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HORMONAL PHEROMONES IN MALE GOLDFISH: NEUROENDOCRINE MECHANISMS AND EFFECTS ON FERTILITY

by

Wenbin Zheng

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: **HORMONAL PHEROMONES IN MALE GOLDFISH: NEUROENDOCRINE MECHANISMS AND EFFECTS ON FERTILITY** submitted by Wenbin Zheng in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

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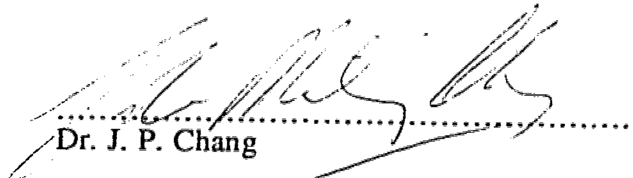

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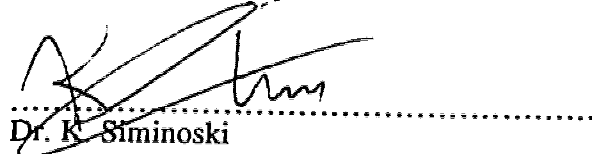

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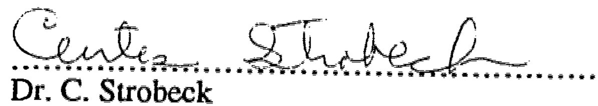

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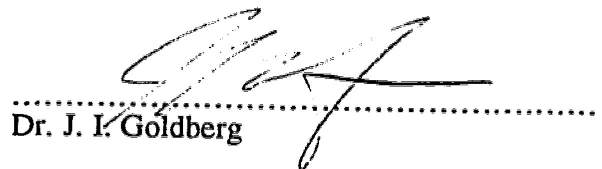

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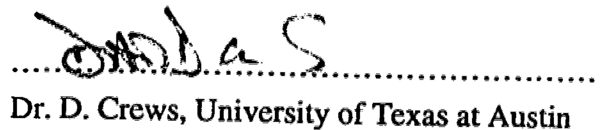

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ABSTRACT

This study examined the effects and the neuroendocrine mechanisms of two sex pheromones, 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) and prostaglandin $F_{2\alpha}$ including its metabolites (PGF), affecting fertility in the male goldfish *Carassius auratus*. The steroidal pheromone, 17,20ß-P, is released by female goldfish for several hours before ovulation, and increases male reproductive success through neuroendocrine mechanisms distinct from the effects of PGF, which is released by females after ovulation.

Responses of milt volume to 17,20ß-P and PGF were compared using hypophysectomy, injection of human chorionic gonadotropin, and differential temperatures. 17,20ß-P-induced increase in milt volume is dependent on gonadotropin-II (GtH-II) whereas PGF-induced milt increase is GtH-II-independent. Using dopamine agonists and a GtH-releasing-hormone (GnRH) antagonist, this study showed that 17,20ß-P increases blood GtH-II primarily through decreasing dopaminergic inhibition whereas PGF does so by stimulating GnRH release. 17,20ß-P exposure increased blood GtH-II concentration, sperm motility, motility duration, and total strippable sperm number, despite a decreased sperm concentration.

A microsatellite DNA fingerprinting technique was developed in this study for goldfish paternity analysis. Male goldfish previously exposed to pheromonal 17,20ß-P significantly increased fertilization rates in pair spawning (one male-one female), particularly at the onset of spawning activity. Paternity analysis revealed that 17,20ß-P also increased male fertility during competitive spawning (two males-one female). Furthermore, sperm from 17,20ß-P-exposed males sired more offspring when competing with sperm from control males in *in vitro* fertilization. The results indicate that 17,20ß-P increases male fertility through a variety of effects including increased male spawning activity, total releasable sperm number, and sperm motility. However, it is not known what changes in

sperm function account for the effect of 17,20ß-P on *in vitro* fertilization.

This study has provided the first direct evidence for vertebrates that a sex pheromone enhances fertility by increasing sperm quality and quantity. A model for the neuroendocrine and gonadal responses to pheromonal 17,20ß-P and PGF is proposed for future studies on sex pheromones.

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CHAPTER 1

GENERAL INTRODUCTION

Recent studies demonstrate that a variety of fresh water fish use released gonadal hormones and their metabolites or conjugates as sex pheromones (hormonal pheromones) that have potent effects on both reproductive physiology and behavior (Stacey *et al.*, 1991; Stacey *et al.*, 1994a, Sorensen *et al.*, 1995b). The goldfish (*Carassius auratus*) is the best understood model for hormonal pheromones in teleosts, and possibly all vertebrates, because several of its hormonal pheromones have been identified and responses to these pheromones have been characterized. In particular, two hormonal pheromones have been studied in great detail. One is a female preovulatory pheromone consisting of 17α,20ß-dihydroxy-4-pregnen-3-one and its glucuronated and sulfated metabolites (hereafter referred to as 17,20ß-P; Scott and Sorensen, 1994; Scott and Vermeirssen, 1994; Sorensen *et al.*, 1995); the other is a female postovulatory pheromone consisting of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolites (This mixture of prostaglandins released by ovulated or PGF2α-injected females is hereafter referred to as PGF; Sorensen and Stacey, 1990; Sorensen and Goetz, 1993; Sorensen *et el.*, 1995a). 17,20ß-P, which is released from female goldfish for approximately 10 hours before ovulation, synchronizes spawning readiness by rapidly increasing blood gonadotropin-II (GtH-II) concentration and milt volume in the males. PGF is released by females after ovulation and stimulates sexual arousal in the males, which in turn stimulates increases in both GtH-II and milt volume. However, it is not known whether this increase of GtH-II and milt volume is similar to the one caused by 17,20ß-P. Also, it is not known whether these two pheromones increase male fertility during spawning, an ultimate question about the biological significance of the goldfish pheromone system.

The first objective of this thesis is to determine whether 17,20ß-P and PGF act through different mechanisms to increase blood GtH-II and milt volume. The second objective is to examine the effect of 17,20ß-P on male fertility. These objectives are important because they not only increase our understanding about the mechanisms mediating pheromonal effects, but also provide information relevant to the culture of many kinds of fish which appear to use similar steroids and PGF as sex pheromones (Stacey and Cardwell, 1995).

1-1: DEFINITION OF PHEROMONE

The term "pheromone" was first used to describe chemical messages for sexual attraction among insects (Karlson and Luscher, 1959). Pheromones are defined as "substances that are excreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example a definite behavior or developmental process". Wilson and Bossert (1963) later modified this definition by classifying different pheromones as either "releaser" or "primer". Sex pheromones are categorized as releasers if they stimulate an immediate behavioral response in the pheromone recipient, or as primers if they induce a physiological change in the receiver. In general, physiological responses to primer pheromones are thought to occur only after a relatively long time delay (e.g. hours or days). Although the terms "releaser" and "primer" are commonly used in both terrestrial and aquatic organisms, they can be misleading because one pheromone could have both primer and releaser effects (Wilson and Bossert, 1963). In goldfish, for example, 17,20ß-P and PGF initially were classified as "primer" and "releaser", respectively, based on the first characterization of their physiological and behavioral functions (Sorensen and Stacey, 1990). However, more recent studies have shown that 17,20ß-P has a releasing effect on behavior in

addition to its priming effect on GtH-II (Defraipont and Sorensen, 1993), whereas PGF has a priming effect on GtH-II in addition to its effects on behavior (Sorensen *et al.*, 1989). In order to better understand the differences in the nature or action of the pheromones themselves, it has been therefore suggested that the terms "primer" and "releaser" should be simply replaced by whatever term best describes the specific response in question (Stacey *et al.*, 1994a). For these reasons, neither "primer" nor "releaser" will be used in this thesis. Instead, "preovulatory pheromone" and "postovulatory pheromone" will be used for the classification of 17,20ß-P and PGF, respectively, based on the natural releasing sequence. The description of the action of these two sex pheromones will be based on their particular effects (e.g., either physiological effect or behavioral effect).

1-2: REPRODUCTIVE PHEROMONES IN TETRAPODS

In mammals, it has been well documented that pheromones influence a variety of behavioral and physiological states (Muller-Schwartz and Silverstein, 1980). In hamsters (*Mesocricetus auratus*), for example, a pheromone from the vaginal discharge of females has potent behavioral effects on male hamsters, which will attempt to copulate with objects and/or other males scented with hamster vaginal secretions (Singer *et al.*, 1982). Numerous studies have been reported about the physiological effects of pheromones. For example, urine from female mice can induce a rapid surge of luteinizing hormone (LH) in adult males whereas the odor of a dominant male may suppress concentrations of LH in subordinate males (Bronson, 1982). Also, in laboratory female mice, male urine can accelerate puberty (Vandenbergh, 1969), induce estrous and ovulation in grouped females (Whitten, 1956; Whitten and Champlin, 1972) and block pregnancy in newly mated females (Bruce, 1960; Bruce and Parrot, 1960). In contrast, exposure of female

mice to female urine delays puberty (Cowley and Wise, 1972), inducing anoestrous and protecting against pregnancy (Whitten, 1959)

Although most sex pheromones are believed to operate through neuroendocrine mechanisms to affect reproduction (Bronson, 1982), information on the endocrine mechanisms mediating pheromonal effect in mammals is scarce. In most species, the action of pheromones has been shown to be mediated through the vomeronasal system and not the olfactory system (Johnston *et al.*, 1987; Johnston, 1990). The vomeronasal organ is a chemosensory organ which projects not to the main olfactory bulbs but to the accessory olfactory bulbs (Keverne and Rosser, 1985; Halpern, 1987). Lesions to the vomeronasal organ or to the accessory olfactory bulb prevent female odors from inducing a state of pseudopregnancy, and male odors from inducing estrous, accelerating puberty and blocking pregnancy (Keverne and Rosser, 1985). Removal of the vomeronasal organ in male hamsters results in mating behavior deficits (Meredith, 1986, 1991).

Although exposure to female pheromones and mating behavior often cause an immediate and dramatic increase in plasma concentrations of LH and testosterone in many mammalian species (Bronson and Desjardins, 1982; Coquelin *et al.*, 1984; Sellers and Bartke, 1987), the sex pheromones may not be the sole stimuli causing physiological change. In some cases, sexual interaction *per se* has similar physiological effect. There is evidence that increase of blood LH and steroid hormones is related to sexual motivation (copulation) in rats and mice (Harding, 1981; Kamel and Frankel, 1978; Sellers and Bartke, 1987). Similar responses were reported in a Japanese toad (*Bufo japonicus*): males performing courtship interaction (amplexus) without the presence of sex pheromone rapidly increase LH and gonadal steroids (Ishii and Itoh, 1992). Increase in serum testosterone is also found during male social interaction in birds and fish (Wingfield and Wada, 1989; Cardwell and Liley, 1991; Cardwell *et al.*, 1996).

Progress in studying mammalian pheromonal functions has been greatly impeded by the lack of information on chemical identities. The major sources of sex pheromones have been found to be the cutaneous glands (Mykytowycz, 1976; Blazquez *et al.*, 1987), salivary and preputial glands (Marchlewska-koj *et al.*, 1990), urine (Bronson and Whitten, 1968; Vandenbergh, 1969; Novotny *et al.*, 1982), vaginal secretions (Singer *et al.*, 1982), and body fluids (Rivard and Klemm, 1989). However, due to the vast array of compounds contained in urine, and due to the physical properties of cervical-vaginal mucus, which is easily contaminated, attempts to isolate and identify mammalian pheromones have not been very successful (Nishimura *et al.*, 1984; Preti, 1984; Albone *et al.*, 1986; Klemm *et al.*, 1987). From studies reported so far, activity of both volatile substances (e.g. acids, alcohol and dimethyl disulfide) and non-volatile protein fractions have been found in the urine, estrous vaginal excretions and cervical-vaginal mucus (Singer *et al.*, 1982; Novotny *et al.*, 1982; Jemiolo *et al.*, 1986; Preti, 1984; Klemm *et al.*, 1987). Unfortunately, most identified compounds are not as effective as fresh vaginal discharge when tested in the attractant bioassay (Singer *et al.*, 1986).

Despite the uncertainty whether specific compounds function as pheromones in mammals or whether the pheromones are mixtures of volatile and non-volatile compounds, studies in some species have shown that identified compounds can elicit specific behavior and physiological responses in conspecifics. In pig (*Sus scrofa*), for example, a steroid, 5α-androst-16-en-3-one which is produced by the testes and released in saliva (Booth, 1980), can elicit female receptive behavior (Signoret, 1970). Studies in reptiles also provide valuable information about identified sex pheromones affecting mating behavior. In garter snake (*Thamnophis sirtalis parietalis*), for example, a female attractiveness pheromone consisting of a series of saturated and monounsaturated methyl ketones (Mason *et al.*, 1989) is present in the liver and secreted through circulation to the skin (Garstka and Crews, 1981). Synthetic ketones were found to elicit male courting

behavior although they are not as effective as the mixture of naturally occurring methyl ketones (Mason *et al.*, 1989).

Although it is well established that sex pheromones in tetrapods have potent effects on physiology and sexual behavior, no study has examined the effects of these pheromones on sperm function.

1-3: REPRODUCTIVE PHEROMONES IN AQUATIC ORGANISMS

Unlike most terrestrial organisms, aquatic organisms could use released hormones for reproductive pheromones because the released hormones and their metabolites are water soluble and can be detected by the olfaction of aquatic animals (Liley and Stacey, 1983; Cardwell *et al.*, 1991; Stacey *et. al.*, 1991). The speculation that aquatic animals might commonly use hormones as sex pheromones was first proposed by Kittredge *et al.* (1971), who suggested that the female crustacean moulting hormone, crustecdysone, has potent releasing effects on males in some crab species. Although this idea has unfortunately not been confirmed in crabs (Gleeson *et al.*. 1984), it has led to the proposal that aquatic animals may readily have evolved hormonal pheromones (Kittredge and Takahashi, 1972), and the discovery of hormonal pheromones in several fish species.

In support of the hypothesis proposed by Kittredge *et al* (1971), recent evidence suggests that unlike the highly volatile insect and/or proteinaceous mammalian pheromones (Singer *et al.*, 1986), sex pheromones of some teleosts are reproductive hormones and their metabolites (Sorensen and Stacey, 1990). Evidence that fish use hormones and their metabolites as pheromones has been reported for dozens of species from 7 teleost families (Cyprinidae, Gobiidae, Clariidae, Catostomidae, Cottidae, Cobitidae and Salmonidae) (Cardwell *et al.*, 1991; Sorensen *et al.*, 1991a; Stacey and

Sorensen, 1991; Stacey *et al.*, 1995). In fact, in all cases where teleost pheromones have been identified, they are reproductive hormones or their metabolites that are subsequently released into the water where they have pheromonal activity (Stacey and Sorensen, 1991; Stacey *et al.*, 1994a).

The first hormonal pheromone in fish was reported in the black goby, *Gobius jozo* (Colombo *et al.*, 1982). Male *Gobius* are territorial, defending a nest-site from which they release a sex pheromone to attract ovulated females for spawning. This pheromone was found to be etiocholanolone glucuronide (3α-hydroxy-5ß-androstan-17-one-glucuronide), a 5-ß reduced androgenic steroid conjugated with glucuronic acid. Etiocholanolone glucuronide was found to be the major steroid product of the mesorchial gland, a Leydig cell-rich testicular component common among the gobiids (Colombo *et al.*, 1980, 1982). Although the major route for the release of this pheromone is suggested to be urine (Colombo *et al.*, 1982), neither patterns of release nor the sensory system that mediates responsiveness has been examined.

Studies on zebrafish, *Danio rerio*, and African catfish, *Clarias gariepinus* have provided excellent evidence for hormonal pheromones. In the zebrafish, spawning involves neither territoriality or post-spawning care of the scattered eggs. Pheromones from both sexes have been reported. There is good evidence that males release a hormonal pheromone to stimulate ovulation of the females, and ovulated females release a hormonal pheromone to attract males (Van Den Hurk and Resink, 1992). Female zebrafish do not ovulate in the absence of males, but resume ovulation when exposed to the water from a male's tank providing the female has not been made anosmic (Van Den Hurk *et al.*, 1987a). Testicular extracts were found to be as effective as male water at triggering ovulation and the activity of these extracts is associated with a fraction containing glucuronated steroid (Van Den Hurk *et al.*, 1987a). Male zebrafish are able to discriminate ovulated from nonovulated females, and do not court ovulated females if

they are made anosmic (Van Den Hurk and Lambert, 1983). Pheromones from ovaries may be responsible for this effect because ovarian extracts from ovulated females are more attractive to males than are extracts from females in mid-cycle; neither extract affects females or anosmic males. Males also are attracted to a fraction of the ovarian extract containing steroid glucuronides, and to a mixture of estradiol and testosterone glucuronides, suggesting these compounds may be components of the female pheromone.

