 injeções de cPS e 12 h de período de latência. Tratamentos com injeção única de cPS, Clarias-PS, LHRHa, LHRHa-PIM ou ovaprim, não facilitou a extrusão manual de sêmen, nem aumentou o número de espermatozóides coletados por kg. Baseado nesses resultados, provavelmente a falha na extrusão manual de sêmen no peixe-gato Africano mantido em cativeiro tem outra causa além da ausência do pico de LH.

Várias características anatômicas dos peixes-gatos têm sido consideradas como responsáveis pela impossibilidade de se extruir sêmen manualmente. Uma dessas características é a presença das extensões filiformes das vesículas seminais (SV) ao

redor dos ductos espermáticos que podem reter o transporte de sêmen durante a massagem abdominal. Assim, no **Capítulo 3**, os efeitos de dietas contendo 17α-metiltestosterona (MT) no desenvolvimento das SV foram avaliados. Larvas foram tratadas com dieta contendo 50 ppm MT durante os dias 12-33 ou 12-40 após a eclosão, ou 20 ppm durante os dias 12-26, 12-33, 12-40 ou 12-47 após a eclosão. Embora não tenha havido alteração na proporção de machos e fêmeas, todos os tratamentos foram efetivos na supressão do desenvolvimento das SV, mas em diferentes intensidades. A mínima dose efetiva para suprimir o desenvolvimento das SV em 100% dos machos tratados foi 20 ppm MT durante os dias 12-40 após a eclosão. Os machos desse grupo desenvolveram testículos normais que eventualmente continham alguns oócitos que, em sua maioria, desapareceram antes da maturidade sexual. Após a maturidade sexual, a resposta desses machos à extrusão manual de sêmen foi avaliada. Fluido contendo alguns espermatozóides viáveis foi obtido após tratamento com 2 injeções de cPS (o melhor tratamento descrito no Capítulo 2) em 10 dos 19 machos previamente tratados com 20 ppm MT durante os dias 12-40 ou 12-47, mas em apenas 4 dos 15 machos do grupo controle. Quando testado para fertilidade, o sêmen extruído manualmente dos machos tratados com MT produziu taxas de eclosão similares ao sêmen intra-testicular, indicando boa qualidade de ambas amostras após tratamentos com MT e cPS. Esses resultados demonstram que a ausência das extensões filiformes das SV combinada com o tratamento com cPS facilitam a extrusão manual de sêmen fértil nessa espécie.

Como a extrusão manual de espermatozóides foi possível apenas após tratamentos com cPS e os extratos de pituitárias contêm todos hormônios, fatores, etc. produzidos ou armazenados na hipófise, talvez um desses hormônios além do LH, tenha interferido na funcionalidade dos testículos e facilitado a extrusão manual de sêmen. Isotocina (o peptídeo do peixe homólogo a oxitocina) foi isolada do cérebro e hipófise de peixe-gato em altas concentrações. Além disso, os núcleos para-ventriculares do hipotálamo, que utilizam a oxitocina como neuro-transmissor, têm uma função chave na reprodução de mamíferos. Em peixes, entretanto, o papel da oxitocina no comportamento reprodutivo dos machos e na emissão de sêmen ainda é obscuro. No **Capítulo 4**, os efeitos *in vitro* e *in vivo* de alguns contratores musculares na extrusão manual de sêmen foram investigados. Durante os experimentos *in vitro*, fatias de testículos foram incubadas com oxitocina, vasopressina, epinefrina e prostaglandina F2α, assim como LH e extrato de pituitária de Clarias, por 15 e 30 minutos. Oxitocina foi a única droga que elevou a densidade óptica e a concentração espermática do meio de incubação, comparado com o controle. Durante experimentos *in vivo*, oxitocina foi testada em 2 grupos de peixes: machos normais, e machos previamente tratados com dieta MT para inibir o desenvolvimento das SV, como descrito no Capítulo 3. Cada grupo de machos recebeu 2 tratamentos: a) 2 injeções de cPS seguidas por um período de latência de 12 h, como no Capítulo 2; e b) 2 injeções de cPS, seguida de uma injeção de oxitocina, 12 h depois; esses peixes foram amostrados 30 minutos após o tratamento com oxitocina. Não houve efeito da oxitocina na extrusão manual de sêmen, mas a ausência das SV combinada com o tratamento com cPS facilitou esse processo. Juntando os tratamentos cPS e cPS-oxitocina, foi possível extruir sêmen manualmente em 87% dos machos tratados com MT e em 60% dos machos normais. O volume de sêmen extruído foi reduzido em ambos os grupos de machos, entretanto os machos tratados com MT produziram taxas de eclosão de ovos (63.1%) significativamente maiores ($P<0.001$) do que os machos normais (2.1%). O único efeito "positivo" do tratamento cPS-oxitocina foi que os

valores obtidos no espermatócrito do sêmen intra-testicular foram semelhantes ao grupo controle, enquanto que após o tratamento cPS, esses valores foram menores. Esse efeito esteve presente em machos normais e em machos tratados com MT. Embora não significante, houve uma tendência de maiores taxas de eclosão de ovos serem obtidas após o tratamento cPS-oxitocina, tanto com sêmen extruído manualmente, quanto com sêmen intra-testicular, e em ambos os grupos de machos. Um ajuste na dose e período de latência se faz necessário para que o papel da oxitocina no transporte e na extrusão manual de sêmen no peixe-gato Africano seja devidamente investigado.