Research on the biological responses to sex pheromones in zebrafish has not been very successful because of the slow progress in identifying the hormonal pheromones. Although a variety of steroid glucuronides have been produced from *in vitro* testis incubations, only one of these products has been identified in male holding water. However, exposing isolated females to known synthetic glucuronides does not induce ovulation (Van Den Hurk *et al.*, 1987b). Furthermore, recent studies using electro-olfactogram (EOG) recordings show that *Danio* does not detect the steroid glucuronides suggested by Van Den Hurk and Lambert (1983) to have behavioral effects on males, but does detect $17\alpha,20\beta$-P-sulfate and PGF (Stacey and Cardwell, 1995). However, whether these compounds would elicit biological responses in this species is still unknown.

In the African catfish, factors associated with flooding appear to trigger spawning by inducing synchronous ovulation. Males then compete for access to a spawning partner, and the spawning pair enters submerged vegetation where the eggs are scattered and fertilized (Lambert *et al.*, 1986; Van Oordt *et al.*, 1987). Although there is evidence for an unidentified male pheromone stimulating ovulation (Resink *et al.*, 1989), research has focused on female behavioral responses to pheromones released by the males, which have large seminal vesicles demonstrated to synthesize and store a variety of free and conjugated steroids (Lambert *et al.*, 1986; Schoonen *et al.*, 1988). Ovulated females

prefer male odor to female odor, whereas ovulated and anosmic females show no preference (Resink *et al.*, 1987). Fractions of seminal vesicle fluid containing steroid glucuronides attract ovulated females, and the effectiveness of these fractions can be eliminated with glucuronidase treatment (Resink *et al.*, 1989). Ovulated *Clarias* are attracted to a mixture containing 7 of the 8 glucuronated steroids known to be synthesized *in vitro* by the seminal vesicle (Schoonen *et al.*, 1988; Resink *et al.*, 1989). Unfortunately, it remains to be determined what levels of these steroid glucuronides are released *in vivo*, and what biological response they might effect.

Evidence for hormonal pheromones also comes from many other species, including loach, *Misgurnus anguillicaudatus* (Kitamura *et al.*, 1994), Atlantic salmon, *Salmo salar* (Moore, 1991; Moore and Scott, 1991, 1992), yellowfin Baikal sculpin, *Cottocomephorus grewingki* (Dmitrieva and Ostroumov, 1986; Dmitrieva *et al.*, 1988; Katsel *et al.*, 1992), Pacific herring, *Clupea harengus pallasi* (Carolsfeld *et al.*, 1992), and tinfoil barb, *Bardodes schwanenfeldi* (Cardwell *et al.*, 1995). Recently, Stacey *et al.* (1995) have conducted EOG recording on over one hundred species that detect a wide range of steroids and prostaglandins. Although the biological responses to these detected compounds have not been tested, the fact that the majority of these fish detect at least one steroid or prostaglandin provides strong evidence that hormonal pheromones are widespread in fish.

1-4. HORMONAL PHEROMONES IN GOLDFISH

Goldfish provide the best understood model for the functions of hormonal pheromones in fish. In temperate climates, female goldfish ovulate in the spring in response to rising water temperatures and emerging aquatic vegetation (Stacey *et al.*, 1979). In response to the preovulatory GtH-II surge commencing approximately 10

hours prior to ovulation, goldfish follicles synthesize 17,20ß-P which induces final oocyte maturation (Goetz, 1983; Nagahama, 1990). 17,20ß-P and its metabolites are then released to the water where they function as a preovulatory pheromone. Male goldfish exposed to water-borne 17,20ß-P show significant increase in blood GtH-II concentration within 15 minutes and milt volume (sperm and seminal fluid) within 6 hours (Dulka *et al.*, 1987; Sorensen *et al.*, 1987; Stacey *et al.*, 1989; Stacey and Sorensen, 1986). At ovulation, $PGF_{2\alpha}$ is synthesized in the female reproductive system and enters the circulation. The $PGF_{2\alpha}$ acts within the brain as a hormone to trigger female spawning behavior (Stacey and Goetz, 1982; Stacey, 1987). A mixture of $PGF_{2\alpha}$ and it metabolites is then released to the water (Sorensen *et al.*, 1995a) and functions as a postovulatory pheromone to trigger male courtship behavior via olfactory receptors which are distinct from the receptors for 17,20ß-P (Sorensen *et al.*, 1988; Stacey *et al.*, 1994a).

Recent studies have demonstrated that both preovulatory and postovulatory pheromones are not a single compound, but a complex mixture. At the time when 17,20ß-P is released by ovulatory females, other C21 steroids such as 17α-hydroxyprogesterone, the precursor of 17,20ß-P (Van Der Kraak *et al.*, 1989) and two 17,20ß-P conjugates, 17,20ß-P-glucuronide (17,20ß-P-G) and 17,20ß-P-sulfate (17,20ß-P-S) (Stacey *et al.*, 1989; Scott and Sorensen, 1994; Sorensen *et al.*, 1995b) are also released by the ovulatory female goldfish. Electrophysiological measurements of olfactory activity strongly indicate that goldfish use mixtures of unmodified steroid hormones and steroids conjugated with sulfate and glucuronic acid as hormonal pheromones. Although 17,20ß-P is the most potent olfactory stimulant, the male olfactory organ is also very sensitive to 17,20ß-P-G and 17,20ß-P-S (Sorensen *et al.*, 1987, 1990, 1991a, 1995b). In the case of the postovulatory pheromone, studies show that the most potent known prostaglandin is 15-keto-$PGF_{2\alpha}$ which is released by $PGF_{2\alpha}$-injected females (Sorensen *et al.*, 1988; Sorensen and Goetz, 1993). But neither

15-keto-$PGF_{2\alpha}$ nor $PGF_{2\alpha}$ itself appears to be the only active component released by female goldfish. Identification of several radiolabeled olfactory stimulants released by female goldfish after injection with labeled $PGF_{2\alpha}$ confirms that the postovulatory female pheromone is comprised of metabolites of $PGF_{2\alpha}$ (Sorensen and Goetz, 1993; Sorensen *et al.*, 1988, 1995a). Behavioral tests also shown that the odor from $PGF_{2\alpha}$-injected females, and not $PGF_{2\alpha}$ itself can rapidly induce in males behavioral responses similar to those induced by the odor from ovulated females (Sorensen *et al.*, 1986).

In addition to 17,20ß-P-like pheromones and PGF pheromones, androstenedione -like steroids also act as pheromones in male goldfish. Unlike 17,20ß-P and PGF, androstenedione (AD) and testosterone are considered as inhibitory pheromones because they inhibit the milt response to 17,20ß-P (Stacey and Sorensen, 1991; Stacey, 1991). Because female goldfish release both 17,20ß-P and AD (Sorensen and Scott, 1994) and the 17,20ß-P : AD ratio increases as the ovulatory GtH-II surge progresses (Scott and Sorensen, 1994), it is hypothesized that male goldfish have evolved to use the 17,20ß-P : AD ratio as an indicator for impending ovulation (Stacey *et al.*, 1991).

EOG cross-adaptation studies indicate that the olfactory organ of male goldfish has at least five distinct classes of receptors for steroids and prostaglandins. 17,20ß-P acts through a class of receptors that also detects 17P and 17,20ß-P-G (Sorensen *et al.*, 1987, 1990, 1991b) whereas 17,20ß-P-S appears to act through another class of receptors (Sorensen *et al.*, 1991). AD appears to act on a third class of olfactory receptors distinct from the above two (Sorensen *et al.*, 1991b) although AD has been found to displace 17,20ß-P from 17,20ß-P receptors when applied at 50 times the concentration of 17,20ß-P (Rosenblum *et al.*, 1991). Finally, there are two distinct classes of receptors for $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ (Sorensen *et al.*, 1988). Although morphological studies have shown that there are at least two types of sensory receptor cells on the olfactory epithelium of goldfish (Ichikawa and Ueda, 1977), there is no

evidence showing that different receptors may distribute on different types of olfactory neurons. The adaptation of different types of receptors in the goldfish may not indicate functional specificity because $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ appear to have the same actions (Sorensen *et al.*, 1989). Instead, the receptor diversification may be related to the releasing pattern of different pheromones. In goldfish, 15-keto-$PGF_{2\alpha}$ is released exclusively in the urine whereas $PGF_{2\alpha}$ is released both in the urine and across the gill (Appelt and Sorensen, 1995). The excretion routes of steroidal pheromones are not clear in goldfish, but studies in rainbow trout (*Oncorhynchus mykiss*) indicate that 17,20ß-P is released across the gill whereas 17,20ß-P-S is released in the urine (Scott and Liley, 1994; Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996). The diversity of olfactory receptors for hormonal pheromones makes it possible that their effects are mediated by different mechanisms.

Both 17,20ß-P-like steroidal pheromones and PGF have been reported to exert physiological and behavioral effects. Although most research on 17,20ß-P has focused on its ability to rapidly increase blood GtH-II concentration (Dulka *et al.*, 1987; Stacey *et al.*, 1989; Sorensen *et al.*, 1987; Stacey and Sorensen, 1986), this steroid also exerts behavioral effects, as male goldfish exposed to 17,20ß-P significantly increase swimming speed (Bjerselius *et al.*, 1995) and performed more spawning acts with females than do control males (Defraipont & Sorensen 1993). On the other hand, PGF, the postovulatory pheromone, not only triggers male courtship behavior (Sorensen and Stacey, 1991), but also has physiological effects, stimulating a rapid increase in GtH-II and milt volume by stimulating courtship behavior (Kyle *et al.*, 1985; Sorensen *et al., 1989*).

1-5: MECHANISMS MEDIATING ENDOCRINE AND BEHAVIORAL RESPONSES TO HORMONAL PHEROMONES IN MALE GOLDFISH

Although studies in goldfish have provided the most detailed information about function of chemically identified pheromones in teleosts, relatively little is known about the neuroendocrine mechanisms that mediate the pheromonal effects on milt and spawning behavior. In particular, nothing is known about whether these pheromones influence male fertility through different neuroendocrine mechanisms.

Neuroendocrine responses to sex pheromones in goldfish are mediated by the olfactory system, which has specific membrane bound receptors in the olfactory epithelium (Rosenblum *et al.*, 1991; Sorensen *et al.*, 1989). Olfactory tract section (Dulka and Stacey, 1991; Stacey and Kyle, 1983) and electrical recording from the olfactory bulb (Sorensen *et al.*, 1991b; Fujita *et al.*, 1991) indicate that pheromonal responses in goldfish are mediated by the medial olfactory tract which is believed to be analogous to the mammalian vomeronasal system (Dulka, 1993).

Considerable evidence indicates that endocrine responses to pheromonal 17,20ß-P and PGF are mediated by different mechanisms (Stacey *et al.*, 1994a). 17,20ß-P and PGF have distinct olfactory receptors on the olfactory epithelium (Sorensen *et al.*, 1988, 1990). 17,20ß-P increases GtH-II and milt volume directly, but PGF does so indirectly by stimulating sexual interactions among conspecifics (Sorensen *et al.*, 1989) and this effect depends on a circadian rhythm (Dulka, 1989; Dulka *et al.*, 1987). The increase of milt volume induced by 17,20ß-P takes 4-6 hours and can be blocked by hypophysectomy (Dulka *et al.*, 1987) whereas the increase induced by PGF can occur within 30 minutes even without observable change in GtH-II (Kyle *et al.*, 1985). The observation that water-borne 17,20ß-P, and the stimuli from spawning with $PGF_{2\alpha}$-injected females, have additive effects on blood GtH-II concentrations (Sorensen *et al.*,

1989) also indicates the endocrine response to these pheromones may be mediated by different mechanisms.

In goldfish, the regulation of pituitary GtH-II release is under a dual control of stimulatory GtH-releasing hormone (GnRH) neurons and inhibitory dopamine neurons (Peter *et al.*, 1991a, b). There are two native forms of GnRH, the salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II) found in the goldfish brain (Yu *et al.*, 1988). Both sGnRH and cGnRH-II immunoreactive fibers are located widely in the brain and the pituitary (Kah *et al.*, 1986a, 1987), and both of these GnRH peptides stimulate GtH-II synthesis (Khakoo *et al.*, 1994) and release (Chang *et al.*, 1990), although cGnRH-II has been found to be more potent than sGnRH in stimulating GtH-II release *in vitro* (Chang *et al.*, 1990; Habibi, 1991). Direct dopaminergic innervation of gonadotrophic cells has been demonstrated by immunocytochemical studies (Kah *et al.*, 1986b). Dopamine not only inhibits both spontaneous and GnRH-stimulated GtH-II release (Chang and Peter, 1983; Omeljaniuk *et al.*, 1987), but also inhibits the release of GnRH from pituitary fragments and preoptic-anterior hypothalamic slices (Yu and Peter, 1992). Other factors such as norepinephrine, serotonin, neuropeptide Y, sex steroids, γ-aminobutyric acid and gonadal proteins (inhibin and activin) also influence GtH-II secretion (Ge *et al.*, 1992; Peter *et al.*, 1991c; Trudeau *et al.*, 1993).

Pheromonal 17,20ß-P appears to stimulate GtH-II release by reducing pituitary dopamine turnover rate (Dulka *et al.*, 1992). Whether GnRH is also involved in the regulation of 17,20ß-P-induced GtH-II release is still not entirely clear. The fact that 17,20ß-P does not increase serum growth hormone (Dulka, 1989) provides indirect evidence that GnRH, which is known to be a GH-releasing hormone in goldfish (Peter *et al.*, 1991c), may not be involved in 17,20ß-P-induced GtH-II increase. On the other hand, evidence that GnRH is involved in 17,20ß-P-induced GtH-II release is the finding that a GnRH antagonist blocks the 17,20ß-P effect (Murthy *et al.*, 1994). However,

there is no direct evidence showing the effect of 17,20ß-P on GnRH concentration. In contrast, there is direct evidence suggesting that GnRH is involved in spawning-induced GtH-II release: spawning with PGF-injected females increases brain GnRH concentration in male goldfish (Yu and Peter, 1990). Interestingly, spawning with spontaneous ovulatory females decreases brain GnRH concentration in male goldfish (Yu *et al.*, 1991). It is not clear why male goldfish show different changes in brain GnRH concentration in response to $PGF_{2\alpha}$-injected females and ovulatory females. However, these changes in GnRH concentration during spawning suggest that GnRH may be the main neuroendocrine factor regulating spawning-induced GtH-II release.

The possible existence of different neuroendocrine mechanisms mediating different pheromone effects could have evolved to enhance male reproductive success. Because goldfish is a group spawning species, males with more milt available and being capable of releasing more sperm are likely to fertilize more eggs. The increase of blood GtH-II in response to pheromonal stimuli appears to increase sperm number because GtH-II is well known to increase the volume of milt which can be stripped from a variety of fish species (Billard *et al.*, 1982; Courtois *et al.*, 1986; Saad and Billard, 1987). In some species, successive spawning results in reduced male fertilization rates due to possible reduction of released sperm (e.g. lemon tetra, *Hyphessobrycon pulchripinnis*, Nakatsuru and Kramer, 1981). In some other teleosts such as goldfish and a coral reef fish (*Thalossoma bifasciatum*), milt appears not to be depleted during the spawning period (Kyle *et al.*, 1985; Sorensen *et al.*, 1989; Shapiro *et al.*, 1994). The fact that spawning stimuli usually induce blood GtH-II increase (Kyle *et al.*, 1985) suggests that spawning behavior may also enhance male fertility not only by rapidly increasing sperm production, but also by increasing GtH-II concentration which in turn stimulates more milt production in the ongoing spawning. Indeed, pheromonal 17,20ß-P has been shown to increase the sperm quantity and motility in goldfish (Defraipont and Sorensen, 1993).

However, whether the increase of motility has anything to do with fertility has not been examined.

1-6: OBJECTIVES OF THIS STUDY

My research project has two primary objectives: (1) to test the hypothesis that there are different pathways for 17,20ß-P-induced and PGF/spawning-induced increases in GtH-II concentration and milt volume; and (2) to examine the effect of pheromonal 17,20ß-P on male reproductive success including sexual behavior, sperm quantity, sperm quality, and fertility. Results of this study will not only increase our understanding of the proximate (physiological) mechanisms of sex pheromone response in male goldfish, but also the ultimate (fertility) mechanisms for rapidly adjusting sperm allocation to imminent spawning requirement. This information will also provide insights for application of pheromones in aquaculture. Stacey *et al.* (1994a) have shown that many commercially important cyprinids detect PGF and 17,20ß-P-like steroids. Because 17,20ß-P has been shown to induce the same blood GtH-II and milt volume in common carp *Cyprinus carpio* (Stacey *et al.*, 1994b), it is expected the basic elements of the goldfish hormonal pheromone system exist in many cyprinids.

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CHAPTER 2

TWO MECHANISMS FOR INCREASING MILT VOLUME IN MALE GOLDFIS: COMPARISON BETWEEN PHEROMONAL 17,20ß-P AND SPAWNING STIMULI[1]

INTRODUCTION

As with final oocyte maturation (Nagahama, 1990; Nagahama *et al.*, 1995; Swanson, 1994), the increase in milt (sperm and seminal fluid) volume that typically accompanies the final stage of testicular maturation in teleosts is generally attributed to increased circulating gonadotropin-II (GtH-II) (Nagahama, 1994; Yoshikuni and Nagahama, 1991). Such a causal relationship is supported by numerous *in vivo* studies showing either that spontaneous GtH-II increase is associated with an increase in milt volume (Liley *et al.*, 1986;) or that GtH-II injection rapidly increases milt volume (Billard *et al.*, 1982; Courtois *et al.*, 1986; Saad and Billard, 1987). Although the testicular mechanisms mediating the proposed GtH-II effect are less well studied, there is evidence that sperm duct ion regulation (Marshall *et al.*, 1989) and testicular steroidogenesis both may be involved (Ueda *et al.*, 1985). In comparison to ovulation, milt volume regulation has received relatively little attention, a contributing factor likely being that the male processes are perceived to be less discrete and more protracted than those in the female, and therefore more difficult to study in physiological situations. However, studies in goldfish (*Carassius auratus*) showing that milt volume is rapidly and predictably increased by pheromonal and behavioral stimuli associated with spawning

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(Stacey *et al.*, 1994) provide relatively simple methodologies for investigating the mechanisms of milt volume regulation.

In goldfish, the oocyte maturation-inducing steroid 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) appears to function as a female preovulatory pheromone that increases milt volume through a chain of events that begins with binding to olfactory receptor membranes (Rosenblum *et al.*, 1991; Sorensen *et al.*, 1990) and includes activation of the medial olfactory tracts (Sorensen *et al.*, 1991), reduction of tonic dopaminergic inhibition of GtH-II release (Dulka *et al.*, 1992), and rapid (within 15 min) increase in plasma GtH-II and 17,20ß-P (Dulka *et al.*, 1987a) which also is produced by the testes (Ueda *et al.*, 1985; Sakai *et al.*, 1989). In addition to the well documented effects of 17,20ß-P on milt quantity, there also is evidence that the pheromone affects milt quality: 17,20ß-P-exposed males have sperm with longer duration of motility (DeFraipont and Sorensen, 1993), and in competitive spawning are more fertile than non-exposed males, as revealed by microsatellite DNA fingerprinting studies (Zheng W., Stacey, N.E. and Strobeck, C., unpublished results; Chapter 4).

Male goldfish also increase GtH-II and milt volume during interaction either with ovulated females or with nonovulated females in which sexual activity has been induced by injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Kyle *et al.*, 1985; Sorensen *et al.*, 1988, 1989). There are several reasons for believing that these GtH-II and milt responses to spawning stimuli are mediated by different mechanisms than those involved in responses to 17,20ß-P. GtH-II response to spawning stimuli differs with time of day whereas response to 17,20ß-P does not (Dulka *et al.*, 1987b). As well, the milt increase in response to 17,20ß-P has a latency of 4-6 h (Dulka *et al.*, 1987a), whereas the response to spawning stimuli can occur within 30 min (Kyle *et al.*, 1985), a response believed to be too rapid to be induced by GtH-II increase. Indeed, an increase of milt volume in

spawning males can occur even without significant change in blood GtH (Kyle *et al.*, 1985).

The following experiments provide additional evidence that 17,20ß-P and spawning stimuli increase milt volume through different mechanisms. I compared the general time course of milt increase induced by 17,20ß-P and spawning stimuli. I also examined the effects of hypophysectomy, human chorionic gonadotropin (hCG) injection and water temperatures on milt increase induced by these two stimuli.

MATERIALS AND METHODS

Animal source

Goldfish were purchased from Ozark Fisheries Co., Inc., Stoutland, Missouri, kept for various times in 1000 liter or 3400 liter flowing-water tanks at 17-20 °C under a 16L: 8D photoperiod (light on at 0800 h) and fed twice daily with commercial fish food (Nutrafin). Mature male goldfish, identified by the presence of expressible milt and tubercles on the pectoral fins or the opercula, were moved in groups of 2 or 3 fish to 65 liter flowing-water experimental aquaria at 20 °C for at least two days before all experiments (for Experiment 4, fish were acclimated to 11 °C for one week). All aquaria contained artificial floating vegetation and gravel, and were opaque with only a small window at one end for behavioral observation.

Milt sampling

Milt was collected and its volume was measured as described by Kyle *et al.* (1985) and Stacey and Sorensen (1986). Briefly, fish were anesthetized with 2-phenoxyethanol (Syndel; 0.05%) and milt was collected using pre-weighed hematocrit

tubes by applying a gentle pressure to the abdomen. Contamination of milt with urine was carefully avoided by pressing the posterior abdomen where the urinary bladder is located, and wiping with a piece of tissue before milt sampling (Perchec *et al.*, 1995). Milt density is assumed to be 1.0 and milt volumes are expressed as volumes (μl) rather than as weight.

Chemicals

hCG (Sigma) was dissolved in 0.6% NaCl at a concentration of 0.4 IU/μl and injected intraperitoneally at a dose of 2 IU/g. 17,20ß-P (Sigma) was diluted in ethanol (EtOH) at a concentration of 0.1 μg/μl. At the time of pheromone exposure, 100 μl of 17,20ß-P solution was added to the aquaria (65 liters) to give a final concentration of approximately 5×10^{-10} M 17,20ß-P (Dulka, 1987a). Prostaglandin $F_{2\alpha}$ (veterinary grade, 5 mg/ml prostaglandin $F_{2\alpha}$ tromethamine salt, Lutalyse, Upjohn,) was used to induce spawning behavior by intramuscular injection at an approximate dose of 250 ng/g body weight (Kyle *et al.*, 1985) . Female goldfish injected in this way become sexually active for several hours (Stacey and Goetz, 1982) and release an odor that rapidly induces in males behavioral responses similar to those induced by the odor from ovulated females (Sorensen *et al.*, 1986).

Statistical analyses

Milt data in all experiments were analyzed in two steps. First, to determine whether groups had equivalent initial milt volumes, differences between groups in milt volume were analyzed by Mann-Whitney *U*-test. If initial volumes were not different ($P > 0.05$), then the effect of each treatment was evaluated by comparing post-treatment milt volumes using *U*-test with *P* set at 0.05. When post-treatment milt was taken more than one time (experiment 1 and 4) from the same group of fish, a *U*-test (comparing

experimental and control groups) was used for each sample time, and the *Bonferroni correction* was applied and *P* reduced to 0.05/*n* where *n* is the number of post-treatment milt samples taken from each fish (Kleinbaum *et al.*, 1988).

Experiment 1. Do spawning-induced, 17,20ß-P-induced, and hCG-induced increase in milt volume have the same time course?

These experiments compared the latencies to milt increase in response to 17,20ß-P exposure, hCG injection and stimuli provided by spawning.

To determine the latency to milt increase during spawning, mature males were randomly divided on day 1 into control and treatment groups. At 0 hour on day 2, all fish were anesthetized and stripped to obtain initial milt volumes. After half an hour of recovery, the treatment group (n = 10) received two $PGF_{2\alpha}$-injected females per tank and the control group (n = 10) was left untreated. No saline-injected females were included in the control group because they would have caused uncontrolled chasing behavior in the males (Defraipont and Sorensen, 1993) which could influence the results. All fish were stripped again 2 hours after spawning. To determine the latency of 17,20ß-P-induced increase in milt volume, mature males were randomly divided into 17,20ß-P-exposed and control groups. At 0 hour on day 2, all fish were stripped for initial milt. After half an hour of recovery, fish were exposed to either 5×10^{-10} M 17,20ß-P (n = 12) or an equivalent amount of EtOH (n = 12), and stripped again after 2 and 6 h of exposure. To determine the latency to milt increase following hCG injection, mature males were randomly divided into hCG-injected and saline-injected groups. At 0 h on day 2, all fish were stripped, weighed and injected intraperitoneally with either hCG (2 IU/g; 5 µl/g; n = 11) or an equivalent volume of 0.6% NaCl saline (n = 11). All fish were stripped again at 2, 6 and 12 h after injection. Water temperature was 18 - 20 °C in all these three experiments.

Experiment 2. Does hypophysectomy block spawning-induced and 17,20ß-P-induced increase in milt volume?

These experiments were conducted to determine whether $PGF_{2\alpha}$-induced spawning behavior can stimulate milt increase without the presence of the pituitary, a possibility suggested by the fact that spawning-induced increase in milt volume can occur without observable GtH-II increase.

To determine the effect of hypophysectomy on spawning-induced increase in milt volume, mature males were either hypophysectomized (n = 16) by the opercular approach (Yamazaki, 1961) or given a sham operation (n = 16) and then held in 0.6% NaCl. The next morning, all fish were stripped of milt. After 30 min of recovery, fish were either left untreated or allowed to spawn with $PGF_{2\alpha}$-injected females, and stripped again after 2 hours of spawning, which was verified by observation. To determine the effect of hypophysectomy on 17,20ß-P-induced increase in milt volume, another group of males were either hypophysectomized (n = 16) or given a sham operation (n = 16) and held in 0.6% NaCl. The next morning, all fish were stripped for initial milt. After 30 min of recovery, fish were exposed to either 17,20ß-P (5×10^{-10} M) or equivalent EtOH, and stripped again after 6 h of exposure.

To determine whether the lack of stimulatory effect of 17,20ß-P on hypophysectomized fish might have resulted from insufficient milt stores, a further experiment was conducted on hypophysectomized fish injected with hCG. Fourteen males were hypophysectomized, weighed and injected with hCG (2 IU/g; 5 µl/g), and then held in 0.6% NaCl. The next morning, all fish were stripped of milt, exposed to either 17,20ß-P (5×10^{-10} M) or an equivalent amount of EtOH 30 min later, and stripped again after 6 h of exposure. Another 24 mature males were also hypophysectomized, weighed and injected with hCG (2 IU/g; 5 µl/g) and then held in 0.6% NaCl. The next morning, all fish were stripped for initial milt. After 30 minutes for recovery, fish were

either left untreated or allowed to spawn with two $PGF_{2\alpha}$-injected females, and stripped again after 2 h of spawning.

Experiment 3. Does hCG treatment block spawning-induced and 17,20ß-P-induced increase in milt volume?

Having demonstrated that hypophysectomy blocks 17,20ß-P-induced increase in milt volume, presumably by removal of gonadotrophs, this experiment was conducted to determine whether hCG injection would block 17,20ß-P-induced milt increase in intact fish. The rationale was that if 17,20ß-P-induced milt increase is due solely to increase in blood GtH-II, exogenous hCG treatment should swamp this effect. In contrast, spawning-induced milt increase, which does not require the pituitary, should be unaffected.

To determine the effect of hCG pre-treatment on 17,20ß-P-induced increase in milt volume, mature males were anesthetized, weighed, and injected with either hCG (2 IU/g; 5 µl/g; n = 30) or an equivalent volume of 0.6% NaCl (n = 30) on day 1. On day 2, all fish were stripped of milt. After 30 min of recovery, fish in each group were exposed to either $5x10^{-10}$ M 17,20ß-P (n = 15), or equivalent EtOH (n = 15), and stripped again 6 h later. To determine the effect of hCG pre-treatment on spawning-induced increase in milt volume, mature males were anesthetized, weighed, and injected with either hCG (2 IU/g; 5 µl/g; n = 30) or an equivalent volume of 0.6% NaCl (n = 30) on day 1. On day 2, all fish were stripped of milt. After 30 minutes of recovery, half the fish in each treatment group received $PGF_{2\alpha}$-injected females (two females per tank) while the other half did not receive females. Two hours later, all fish were again stripped of milt. Using another 60 fish, this experiment was repeated but males were allowed to spawn with the $PGF_{2\alpha}$-injected females for 6 hours before being stripped of milt.

Experiment 4. Does temperature affect milt increase induced by spawning behavior, 17,20β-P and hCG?

Low temperature reduces gonadotropin-induced milt production in goldfish and common carp, *Cyprinus carpio* (Clemens and Grant, 1964; Saad and Billard, 1987) and delays ovulatory response to hCG in goldfish (Stacey *et al.*, 1979). In view of the evidence (Dulka *et al.*, 1987a; this study) that 17,20ß-P-induced milt increase requires endogenous GtH-II and that spawning-induced milt increase does not, the following experiments were conducted to determine whether latencies to 17,20ß-P-induced and hCG-induced milt increases are more affected by temperature than is the latency to spawning-induced milt increase.

Mature males were held at either 11 °C or 21 °C for one week (two fish per tank). To determine latency to spawning-induced milt increase, fish were stripped of milt, and left untreated (n = 8) or placed with two $PGF_{2\alpha}$-injected females (n = 8) after 30 min of recovery. Spawning behavior was verified during the test period and the time when each male started chasing females was recorded. All males were stripped again 2 hours after adding females. To determine the latency to 17,20ß-P-induced increase in milt volume, fish were exposed to 5×10^{-10} M 17,20ß-P (n = 15) or an equivalent volume of EtOH (n = 15). Fish at 11 °C were stripped repeatedly at 4, 8, 12 and 24 h after exposure; fish at 21 °C were stripped repeatedly at 4, 8 and 12 h after exposure. To determine the latency to hCG-induced increase in milt volume, fish were injected with 2 IU/g hCG (n = 12) or an equivalent volume of O.6% NaCl (n = 12) and stripped again every two hours for 12 hours. Fish held at 11 °C were also stripped a final time at 24 h after injection.

RESULTS

Experiment 1. Time course of milt increase induced by spawning behavior, 17,20ß-P and hCG

Within each of the three experiments, milt volumes of experimental and control males were equivalent at 0 h ($P > 0.05$) prior to treatment (Fig. 2.1). In the first experiment, males started chasing behavior within minutes of adding $PGF_{2\alpha}$-injected females, performed spawning behavior throughout the experimental period and had significantly more milt than control males after 2 h of spawning ($P < 0.01$; Fig. 2.1a). In contrast, milt volumes of 17,20ß-P-exposed and hCG-injected males were equivalent ($P > 0.05$) to those of control males 2 h after treatment but significantly larger by 6 h after treatment ($P < 0.001$; Fig. 2.1b, c).

Experiment 2. Effect of hypophysectomy on milt increase induced by spawning and 17,20ß-P

In the first experiment examining the effect of hypophysectomy on spawning-induced milt increase, initial milt volumes of sham operated fish were significantly greater ($P < 0.001$) than those of hypophysectomized fish when stripped at 0 h on day 2, although within each of the operation groups, milt volumes of the control and spawning groups were equivalent ($P > 0.05$; Fig 2.2a). Both in sham and hypophysectomized males, interaction with $PGF_{2\alpha}$-injected females for 2 h significantly increased strippable milt volume ($P < 0.01$, Fig. 2.2a). In contrast to the effect of spawning activity on milt volume, 17,20ß-P exposure increased milt volume only in sham operated males ($P < 0.01$; Fig. 2.2b).

In hypophysectomized fish injected with hCG, 2 h of spawning activity significantly increased milt volume ($P < 0.01$; Fig. 2.3). However, milt volumes in

17,20ß-P-exposed males were equivalent to those of control males after 6 h of exposure (Fig. 2.3).

Experiment 3. Effect of hCG treatment on milt increase induced by spawning stimuli and 17,20β-P

hCG injection significantly ($P < 0.01$) increased initial milt volumes in all three experiments (Fig. 2.4), although within each of the treatment groups, milt volumes of the control and spawning groups (Fig. 2.4b, c) or ethanol and 17,20ß-P groups (Fig. 2.4a) were equivalent ($P > 0.05$). Exposure to 17,20ß-P significantly increased milt volume in saline-injected males ($P < 0.05$) but did not increase milt volume in hCG-injected males (Fig. 2.4a). In contrast, interaction with $PGF_{2\alpha}$-injected females significantly increased milt volume ($P < 0.05$) in hCG-injected fish and saline-injected fish both after 2 h of spawning (Fig. 2.4b) and 6 h of spawning (Fig. 2.4c).