Possivelmente, os métodos descritos nos Capítulos 2, 3 e 4 para facilitar a extrusão manual de sêmen, não podem sem facilmente introduzidos em criações comerciais, ou talvez nem sejam economicamente viáveis. Assim, os criadores precisam maximizar o uso de cada indivíduo através do congelamento do excesso de sêmen intra-testicular, quando um macho é sacrificado para reprodução. No **Capítulo 5**, métodos para a criopreservação do sêmen do peixe-gato foram investigados. Sêmen foi diluído em 5 a 25% de dimetil sulfóxido (DMSO) ou metanol, e congelado em um freezer programável. Para evitar o excesso de espermatozóides por ovo (que produziria altas taxas de eclosão mesmo com métodos de congelamento sub-ótimos), o sêmen descongelado foi diluído 20, 200 ou 2000 vezes antes de ser usado para fertilização. Taxas de eclosão de ovos semelhantes ao sêmen fresco foram obtidas com sêmen congelado em 10% de metanol e diluído no máximo 200 vezes. Em seguida, programas de congelamento em duas etapas usando 3 velocidades (-2, -5 ou -10°C/min) foram avaliados. Amostras de sêmen foram transferidas para o nitrogênio liquido (LN_2) quando o freezer atingiu várias temperaturas entre -25 e -70°C. Taxas de eclosão iguais ao controle ($P>0.05$) foram obtidas quando o sêmen foi congelado a -5°C/min até -45-50°C, ou a -10°C/min até -55°C. Em programas de congelamento em 3 etapas, amostras de sêmen foram congelados a -5°C/min e mantidas no freezer por 0, 2 ou 5 minutos no final do programa (-30, -35 ou -40°C) antes de serem transferidas para o LN_2. Taxas de eclosão iguais ao controle ($P>0.05$) foram produzidas com sêmen mantido a -35°C por 5 minutos, ou mantidos a -40°C por 2 ou 5 minutos. Finalmente, o sêmen congelado com um dos melhores protocolos (10% metanol, -5°C/min, mantidos a -40°C por 5 minutos, transferido para o LN_2 e diluído 200 vezes) foi testado em escala comercial e nas mesmas condições de fertilização encontradas numa criação. Novamente, nenhuma diferença ($P>0.05$) na taxa de eclosão de ovos foi observada entre sêmen descongelado e fresco.

Após desenvolver com sucesso protocolos para congelar o sêmen do peixe-gato, no **Capítulo 6**, experimentos foram conduzidos na tentativa de explicar o aumento na sobrevivência dos espermatozóides em programas de 3 etapas, conforme observações no Capitulo 5. Nós especulamos que, durante o intervalo entre o final do programa de congelamento e o armazenamento no LN_2, as células teriam mais tempo para atingir o nível de desidratação necessária para evitar a formação de cristais de gelo no meio intracelular, que é letal. A velocidade de desidratação de uma célula é determinada pela sua permeabilidade da membrana a água, que por sua vez, é dependente da temperatura. Assim, a variação da temperatura do sêmen (T_{semen}) medida no interior de um dos tubos e a temperatura do freezer ($T_{chamber}$) que regula a velocidade de congelamento foram acessadas simultaneamente. Sêmen diluído em 10% metanol (baseado no Capítulo 5) foi congelado no freezer programável. Primeiramente foram testados programas de

congelamento em 2 etapas com diferentes velocidades (-2, -5 and -10°C/min). Amostras de sêmen foram transferidas para o LN_2 quando o freezer atingiu várias temperaturas entre -35 e -65°C. A diferença entre T_{semen} e $T_{chamber}$ aumentou quando a velocidade mais rápida de -10°C/min foi usada. Em todos os programas, independentes da velocidade de congelamento ou da $T_{chamber}$, a sobrevivência dos espermatozóides (expressada pela taxa de eclosão de ovos) aumentou de perto de zero quando T_{semen} na transferência para o LN_2 estava acima de -30°C, para valores iguais ($P>0.05$) ao controle quando T_{semen} na transferência estava igual ou inferior a -38°C. Manter as amostras de sêmen no freezer por alguns minutos antes da transferência para o LN_2 (programas em 3 etapas), foi benéfico quando as velocidades mais rápidas foram usadas. Durante esse tempo, $T_{chamber}$ permaneceu constante, enquanto T_{semen} esfriou e equilibrou com $T_{chamber}$, aumentando a sobrevivência dos espermatozóides. As amostras de sêmen puderam ser transferidas para o LN_2 a partir de temperaturas tão altas quanto -36°C quando congelados a -10°C/min, sem comprometer a viabilidade espermática. O uso da velocidade mais lenta de -2°C/min em combinação com programas em 3 etapas, por outro lado, reduziu a sobrevivência dos espermatozóides após o descongelamento. Nós concluímos que, dentro das velocidades testadas aqui, os espermatozóides atingem o nível de desidratação necessário para sobreviver aos processos de congelamento e descongelamento quando a temperatura do sêmen alcança -38°C, desde que haja tempo suficiente para tal. Para que as taxas de eclosão de ovos sejam máximas, o sêmen deve ser congelado a velocidades de -2, -5 ou -10°C/min até -40°C, e transferido para o LN_2 tão logo a T_{semen} atinja -38°C.