Experiment 4. Effect of temperature on milt increase induced by spawning behavior, 17,20β-P and hCG

At both 11 °C and 21 °C, male courting behavior began within several minutes of adding $PGF_{2\alpha}$-injected females. After 2 h of spawning, males at both temperatures had significantly higher milt volumes ($P < 0.05$) than their respective control groups (Fig. 2.5a). At 11 °C, 17,20ß-P did not increase milt volume until 12 h of exposure, whereas at 21 °C, 17,20ß-P induced an increase in milt volume by 4 h of exposure ($P < 0.01$; Fig. 2.5b). At 11 °C, milt volume of hCG-injected fish and saline-injected fish did not differ ($P > 0.05$) until 24 h after injection (Fig. 2.5c), whereas at 21 °C, milt volumes from hCG-injected fish and control fish differed significantly from 6 h after injection ($P < 0.001$; Fig. 2.5c).

DISCUSSION

The results of this and previous studies indicate that in goldfish, the increase in milt volume induced by pheromonal 17,20ß-P is mediated by an increase in circulating GtH-II, whereas the increase in milt volume induced by spawning activities is not. The results also indicate that pheromonal 17,20ß-P and hCG injection increase milt volume by similar mechanisms.

The effect of 17,20ß-P on milt volume is believed to require endogenous GtH-II release because hypophysectomy abolishes 17,20ß-P-induced milt increase (Dulka *et al.*, 1987a; this study), and because hCG injection abolishes the effect of a subsequent 17,20ß-P-exposure. In contrast, this study has shown that hypophysectomy did not abolish spawning-induced milt increase, even in fish that have received hCG injection. The additive effect of spawning stimuli on milt increase in both hCG injected fish (Fig. 2.4b) and 17,20ß-P-exposed fish (Stacey *et al.*, 1987) indicates that the rapid effect of spawning stimuli on milt production does not involve gonadotropin.

In the present study, milt volume of males held at 21 °C consistently increased within 2 h of spawning stimuli, but did not increase until 4 - 6 h after 17,20ß-P exposure or hCG injection. This rapid milt response to spawning stimuli was not affected if males were held at 11 °C, whereas the milt response to 17,20ß-P and hCG was considerably delayed at low temperature. This obvious temperature-dependence of the milt response to 17,20ß-P and hCG is consistent with earlier reports that low temperature reduces the milt response to hormonal treatment in common carp (Clemens and Grant, 1964) and delays ovulatory response to hCG in female goldfish (Stacey *et al.*, 1979), and provides additional strong evidence that spawning stimuli and pheromonal 17,20ß-P act via separate mechanisms. As well, the similar effect of low temperature on the latency to 17,20ß-P-induced and hCG-induced milt increase suggests that pheromonal 17,20ß-P

increases milt by increasing endogenous GtH-II release. Low temperature (12 °C) has been reported to reduce *in vivo* GtH-II secretion rate and magnitude in goldfish (Cook and Peter, 1980; Sokolowska *et al.*, 1985). In common carp, low temperature (10 °C) was also found to suppress GtH-II release from pituitary fragments stimulated by GnRH superagonist (Lin *et al.*, 1996). Whether the latency to 17,20ß-P-induced increase in milt volume is due to the suppression and/or delay of GtH-II release remains to be examined. However, the fact that hCG-induced milt increase is delayed by low temperature suggests that low temperature delays milt increase possibly by influencing the GtH-II-induced gonadal steroid production, as demonstrated in common carp (Kime and Manning, 1986).

Although spawning interactions are well known to increase plasma GtH-II concentrations in male goldfish (Kyle *et al.*, 1985; Sorensen *et al.*, 1989), the fact that spawning-induced milt increase is not blocked by hypophysectomy or hCG injection, and is not obviously affected by low temperature, indicates that the rapid milt increase in spawning males is not dependent on GtH-II increase. The mechanism mediating this rapid milt increase is unknown, although it may involve the neuromuscular mechanism shown by Dulka and Demski (1986) to induce testis and sperm duct contraction. In mammals, oxytocin and related peptides induce *in vitro* contraction of prostate and seminal vesicle, and stimulate sperm release (Boer *et al.*, 1983; Sharaf *et al.*, 1992). If similar mechanisms exist in fish, they could mediate the rapid effects of spawning stimuli on milt volume.

The current understanding of the two mechanisms which mediate milt production induced by 17,20ß-P and spawning stimuli is presented in Figure 2.6. During the preovulatory period, pheromonal 17,20ß-P increases the concentrations of plasma GtH-II which in turn acts on the testes to increase milt production and movement of milt into the sperm ducts. At ovulation and the onset of sexual activity, pheromonal and behavioral

cues from females activate neural inputs to the reproductive tract, further increasing the movement of milt to the ducts. Spawning stimuli also can increase the concentration of plasma GtH-II, but this may be achieved by different neuroendocrine pathways. However, the resulting increase in GtH-II may act via the same endocrine pathway as the 17,20ß-P-induced GtH-II response to increase milt volume, and may serve to replenish milt stores depleted by spawning activity.

Figure 2.1. Time course of milt increase induced by spawning behavior (a), pheromonal 17,20ß-P (b) and hCG injection (c). a: fish were stripped of milt, placed with $PGF_{2\alpha}$-injected females or left untreated, and stripped again 2 h after adding females (n = 10 in each group). b: fish were stripped of milt, exposed to 17,20ß-P (5×10^{-10} M) or an equivalent volume of EtOH, then stripped again after 2 and 6 h of exposure (n = 12 in each group). c: fish were stripped of milt, injected with hCG (2 IU/g) or an equivalent volume of 0.6% NaCl, and stripped again at 2, 6 and 12 h after injection (n = 11 in each group). * indicates significant difference between treatment group and control group.

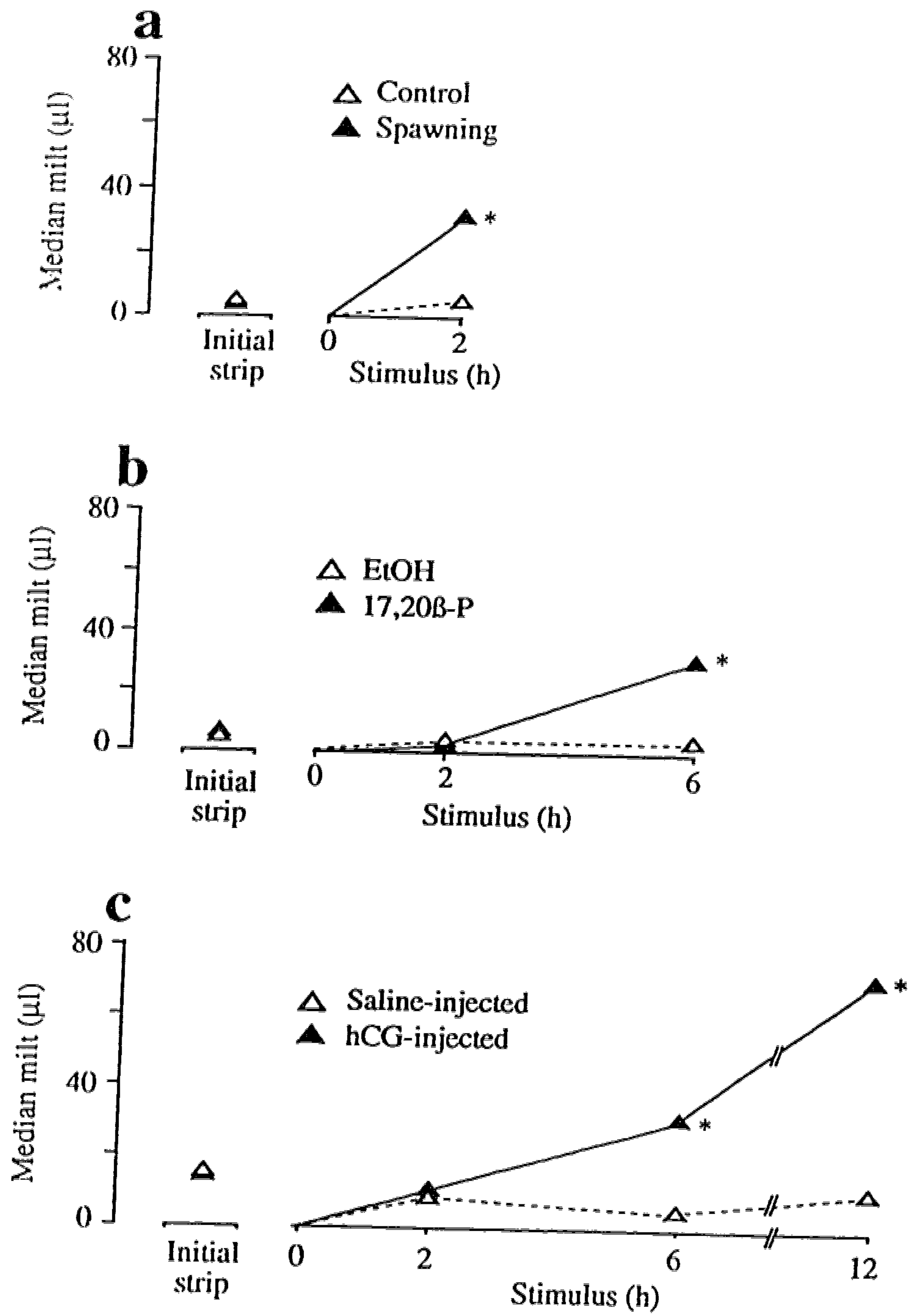
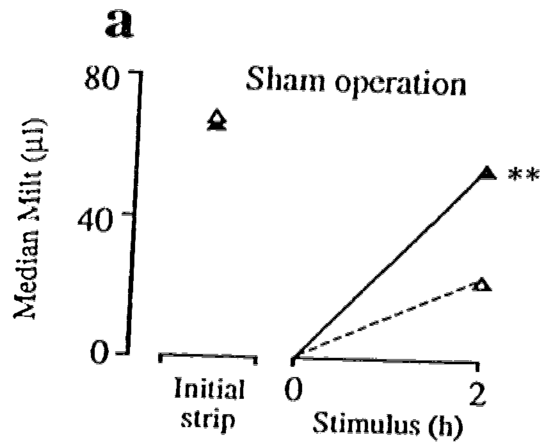
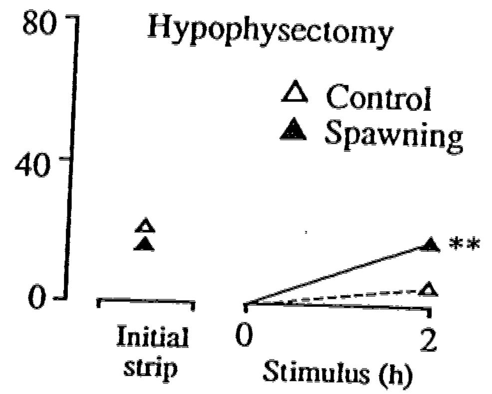

a
80
40
0
Median milt (µl)
Control
Spawning
*
Initial strip
0
2
Stimulus (h)
b
80
40
0
Median milt (µl)
EtOH
17,20ß-P
*
Initial strip
0
2
6
Stimulus (h)
c
80
40
0
Median milt (µl)
Saline-injected
hCG-injected
*
*
Initial strip
0
2
6
12
Stimulus (h)

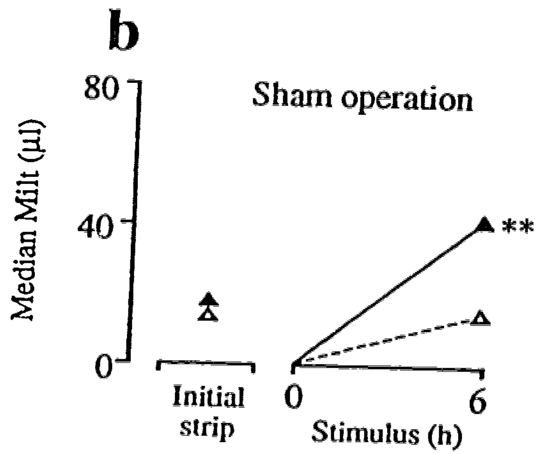
Figure 2.2. Effect of hypophysectomy on milt increase induced by spawning (**a**) and pheromonal 17,20ß-P (**b**) in goldfish. **a**: fish were hypophysectomized (n = 16) or sham operated (n = 16) on day 1. On day 2, all fish were stripped of milt and then left untreated or placed with $PGF_{2\alpha}$-injected females (two females per tank), and stripped again after 2 h of spawning. **b**: fish were hypophysectomized (n =16) or sham operated (n = 16) on day 1. On day 2, all fish were stripped of milt, exposed to either 17,20ß-P (5×10^{-10} M) or an equivalent volume of EtOH, and stripped again after 6 h of exposure. **= $P < 0.01$ (treatment vs. control).

a





b



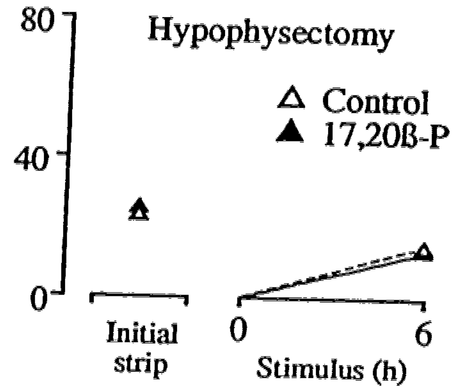


Figure 2.3. Effect of hypophysectomy on milt increase induced by spawning and pheromonal 17,20ß-P in hCG-injected goldfish. All fish were hypophysectomized and injected with hCG (2 IU/g) on day 1. On day 2, all fish were stripped of milt and then left untreated ($n = 12$), placed with $PGF_{2\alpha}$-injected females ($n = 12$), exposed to 5×10^{-10} M 17,20ß-P ($n = 7$), or exposed to an equivalent volume of EtOH ($n = 7$), and stripped again after 2 h of spawning or 6 h of exposure. **= $P < 0.01$ (treatment vs. control)

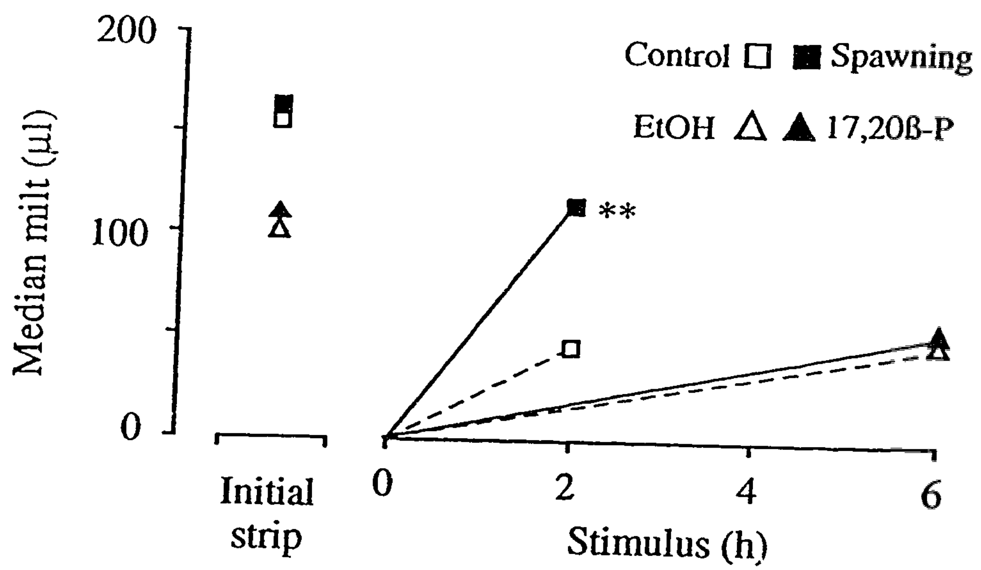

200
100
0
Median milt (µl)
Control
Spawning
EtOH
17,20ß-P
**
Initial
strip
0
2
4
6
Stimulus (h)

Figure 2.4. Effect of hCG pre-treatment (2 IU/g) on milt volume in response to 17,20ß-P exposure (**a**) and spawning stimuli (**b**, **c**). **a**: all fish were injected with hCG or an equivalent volume of 0.6% NaCl on day 1. On day 2, fish were stripped of milt, exposed to either 5×10^{-10} M 17,20ß-P (n = 15) or an equivalent volume of EtOH (n = 15), and stripped again after 6 h of exposure. **b** and **c**: all fish were injected with hCG or an equivalent volume of 0.6% NaCl on day 1. On day 2, fish were stripped of milt, placed with $PGF_{2\alpha}$-injected females (n = 15) or left untreated (n = 15), and stripped again after 2 or 6 h of spawning. * indicates significant difference between treatment group and its control group.

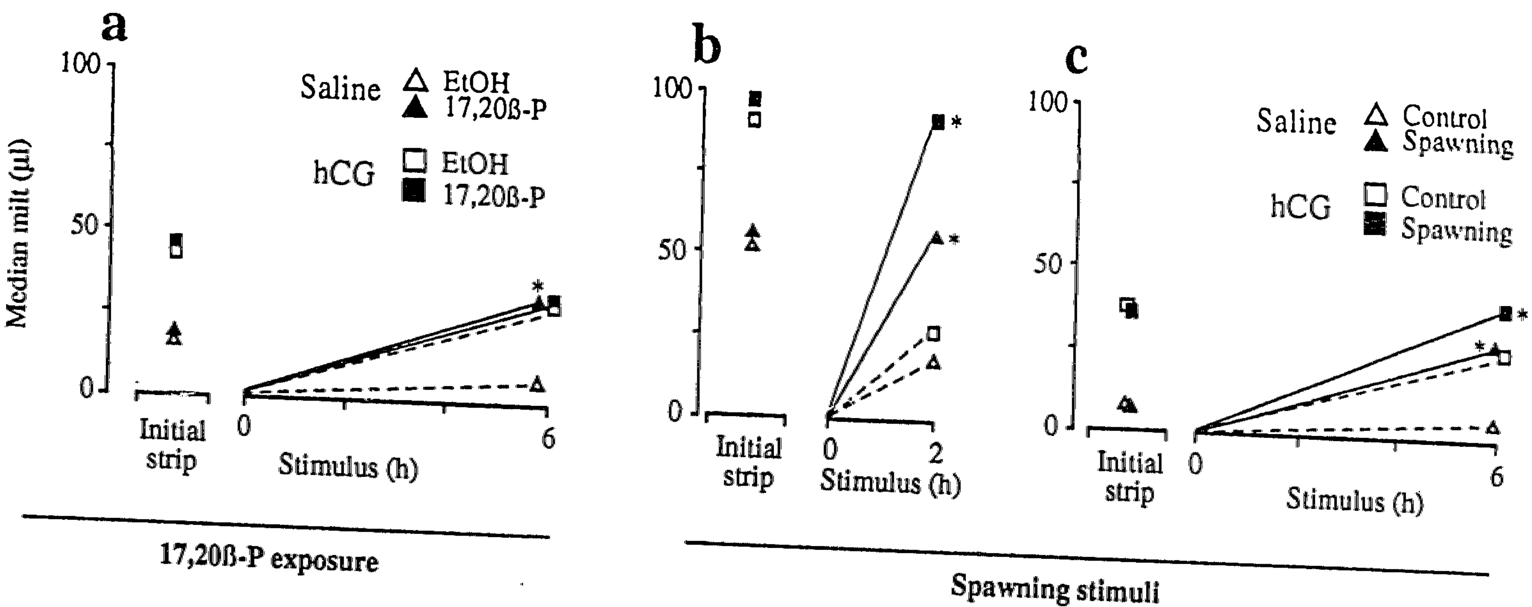
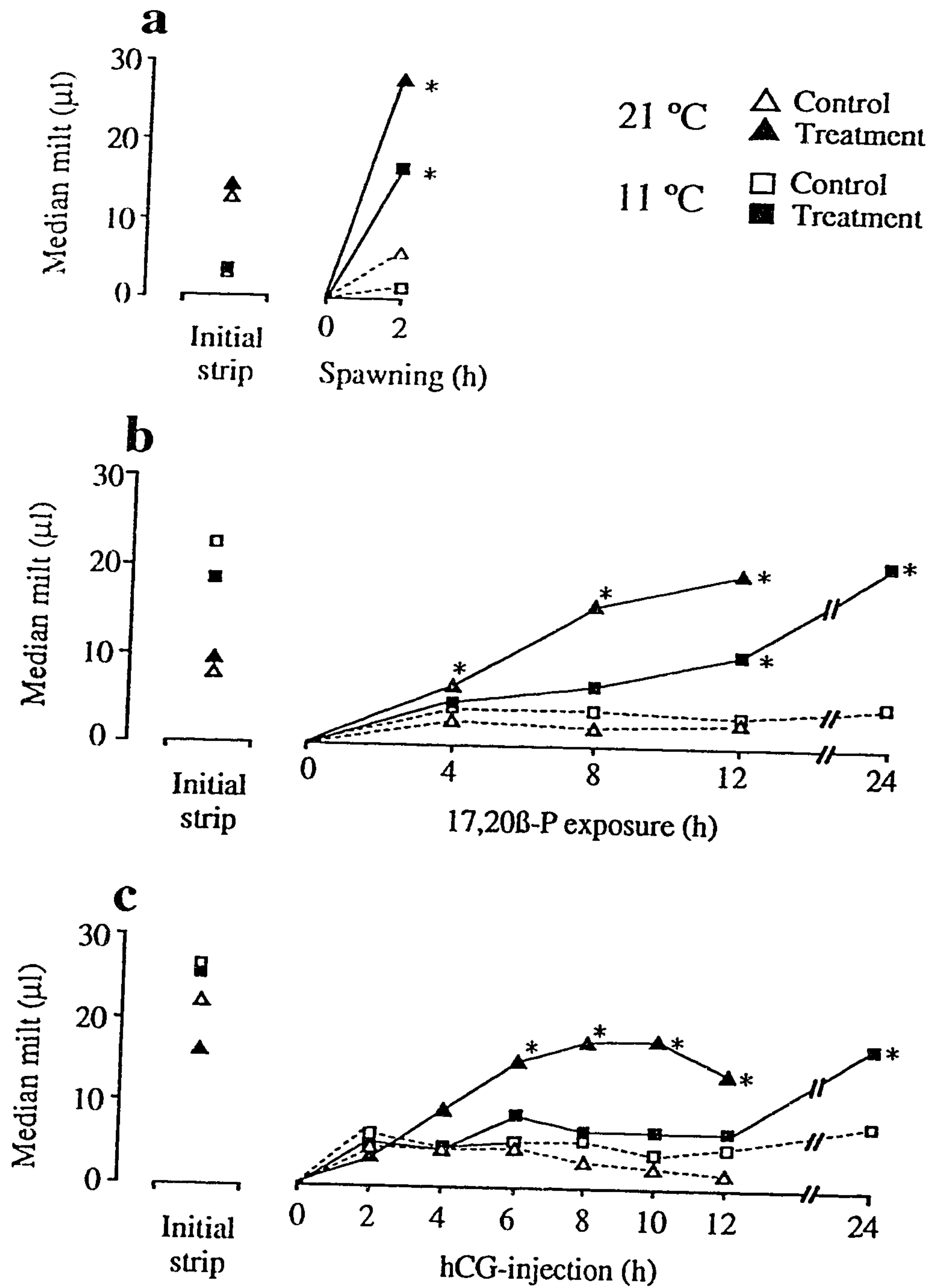
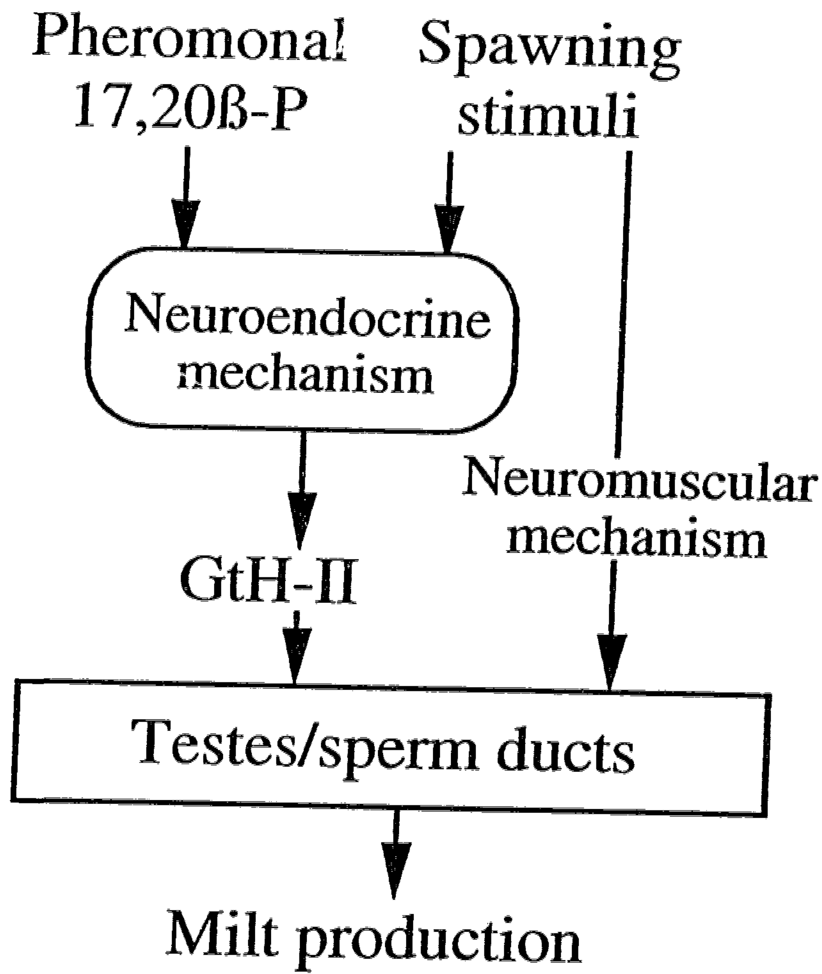

a
b
c
Median milt (µl)
100
50
0
Saline
hCG
EtOH
17,20ß-P
Control
Spawning
Initial strip
Stimulus (h)
0
2
6
*
17,20ß-P exposure
Spawning stimuli

Figure 2.5. Effect of temperature (11 °C and 21 °C) on milt increase induced by spawning (**a**), 17,20ß-P (**b**) and hCG (**c**). **a**: fish were stripped of milt, placed with $PGF_{2\alpha}$-injected females (n = 8) or left untreated (n = 8), and stripped again after 2 h of spawning. **b**: all fish were stripped of milt, exposed to 5 x 10^{-10} M 17,20ß-P (n = 15) or an equivalent amount of EtOH (n = 15), and repeatedly stripped again at 4, 8 and 12 h after exposure. Fish held at 11 °C received a final strip after 24 h of exposure. **c**: all fish were stripped of milt, injected with 2 IU/g hCG (n = 12) or an equivalent volume of 0.6% NaCl (n = 12), and repeatedly stripped again every two hours for 12 hours. Fish held at 11 °C received a final strip 24 h after injection. * indicates significant difference between treatment group and its control group.



a
Median milt (µl)
30
20
10
0
Initial strip
0
2
Spawning (h)
*
*
21 °C
△ Control
▲ Treatment
11 °C
□ Control
■ Treatment
b
Median milt (µl)
30
20
10
0
Initial strip
0
4
8
12
24
17,20ß-P exposure (h)
c
Median milt (µl)
30
20
10
0
Initial strip
0
2
4
6
8
10
12
24
hCG-injection (h)

Figure 2.6. A proposed model of two mechanisms mediating milt production induced by 17,20ß-P and spawning stimuli. During the preovulatory period, pheromonal 17,20ß-P increases plasma concentration of GtH-II, which in turn acts on the testes to increase milt production and movement of milt into the sperm ducts. At ovulation and the onset of sexual activity, pheromonal and behavioral cues from females activate neural inputs to the reproductive tract, further increasing the movement of milt to the ducts. Spawning stimuli also can increase plasma GtH-II concentration, which may act via the same endocrine pathway as 17,20ß-P for milt increase and may serve to replenish milt stores depleted by spawning activity.



Pheromonal
17,20ß-P
Spawning
stimuli
Neuroendocrine
mechanism
Neuromuscular
mechanism
GtH-II
Testes/sperm ducts
Milt production

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CHAPTER 3

PHEROMONAL 17,20ß-P AND SPAWNING STIMULI ACT VIA DIFFERENT NEUROENDOCRINE MECHANISMS TO INCREASE GONADOTROPIN AND MILT VOLUME IN MALE GOLDFISH *Carassius auratus* [1]

INTRODUCTION

Ovulatory female goldfish release two distinct sex pheromones that affect male behavior and physiology, a preovulatory pheromone consisting of 17α,20ß-dihydroxy-4-pregnen-3-one and its glucuronated and sulfated metabolites (hereafter referred to as 17,20ß-P; Sorensen *et al.*, 1995b; Stacey *et al.*, 1989), and a postovulatory pheromone consisting of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolites including 15-keto-$PGF_{2\alpha}$ (This mixture of prostaglandins released by ovulated or $PGF_{2\alpha}$-injected females is hereafter referred to as PGF; Sorensen *et al.*, 1988; Sorensen and Goetz, 1993; Sorensen *et al.*, 1995a). Although both 17,20ß-P and spawning stimuli (interaction with a sexually active female releasing PGF) induce qualitatively similar increases in plasma gonadotropin-II (GtH-II) and milt (sperm and seminal fluid) volume, there is evidence (reviewed by Stacey *et al.*, 1994; Zheng and Stacey, in press) that they do so through different mechanisms. Here, I present additional evidence in support of this possibility by showing that the effects of pheromonal 17,20ß-P and spawning stimuli are disrupted differentially by pharmacological agents that influence GtH-II release.

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Evidence for different mechanisms mediating responses to pheromonal 17,20ß-P and spawning stimuli comes from a variety of studies. 17,20ß-P and PGF are detected by separate olfactory receptor mechanisms (Sorensen *et al.*, 1988). Also, 17,20ß-P and spawning stimuli have additive effects on GtH-II increase (Sorensen *et al.*, 1989) and differ in how they elicit responses, 17,20ß-P acting directly on individuals (Stacey and Sorensen, 1986; Sorensen *et al.*, 1989), and PGF acting indirectly by stimulating socio-sexual behaviors (Sorensen *et al.*, 1989). In this study, I use the term *spawning stimuli* in reference to those stimuli received by the male during interactions with a sexually active female (either ovulated or $PGF_{2\alpha}$-injected), acknowledging that it is as yet unclear which component(s) of the spawning stimuli (behavioral, pheromonal or other) increase(s) the male's blood GtH-II concentration and milt volume. The milt responses induced by 17,20ß-P and spawning stimuli also differ in several important aspects. The latency to 17,20ß-P-induced increase in milt volume (> 3 h; Dulka *et al.*, 1987b) is longer than the latency to spawning-induced increase in milt volume (< 1 h; Kyle *et al.*, 1985; Sorensen *et al.*, 1989), is influenced more by the temperature at which experiments are conducted (Zheng and Stacey, in press), and is comparable to the latency to milt increase induced by exogenous GtH injection (Zheng and Stacey, in press). Finally, it appears that the milt response to 17,20ß-P is dependent on the induced GtH-II increase, whereas the milt response to spawning stimuli is not. Support for this interpretation is that: (i) hypophysectomy blocks 17,20ß-P-induced but not spawning-induced milt increase (Dulka *et al.*, 1987; Zheng and Stacey, in press); (ii) exogenous GtH intended to swamp pheromone-induced GtH-II increase blocks 17,20ß-P-induced but not spawning-induced milt increase (Zheng and Stacey, in press); and (iii) spawning-induced milt increase can occur without observable increase in GtH-II (Kyle *et al.*, 1985; Sorensen *et al.*, 1989). Together, these differences in GtH-II and milt responses to pheromonal 17,20ß-P and

spawning stimuli suggest these two stimuli likely activate different neuroendocrine mechanisms.

In the goldfish pituitary, GtH-II release is stimulated by gonadotropin-releasing hormone (GnRH) and inhibited by dopamine (DA) (Peter *et al.*, 1991a, b). Although DA involvement in PGF-induced GtH-II increase has not been investigated, GnRH appears to be involved because male goldfish increase brain GnRH concentration during spawning with $PGF_{2\alpha}$-injected females (Yu *et al.*, 1990). In the case of 17,20ß-P-induced GtH-II increase, both DA and GnRH have been suggested to be involved. For example, 17,20ß-P exposure reduces the dihydroxyphenylacetic acid (primary DA metabolite in goldfish) / DA ratio in the pituitary (Dulka *et al.*, 1992), indicating this pheromone reduces tonic DA inhibition of GtH-II release. As well, a GnRH antagonist (analog E) blocks 17,20ß-P-induced GtH-II increase (Murthy *et al.*, 1994), suggesting the pheromone may also act by increasing endogenous GnRH release to the pituitary. In this study, I investigated the roles of GnRH and DA in 17,20ß-P-induced and spawning-induced increases in GtH-II and milt volume through the use of analog E, and also through the use of two DA type-2 (D-2) receptor agonists (bromocryptine and LY171555) which are known to inhibit GnRH-induced GtH-II release in goldfish (Chang *et al.*, 1983, 1984a, b, 1990b). The results indicate that pheromonal 17,20ß-P and spawning stimuli increase GtH-II concentration via separate neuroendocrine mechanisms, and that spawning-induced increases in GtH-II and milt volume are independently regulated events.