Baseado nos resultados apresentados nessa tese, provavelmente não é pela falta do pico de LH que não se pode extruir sêmen manualmente dos peixes-gatos mantidos em cativeiro. Alternativamente, os resultados promissores obtidos com machos tratados com MT nos leva a crer num bloqueio anatômico do transporte do sêmen. A oxitocina provavelmente tem um papel no transporte espermático no peixe-gato, entretanto mais pesquisas são necessárias para otimizar dose e período de latência. O uso de sêmen criopreservado de peixe-gato Africano mostrou-se viável, uma vez que altas taxas de eclosão de ovos foram obtidas. A criopreservação do sêmen é uma ferramenta útil na seleção e conservação da diversidade genética nas espécies de peixes-gatos.

Acknowledgments

This dissertation represents the final outcome of several years of hard work. As such, I want to thanks for direct and indirect help from many people.

First of all, I would like to thanks the financial support I received from Fundação Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior – CAPES, Brazil – that supported both my family's living cost and the department and university fees. My thanks also to the Animal Science Department of Lavras University - UFLA, for finding a temporary replacement for my activities as docent, during the last 4 years.

Very special thanks to my supervisor Dr. Hans Komen, for his encouragement and helpful discussions. Thanks for teaching me how to organize my ideas in a K.I.S.S. way! Many thanks also to my promotor Professor Dr. Bram Huisman for his always clever comments.

Thanks to my M.Sc. students (Mohamed Yagoub, Yonas Fessehaye, Erik-Jan lock and Andreas Jatzkowski) and to Menno ter Veld for your greatful help specially during sampling time. Thanks to Sietze, Aart, Truus and Wilna for taking care of my fish during uncountable weekends and holidays.

Thanks to Michel Tank and Karin van de Braak, who I shared office for such a long time. Thanks to my latin colleagues Pablo Almazán, Beatriz Torres, Armando Garcia, Gustavo Monti, Catarina Martins, Rosane Barreto, Sandra Rodrigues, Beatriz Waltrik and Rodrigo Ozorio, for our good talk during lunch and coffee breaks and discussion during several courses.

Finalmente, mas não menos importante, agradeço a minha família. Viver no estrangeiro foi uma experiência muito enriquecedora, mas que também envolveu sacrifícios de todos nós. Agradeço aos meus filhos Giuliano, Amanda e Felipe por terem compreendido a minha ausência durante os dias e por fazerem tão felizes as minhas noites. Agradeço ao meu marido Ricardo pelo apoio em casa com as crianças e pelo jantar sempre quentinho a minha espera. Agradeço a vocês quatro pela estrutura familiar que sempre me deu apoio para continuar meus estudos, mesmo quando perdi todo o experimento pela terceira vez.

List of Publications

Refereed articles

Viveiros, A.T.M.; So, N., Komen, J., 2000. Sperm cryopreservation of African catfish, Clarias gariepinus: cryoprotectants, freezing rates and sperm:egg dilution ratio. Theriogenology 54(9), 1395-1408.

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Abstracts

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Curriculum vitae

Ana Tereza de Mendonça Viveiros was born on December 4th, 1963 in Rio de Janeiro city, Brazil. She studied Veterinary Medicine at Fluminense Federal University, Rio de Janeiro and graduated in 1985. In 1986-88, she followed MSc course on animal reproduction at Viçosa Federal University, Minas Gerais. In 1989, she worked as veterinarian practician, in Rio de Janeiro. After that, she started her academic duties as docent firstly at the Faculty of Veterinary in Alfenas University (1990-91) and then at the Animal Science Dept at Lavras Federal University (UFLA; 1991 - at present). Meanwhile, her two sons and daughter were born. In November 1997, she arrived in Wageningen for PhD studies, at the Fish Culture and Fisheries Group of Wageningen University, to work on catfish reproduction. The results of this work are presented in this thesis. Ana is returning to her position at the Animal Science Dept, UFLA, in Brazil after graduation.

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Printed by Ponsen & Looijen, Wageningen
Cover design: Giuliano, Amanda and Felipe Viveiros

This PhD thesis was financed by
Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Brazil
Project BEX 1407/96-1.

The author also received financial support from
Universidade Federal de Lavras, Minas Gerais, Brazil.