MATERIALS AND METHODS

Procedures common to more than one experiment are provided below, followed by details of individual experiments. To assist in the clarification and comparisons of experimental designs, the experimental layouts for the eight experiments are also summarized in Table 3.1.

Animal source

Goldfish were purchased from Ozark Fisheries Co., Inc., Stoutland, Missouri, kept for various times in 1000 or 3400-liter flowing-water tanks at 17-20 °C under a 16 L: 8 D photoperiod (lights on at 0800 h) and fed twice daily with commercial fish food (Nutrafin). Mature male goldfish, identified by the presence of expressible milt and tubercles on the pectoral fins or opercula were moved to 65-liter flowing-water tanks at 20 °C for at least two days before all experiments. All aquaria contained artificial floating vegetation and gravel, and had opaque walls except for a small window at one end for behavioral observation.

Milt volume

Milt was collected and its volume was measured as described by Kyle *et al.* (1985) and Stacey and Sorensen (1986). Briefly, fish were anesthetized with 2-phenoxyethanol (0.05%) and milt was collected using pre-weighed hematocrit tubes by applying a gentle pressure to the abdomen. Contamination of milt with urine was carefully avoided by pressing the posterior abdomen where the urinary bladder is located, and wiping the cloaca with a piece of tissue before milt sampling. Milt density is assumed to be 1.0 and milt data are expressed as volumes (µl) rather than as weight.

Reagents

Stimulus females to be used as spawning partners that would release pheromonal PGF were prepared by injecting a female intramuscularly with 5 µl of a commercial prostaglandin preparation (Lutalyse, Upjohn) containing 5 mg/ml $PGF_{2\alpha}$ tromethamine salt (Sorensen *et al.*, 1989). Female goldfish injected in this way become sexually active for several hours (Stacey and Goetz, 1982) and release an odor that rapidly induces in males behavioral responses similar to those induced by the odor from ovulated females (Sorensen *et al.*, 1986). 17,20ß-P (Sigma) was diluted in ethanol (EtOH) at a concentration of 0.1 µg/µl. At the time of pheromone exposure, 100 µl of this solution was added to the aquarium (65 liters) to give a final concentration of 17,20ß-P approximately 5×10^{-10} M (Dulka, 1987b). Bromocryptine (Sigma) was dissolved in a minimal amount of EtOH and subsequently suspended in 0.6% NaCl saline (4 mg/ml). The final concentration of EtOH was less than 0.5%. LY171555 (quinpirole hydrochloride, Research Biochemicals Incorporated) was dissolved directly in 0.6% NaCl saline (40 µg/ml). The GnRH antagonist, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-$Trp^{3,6}$]-mGnRH (termed analog E after Murthy *et al.*, 1993) was a gift from Dr. J.E. Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA) and Dr. R.E. Peter (University of Alberta), was dissolved in a primary solvent containing propylene glycol (Pg) and physiological saline at a ratio of 60:40 and subsequently diluted with saline (0.2 µg/µl). All solutions were made up immediately before use and injected intraperitoneally in a volume of 5 µl/g body weight. Control fish were given an equivalent volume of EtOH-saline (for bromocryptine experiments), saline (for LY171555 experiments) or Pg-saline (for analog E experiments).

GtH-II assay

Serum GtH-II was measured by a validated carp GtH-II specific radioimmunoassay (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992). Blood samples were taken from the caudal vasculature of anesthetized fish with a 25-gauge needle. Samples were put on ice to clot for about 4 hours. After 10 minutes of centrifugation at 10,000 x *g*, serum was separated and stored at minus 20 °C. All samples were measured for GtH-II concentration within two months.

Data analyses

Initial inspection of the GtH-II data revealed heterogeneity of variance among treatment groups. Thus, GtH-II data were log transformed and analyzed by one factor ANOVA followed by Fisher's least significance (LSD) with P set at 0.05. In Experiment 6, the difference between GtH-II concentrations of control and experimental (17,20ß-P-exposed or spawning) males, also were analyzed by t-test. Milt data were analyzed in two steps. First, to ensure groups had equivalent initial milt volumes ($P > 0.05$), pre-treatment milt volumes were analyzed by Kruskal-Wallis nonparametric ANOVA. Milt volumes for post-treatment were compared by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons test.

Experiment 1-4: Effect of Bromocryptine on 17,20β-P-induced and spawning-induced increases in GtH-II and milt volume.

Experiment 1 was conducted to test whether bromocryptine blocks 17,20ß-P-induced GtH-II increase. Fish were randomly placed in trios in 65-liter flow-through aquaria on day 1. At 2000 h on day 3, fish were stripped of milt, and injected with 20 µg/g (same as Chang *et al.*, 1984b) bromocryptine (n = 24) or EtOH-saline (n = 24). Two hours after injection, water was turned off, and half of the fish from each injection

treatment were exposed to 17,20ß-P (5×10^{-10} M) while the other half were exposed to an equivalent concentration of EtOH. At 2400 h, 4 h after injection and 2 h after initial 17,20ß-P exposure, fish were anesthetized and bled. Milt was not taken in this experiment.

In Experiment 2, fish were again randomly distributed in trios in 65-liter flow-through aquaria on day 1. At 2000 h on day 3, fish were stripped of milt, and injected with 20 µg/g bromocryptine (n = 18) or EtOH-saline (n = 18). Two hours later, water supplies were turned off, and half of the fish from each injection treatment were exposed to 17,20ß-P (5×10^{-10} M) while the other half were exposed to an equivalent concentration of EtOH. At 0800 h on day 4, 12 h after injection and 10 h after initial 17,20ß-P exposure, fish were anesthetized, bled and again stripped of milt.

Experiment 3 was conducted to determine whether bromocryptine will block spawning-induced increases in GtH-II and milt volume over the brief experimental time-course used in Experiment 1. On day 1, fish were randomly distributed in trios in 65-liter flow-through aquaria. At 2000 h on day 3, fish were anesthetized, stripped of milt, and injected with 20 µg/g bromocryptine (n = 24) or EtOH-saline (n = 24). At 2200 h, two PGF2α-injected females were added to half of the aquaria containing bromocryptine-injected males and half of the aquaria containing control males. No additional fish were added to the remaining aquaria. All aquaria were observed periodically to ensure that spawning or chasing behavior was occurring. At 2400 h, 4 h after injection and 2 h after adding females, fish were anesthetized, bled and again stripped of milt.

Experiment 4 was conducted to determine whether bromocryptine inhibits spawning-induced GtH-II and milt increases over the extended experimental time-course used in Experiment 2. On day 1, fish were randomly distributed in trios in 65-liter flow-through aquaria. At 2000 h on day 3, fish were anesthetized, stripped of milt, and injected with 20 µg/g bromocryptine (n = 33) or EtOH-saline (n = 18). At 0600 h on day

4, two PGF2α-injected females were added to six of the aquaria containing bromocryptine-injected males and three of the aquaria containing control males. No additional fish were added to the remaining aquaria. All aquaria were observed periodically to ensure that spawning or chasing behavior was occurring. At 0800 h on day 4, 12 h after injection and 2 h after adding females, fish were anesthetized, bled and again stripped of milt.

Experiment 5 and 6: Effect of LY171555 on 17,20β-P-induced and spawning-induced increases in GtH-II and milt volume.

Experiment 5 was conducted to determined whether 17,20β-P-induced increases in GtH-II and milt volume could be inhibited by the dopamine D-2 receptor agonist, LY171555. Fish were randomly distributed into 65-liter flow-through aquaria (3 or 4 fish in each) on day 1. At 2000 h on day 3, fish were stripped of milt, and injected with 0.2 μg/g LY171555 (n = 24) or with saline (n = 16). This dose of LY171555 was chosen because a preliminary experiment indicated that males placed with PGF2α-injected females showed normal spawning behavior when injected at 0.2 μg/g or 2 μg/g but failed to perform spawning behavior when injected at 20 μg/g. At 2200 h, water was turned off, and half of the fish from each injection treatment group were exposed to 17,20β-P (5×10^{-10} M) while the other half were exposed to an equivalent concentration of EtOH. At 0800 h on day 4, 12 h after injection and 10 h after initial 17,20β-P exposure, fish were anesthetized, bled and again stripped of milt.

Experiment 6 was conducted to determine whether LY171555 could inhibit spawning-induced increases in GtH-II and milt volume. Fish were randomly distributed into 65-liter aquaria (3-4 fish in each) on day 1. At 2000 h on day 3, all fish were anesthetized, stripped of milt, and injected with 0.2 μg/g LY171555 (n = 16) or with saline (n = 13). At 2200 h, eight LY171555-treated fish and seven saline-treated fish

received PGF2α-injected females (two PGF2α-females per tank). All aquaria were observed periodically to ensure that spawning or chasing behavior was occurring. At 2400 h, 4 h after injection and 2 h after adding females, all fish were anesthetized, bled and again stripped of milt.

Experiment 7 and 8: Effect of analog E on 17,20β-P-induced and spawning-induced increases in GtH-II and milt volume.

Experiment 7 was conducted to confirm the ability of analog E to block the 17,20ß-P-induced GtH-II increase (Murthy *et al.*, 1994) and determine whether increase in milt vo[illegible]e also is inhibited. Spermiating male goldfish (n = 40) were randomly distributed into 65-liter aquaria with 3 or 4 per tank on day 1. At 2100 h on day 3, all fish were anesthetized, stripped of milt, and injected with 1 µg/g (same as Murthy *et al.*, 1994) analog E (n = 20) or with Pg-saline (n = 20). At 2200 h, half of the fish from each injection treatment were exposed to 5 x 10^{-10} M 17,20ß-P and the other half from the same treatment were exposed to an equivalent concentration of EtOH. At 0800 h, 11 h after injection and 10 h after initial 17,20ß-P exposure, all fish were anesthetized, bled and again stripped of milt.

Experiment 8 was conducted to determine whether analog E could block spawning-induced increases in GtH-II and milt volume. Spermiating male goldfish (n = 40) were randomly distributed into the 65-liter flow-through aquaria with 3 or 4 per tank on day 1. At 2100 h on day 3, all fish were anesthetized, stripped of milt, and injected with either 1 µg/g analog E (n = 20) or an equivalent volume of Pg-saline (n = 20). At 2200 h, half of the fish from each injection treatment were induced to spawn by adding two PGF2α-injected females. No females were added to the control tanks. All aquaria were observed periodically to ensure that spawning or chasing behavior was occurring.

At 2400 h, 3 h after injection and 2 h after spawning, all fish were anesthetized, bled and again stripped of milt.

RESULTS

Experiments 1-4: Effect of bromocryptine on 17,20ß-P-induced and spawning-induced increases in GtH-II and milt volume.

In Experiments 2, 3 and 4, milt volumes at 0 h were equivalent among groups (Kruskal-Wallis ANOVA; $P > 0.05$; data not shown). Milt was not collected in Experiment 1.

In Experiments 1 and 2, serum GtH-II concentrations were significantly greater in control (EtOH-saline injected) males 2 h (Fig. 3.1) and 10 h (Fig. 3.2) after 17,20ß-P exposure. Bromocryptine-injection blocked these 17,20ß-P-induced GtH-II increases, and also blocked the significant increase in milt volume observed in control fish 10 h after 17,20ß-P exposure (Fig. 3.2).

Spawning with PGF2α-injected females for 2 h significantly increased serum GtH-II and milt volume in both saline-injected males and bromocryptine-injected males, regardless of whether bromocryptine was injected 2 h before (Experiment 3, Fig. 3.3) or 10 h before (Experiment 4, Fig. 3.4) placement with PGF2α-injected females. Although spawning behavior was not quantified, bromocryptine-injected males showed apparently normal spawning behavior with PGF2α-injected females.

Experiments 5 and 6: Effect of LY171555 on 17,20β-P-induced and Spawning-induced GtH-II and Milt Increases.

In Experiments 5 and 6, milt volumes at 0 h were equivalent among groups (Kruskal-Wallis ANOVA; $P > 0.05$; data not shown).

In Experiment 5, exposure to 17,20ß-P for 10 h significantly increased serum GtH-II concentrations and milt volumes in saline-injected but not in LY171555-injected males (Fig. 3.5).

In Experiment 6, spawning with PGF2α-injected females significantly increased milt volume in both saline-injected and LY171555-injected males (Fig. 3.6). However, because LY171555 significantly ($P < 0.05$) reduced GtH-II concentration in control (non-spawning) males, treatment effects on GtH-II were evaluated by *t*-test, comparing data of control and spawning males within either the saline-injected or LY171555-injected groups. Spawning stimuli significantly ($P < 0.05$) increased GtH-II concentration in LY171555-injected fish, but not in saline-injected fish ($P = 0.059$).

Experiments 7 and 8: Effect of GnRH antagonist (analog E) on 17,20β-P-induced and spawning-induced increases in GtH-II and milt volume.

In Experiments 7 and 8, milt volumes at 0 h were equivalent among groups (Kruskal-Wallis ANOVA; $P > 0.05$; data not shown).

In Experiment 7, serum GtH-II concentrations and milt volumes were significantly increased 10 h after 17,20ß-P exposure in control (Pg-saline-injected) males (Fig. 3.7). Injection of analog E blocked these 17,20ß-P-induced increases in GtH-II and milt volume.

In Experiment 8, both analog E-injected males and control males showed normal spawning behavior in response to PGF2α-injected females during the whole experimental period. Spawning with PGF2α-injected females for 2 h significantly increased serum

GtH-II in saline-injected males but not analog E-injected males. In contrast, spawning with PGF2α-injected females significantly increased milt volume in both saline-injected and analog E-injected males (Fig. 3.8).

DISCUSSION

Consistent with other studies (Stacey *et al.*, 1989; Stacey and Sorensen, 1986), 17,20ß-P pheromone increased serum GtH-II and strippable milt volume in male goldfish. However, these pheromonal effects were completely abolished by dopamine D-2 receptor agonists, bromocryptine and LY171555. In goldfish, dopamine has been demonstrated to be a GtH-II release inhibitor (Chang and Peter, 1983; Chang *et al.*, 1984a) which acts via D-2 receptors (Chang *et al.*, 1990b; Omeljaniuk *et al*, 1987). It has also been reported that a rapid reduction of DA turnover in the pituitary occurs during 17,20ß-P-induced GtH-II release in male goldfish (Dulka *et al*, 1992). The results from this study provide additional evidence that 17,20ß-P-induced GtH-II release in male goldfish is mediated through dopamine D-2 receptors. The inhibitory effects of both bromocryptine and LY171555 on milt volume also indicate that 17,20ß-P-induced milt increase in male goldfish is mediated via endogenous GtH-II increase. It is also found that LY171555 inhibits 17,20ß-P-induced GtH-II and milt increase in the common carp, *Cyprinus carpio* (Zheng and Stacey, unpublished results).

The failure of bromocryptine and LY171555 to inhibit the increases of serum GtH-II and milt volume induced by spawning behavior in male goldfish indicates that GtH-II release induced by spawning stimuli is regulated by a mechanism different from that mediating the effect of 17,20ß-P pheromone. Different mechanisms also are suggested by the fact that GtH-II response to 17,20ß-P does not differ with time of day

whereas response to spawning stimuli tends to be greater during the scotophase (Dulka *et al.*, 1987a), and that 17,20ß-P and spawning stimuli have additive effects on GtH-II concentration (Sorensen *et al.*, 1989). Because the rapid spawning-induced milt increase in goldfish appears to be GtH-independent (i.e., it is not blocked by hypophysectomy, and can occur without concurrent GtH-II increase; Zheng and Stacey, in press), it perhaps is not surprising that it is not inhibited by bromocryptine or LY171555. However, the failure of bromocryptine and LY171555 to inhibit spawning-induced serum GtH-II increase suggests this response is not mediated by dopamine withdrawal.

Regulation of GtH-II release in goldfish is under a dual control of stimulatory effects of GnRH and a strong inhibitory tone of DA (Peter *et al.*, 1991a, b). The fact that bromocryptine and LY171555 did not block spawning stimuli-induced increases in blood GtH-II and milt volume suggests that spawning stimuli may operate via a DA-independent mechanism. Although many factors are known to stimulate GtH-II release in goldfish (norepinephrine, serotonin, γ-aminobutyric acid, neuropeptide Y, Peter *et al.*, 1991b; inhibin and activin, Ge *et al.*, 1992), the inhibitory effect of analog E on spawning-induced GtH-II increase in this study suggests that GnRH is likely the main factor mediating the effect of spawning stimuli. Indeed, male goldfish accompanying spawning females show a rapid depletion and repletion of brain GnRH concentration (Yu and Peter, 1990; Yu *et al.* 1991) suggesting spawning stimuli do induce GnRH release. In goldfish, a stimulatory GnRH analog is able to stimulate GtH-II release in goldfish injected with bromocryptine at 20 μg/g (as in this study) although the effect was greatly reduced (Chang *et al.*, 1984b). Therefore, if spawning stimuli increase GnRH release, they might be expected to elevate blood GtH-II concentrations in bromocryptine-injected fish. Although dopamine is a potent inhibitor of GtH-II secretion, it appears to be unable to block all the stimulatory pathways of GtH-II secretion. Evidence that dopamine only partially reduces the acute stimulatory effect of inhibin and activin on GtH-II release (Ge

et al., 1992) and does not block the stimulatory effect of arachidonic acid on GtH-II secretion (Chang *et al.*, 1991) strongly suggests that there exist other GtH-II releasing mechanisms independent of dopamine inhibition.

The inhibition by the GnRH antagonist (analog E) of 17,20β-P-induced increases in GtH-II and milt volume not only suggests that GnRH is involved in the regulation of 17,20β-P action, but also provides further evidence that 17,20β-P-induced increase in milt volume is GtH-II dependent. Although the mechanism mediating the effect of 17,20β-P on GtH-II release is not entirely clear, evidence suggests that 17,20β-P exerts this effect by decreasing the relea[illegible]mine (Dulka *et al.*, 1992), which has inhibitory effects on GnRH terminals and gonadotrophs (Chang, *et al.*, 1990b; Yu and Peter, 1992). It is also possible that 17,20β-P might stimulate GnRH release through other mechanisms because some other neurotransmitters (norepinephrine, serotonin, γ-aminobutyric acid, neuropeptide Y) are known to stimulate GnRH release (Peter *et al.*, 1991b). However, it is possible that 17,20β-P is not as potent as spawning stimuli for inducing GnRH release since exposure of male goldfish to 17,20β-P for 12 h significantly increases serum GtH-II concentrations, but does not alter GnRH concentrations in the telencephalon, hypothalamus and pituitary (Dulka, 1989). In contrast, exposure to spawning females for 1 or 2 h significantly increases brain GnRH concentration (Yu and Peter, 1990). Also, exposure to 17,20β-P does not increase serum GtH-II in male goldfish injected with α-methyl-*p*-tyrosine methyl ester which depletes brain and pituitary dopamine level (Dulka, 1989; Zheng W., Murthy, C.K. and Stacey, N., unpublished results), suggesting that 17,20β-P stimulates GtH-II release primarily by decreasing DA inhibition, an effect similar to that of DA antagonist (e.g., pimozide and domperidone, Omeljaniuk *et al.*, 1987). The simplest interpretation of the observed analog E block of 17,20β-P-induced GtH-II increase may be that the GnRH antagonist prevents the stimulatory action of tonic, basal GnRH secretion, thus

preventing the GtH-II increase that normally occurs when 17,20ß-P reduces DA inhibition.

In this study, spawning-induced GtH-II increase was not blocked by DA agonists, but was blocked by the GnRH antagonist, analog E, indicating that spawning stimuli increase GtH-II primarily through release of endogenous GnRH. Furthermore, the fact that spawning-induced increase in milt volume was not blocked by analog E is consistent with the earlier proposal (Zheng and Stacey, in press) that spawning-induced increase in milt volume is GtH-II-independent

The functional interaction between pheromonal stimuli and GnRH systems is also supported by neuroanatomical studies. Two native forms of GnRH, salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II), are found in the goldfish brain and pituitary (Yu *et al.*, 1988); however, this study did not attempt to determine whether one or both of these peptides mediates responses to pheromonal 17,20ß-P and spawning stimuli. There is evidence that both sGnRH and cGnRH-II are involved in the regulation of GtH-II release because both sGnRH and cGnRH-II cell bodies have a similar distribution in the goldfish brain and pituitary (Kah *et al.*, 1986; Kim *et al.*, 1995), Also, both of the GnRH peptides stimulate GtH-II synthesis (Khakoo *et al.*, 1994) and release (Chang *et al.*, 1990a), although cGnRH-II has been found to be more potent than sGnRH in stimulating GtH-II release *in vitro* (Chang *et al.*, 1990a; Habibi, 1991). Both sGnRH and cGnRH-II neurons are found in the terminal nerve, the ventral telencephalon, the preoptic area (POA) and the hypothalamus; cGnRH-II cell bodies also are found in the midbrain tegmentum (Kim *et al.*, 1995).

The ventral telencephalon and the POA are the terminal fields of the medial olfactory tract (MOT; reviewed by Dulka, 1993) which has been shown to mediate pheromonal effects on spawning behavior and GtH-II release (Dulka and Stacey, 1991; Sorensen *et al.*, 1991; Stacey and Kyle, 1983). The GnRH neurons located in the ventral

floor of the POA appear to play an important role in pheromone-stimulated GtH-II release because both GnRH and dopamine neurons located in the POA project directly to the proximal pars distalis of the pituitary and innervate gonadotrophs (Kah *et al.*, 1986, 1987). Brain lesion studies demonstrate that the ventral telencephalon is involved in the direct control of male spawning behavior (Kyle and Peter, 1982; Kyle *et al.*, 1982). It therefore has been suggested (Dulka, 1993) that the GtH-II and milt responses to pheromonal 17,20ß-P is mediated by the MOT projections to the POA, whereas the spawning response to pheromonal PGF is mediated by the MOT projections to the ventral telencephalon.

This proposal is consistent with the present observation that pheromonal 17,20ß-P and PGF/spawning activity act via different neuroendocrine mechanisms to stimulate GtH-II release. Pheromonal 17,20ß-P acts on olfactory receptors (Rosenblum *et al.*, 1991), and this input is conveyed by the medial olfactory tract to the POA where it reduces dopaminergic input to the pituitary. This 17,20ß-P input may also stimulate the GnRH neurons located in the POA. On the other hand, input of pheromonal PGF stimulates the GnRH neurons in the ventral telencephalon, and activates spawning behavior. Spawning stimuli may in turn provide other sensory inputs (e.g., visual, tactile) to stimulate brain GnRH release and sperm release. Evidence supporting this possibility is the demonstration that stimulation of goldfish optic nerve induces sperm release (Demski and Dulka, 1984). In addition, spawning stimuli may provide tactile input to the terminal nerve (Fujita *et al.*, 1991) which sends a great number of sGnRH fibers to the brain areas (Kim *et al.*, 1995). In summary, the evidence suggests that PGF/spawning stimulates GnRH neurons anatomically different from those mediating pheromonal 17,20ß-P input. However, it remains to be determined which GnRH form is involved, and whether the increase in brain GnRH induced by PGF/spawning (Yu and

Peter, 1990) shares the same final pathway with 17,20ß-P, or acts via a different pathway to stimulate GtH-II release.

In conclusion, this study demonstrates: 1) pheromonal 17,20ß-P and spawning activity stimulate GtH-II increase in goldfish via distinct neuroendocrine mechanisms, 2) the effect of pheromonal 17,20ß-P on milt increase is GtH-II-dependent, and 3) the effect of spawning activity on milt increase is GtH-II-independent.

Based on the results from this and previous studies, a model for neuroendocrine mechanisms mediating the effects of pheromonal 17,20ß-P and PGF on GtH-II release is proposed (Fig. 3.9). During the preovulatory period, 17,20ß-P released from female goldfish acts via the male olfactory system to reduce DA release, which results in an increase of GtH-II release by decreasing tonic DA inhibition of D-2 receptors on gonadotrophs (Chang, *et al.*, 1990b) as well as D-1 receptors on GnRH terminals (Yu and Peter, 1992). It is also possible that 17,20ß-P may stimulate GtH-II release by directly increasing GnRH release since the effect of 17,20ß-P on GtH-II release is blocked by GnRH antagonist. At ovulation and the onset of spawning activity, female goldfish stop releasing 17,20ß-P and start releasing PGF, which increases sexual arousal in the males (Stacey *et al.*, 1994). Pheromonal PGF and spawning cues stimulate GtH-II release primarily through GnRH release. According to this model, the additive effects of 17,20ß-P and spawning stimuli on GtH-II concentration (Sorensen *et al.*, 1989) occur because 17,20ß-P decreases the inhibitory action of DA on both GnRH terminals and gonadotrophs, at the same time that spawning stimuli are inducing endogenous GnRH release. Differential involvement of sGnRH and cGnRH-II (see discussion above) may also contribute to the two different mechanisms mediating GtH-II release.

TABLE 3.1. The list of the experimental procedures for the treatments using dopamine type-2 receptor agonists and a GnRH antagonist.

Exp. #	Drug Injected	Pheromone Studied	Procedure at Time (h)							
			0	1	2	3	4	10	11	12
1	Bromocryptine	17,20β-P	inject (strip)[a]		17,20β-P		(GtH)[a]			
2	Bromocryptine	17,20β-P	inject (strip)		17,20β-P					(GtH, milt)
3	Bromocryptine	PGF	inject (strip)		PGF female		(GtH, milt)			
4	Bromocryptine	PGF	inject (strip)					PGF female		(GtH, milt)
5	LY171555	17,20β-P	inject (strip)		17,20β-P					(GtH, milt)
6	LY171555	PGF	inject (strip)		PGF female		(GtH, milt)			
7	Analog-E	17,20β-P	inject (strip)	17,20β-P					(GtH, milt)	
8	Analog-E	PGF	inject (strip)	PGF female		(GtH, milt)				

[a] = samples in parentheses

Figure 3.1. Inhibitory effect of bromocryptine on 17,20ß-P-induced GtH-II increase. Fish were injected with 20 μg/g bromocryptine or EtOH-saline at 0 h, exposed to 5×10^{-10} M 17,20ß-P or EtOH at 2 h, and bled at 4 h. Letters not joined by underlining indicate groups that differ significantly ($P < 0.05$). n = 12 in all groups.

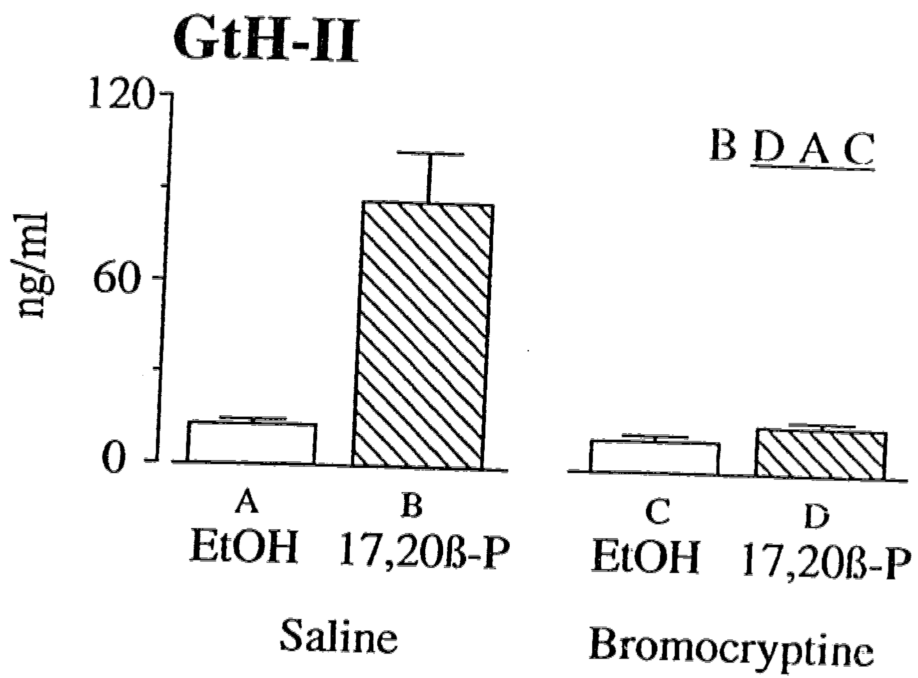
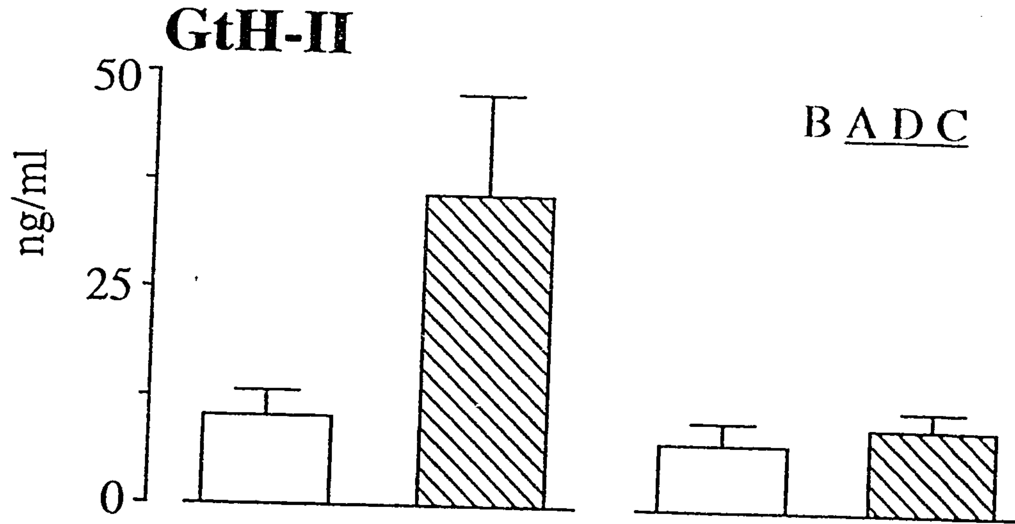

GtH-II
120
60
0
ng/ml
B D A C
A
EtOH
B
17,20ß-P
C
EtOH
D
17,20ß-P
Saline
Bromocryptine

Figure 3.2. Inhibitory effect of bromocryptine on 17,20ßP-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Fish were anesthetized, stripped of milt and injected with 20 µg/g bromocryptine or EtOH-saline at 0 h, exposed to 5 x 10^{-10} M 17,20ß-P or EtOH at 2 h, bled and again stripped of milt at 12 h. Letters not joined by underlining indicate groups that differ significantly. n = 9 in all groups.


GtH-II
50
25
0
ng/ml
B A D C

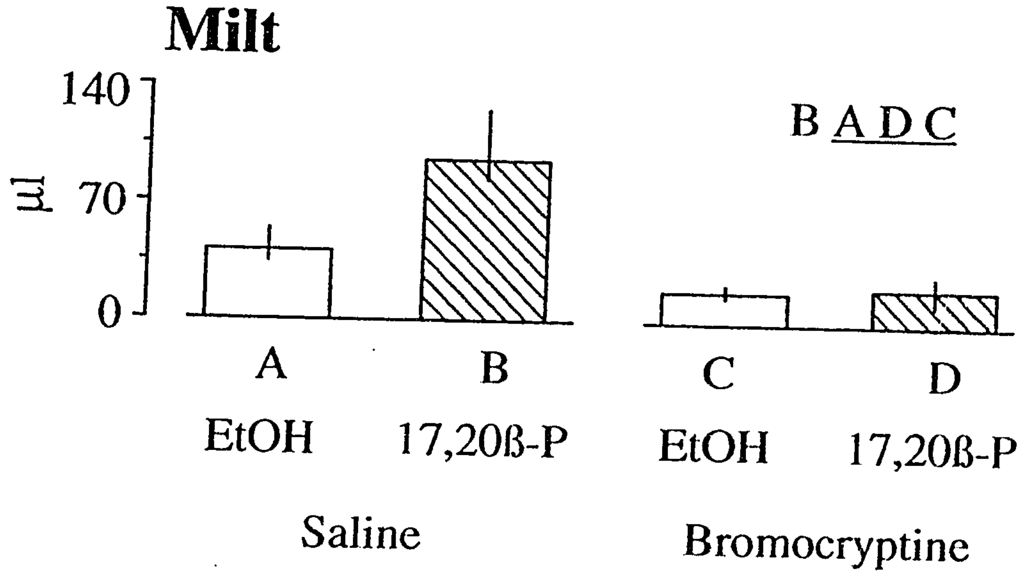
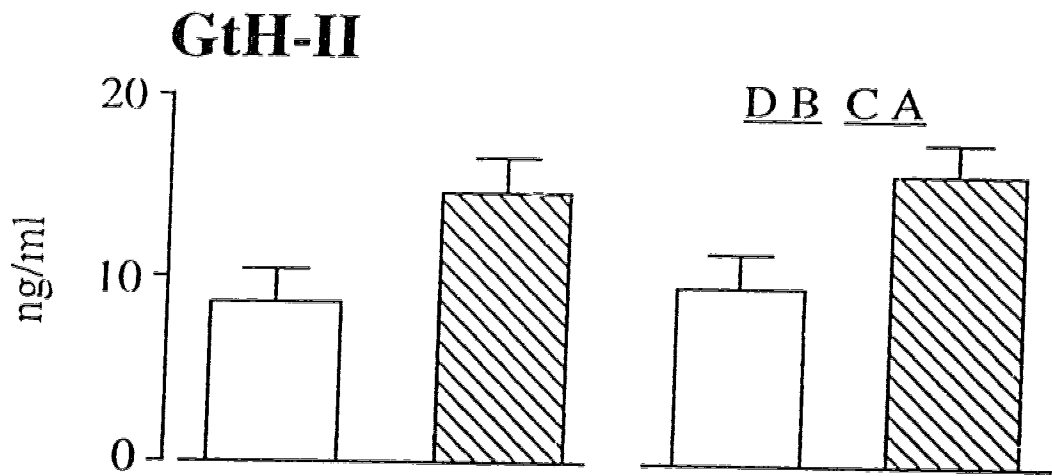

Milt
140
70
0
µl
B A D C
A
B
C
D
EtOH
17,20ß-P
EtOH
17,20ß-P
Saline
Bromocryptine

Figure 3.3. Bromocryptine does not block spawning-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Fish were anesthetized, stripped of milt and injected with either 20 µg/g bromocryptine or EtOH-saline at 0 h. Spawning males were placed with $PGF_{2\alpha}$-injected females at 2 h and control males were untreated. At 4 h, all males were bled and again stripped of milt. Letters not joined by underlining indicate groups that differ significantly. n = 12 in all groups.


GtH-II
20
10
0
ng/ml
D B C A

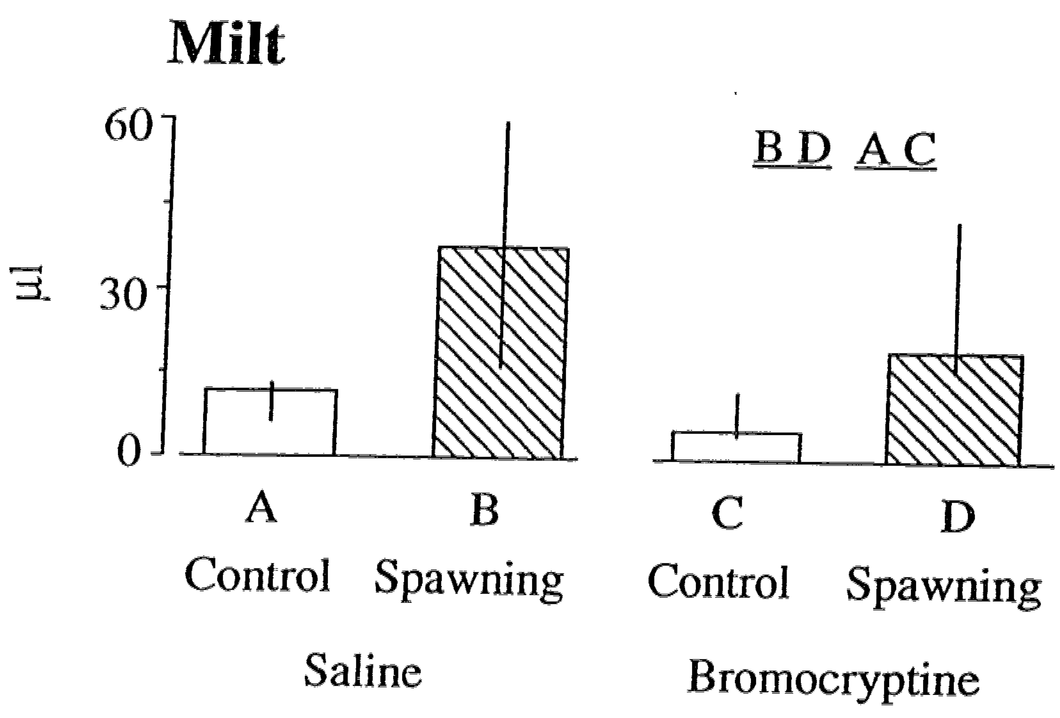
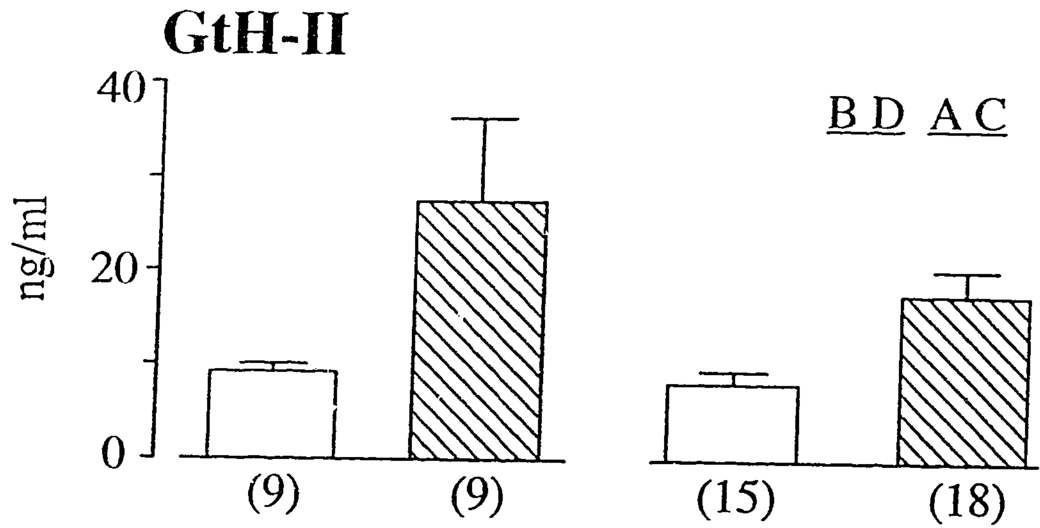

Milt
60
30
0
µl
B D A C
A
B
C
D
Control
Spawning
Control
Spawning
Saline
Bromocryptine

Figure 3.4. Bromocryptine does not block spawning-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Fish were anesthetized, stripped of milt and injected with either 20 μg/g bromocryptine or EtOH-saline at 0 h. Spawning males were placed with $PGF_{2\alpha}$-injected females at 10 h and control males were untreated. At 12 h, males were bled and again stripped of milt. Letters not joined by underlining indicate groups that differ significantly. Sample sizes are indicated below GtH-II data.


GtH-II
40
20
0
ng/ml
B D A C
(9)
(9)
(15)
(18)

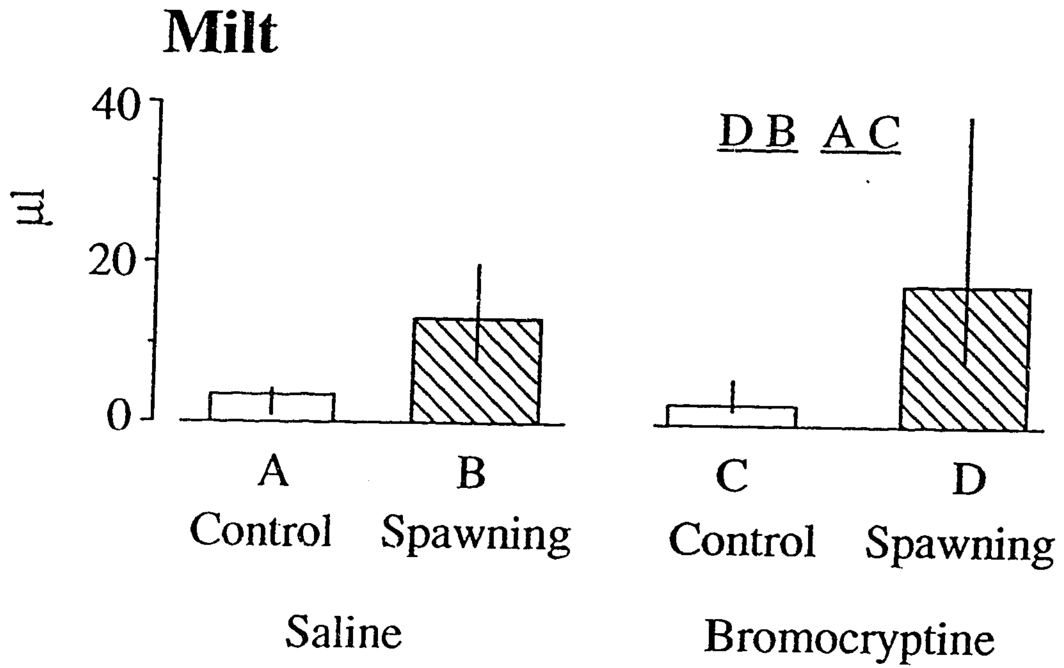
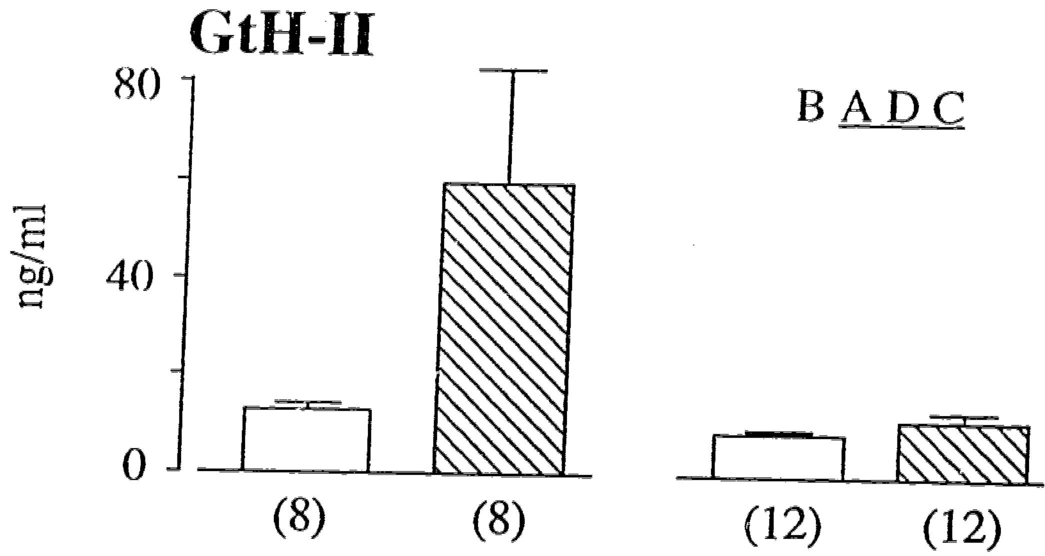

Milt
40
20
0
µl
D B A C
A
Control
B
Spawning
C
Control
D
Spawning
Saline
Bromocryptine

Figure 3.5. Inhibitory effect of LY171555 on 17,20ß-P-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Fish were anesthetized, stripped of milt and injected with either LY171555 (0.2 μg/g) or saline at 0 h, exposed to 5×10^{-10} M 17,20ß-P or EtOH at 2 h, bled and again stripped of milt at 12 h. Letters not joined by underlining indicate groups that differ significantly. Sample sizes are indicated below GtH-II data.


GtH-II
80
40
0
ng/ml
B A D C
(8)
(8)
(12)
(12)

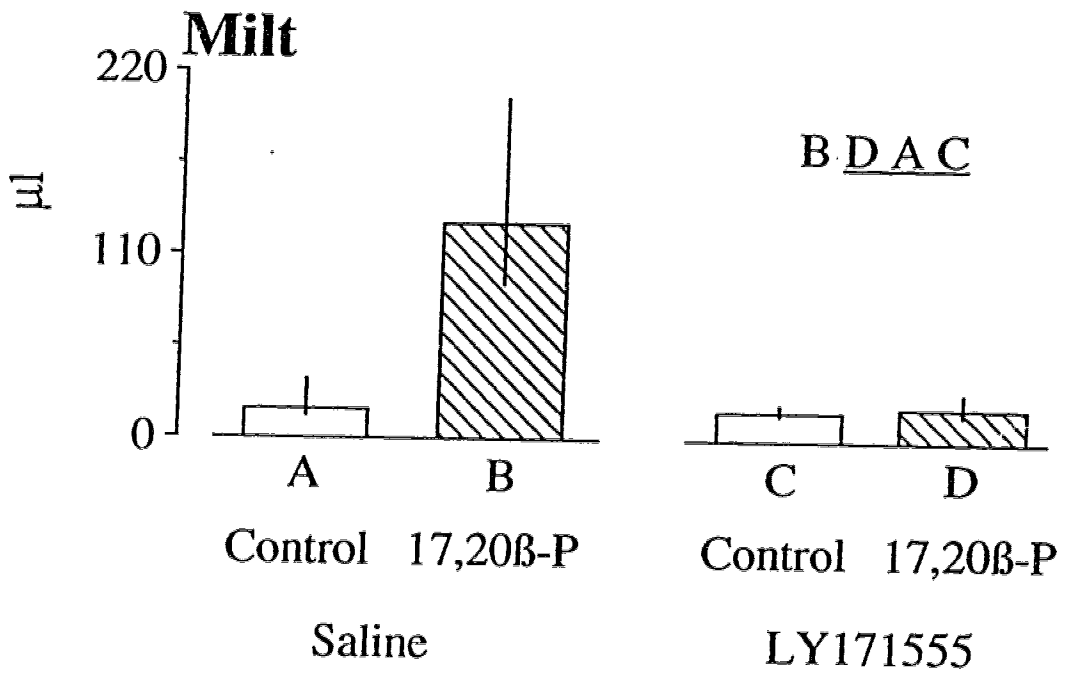
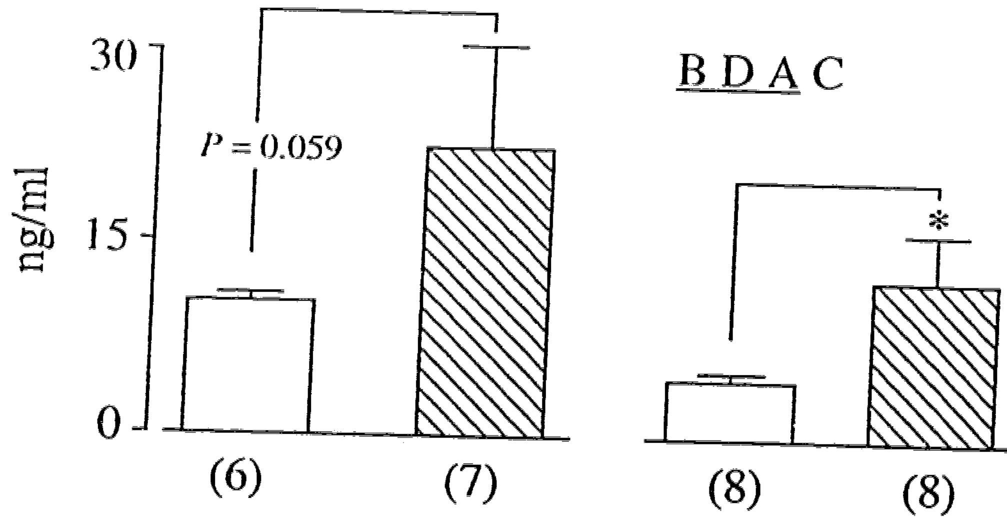

Milt
220
110
0
µl
B D A C
A
B
C
D
Control
17,20ß-P
Control
17,20ß-P
Saline
LY171555

Figure 3.6. LY171555 does not block spawning-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Fish were anesthetized, stripped of milt and injected with either LY171555 (0.2 µg/g) or saline at 0 h. Spawning males were placed with $PGF_{2\alpha}$-injected females at 2 h and control males were untreated. At 4 h, all males were bled and again stripped of milt. Letters not joined by underlining indicate groups that differ significantly. Comparison between spawning and non-spawning males within each injection group was also analyzed by t-test. * = $P < 0.05$. Sample size are indicated below GtH-II data.


GtH-II
30
15
0
ng/ml
P = 0.059
B D A C
*
(6)
(7)
(8)
(8)

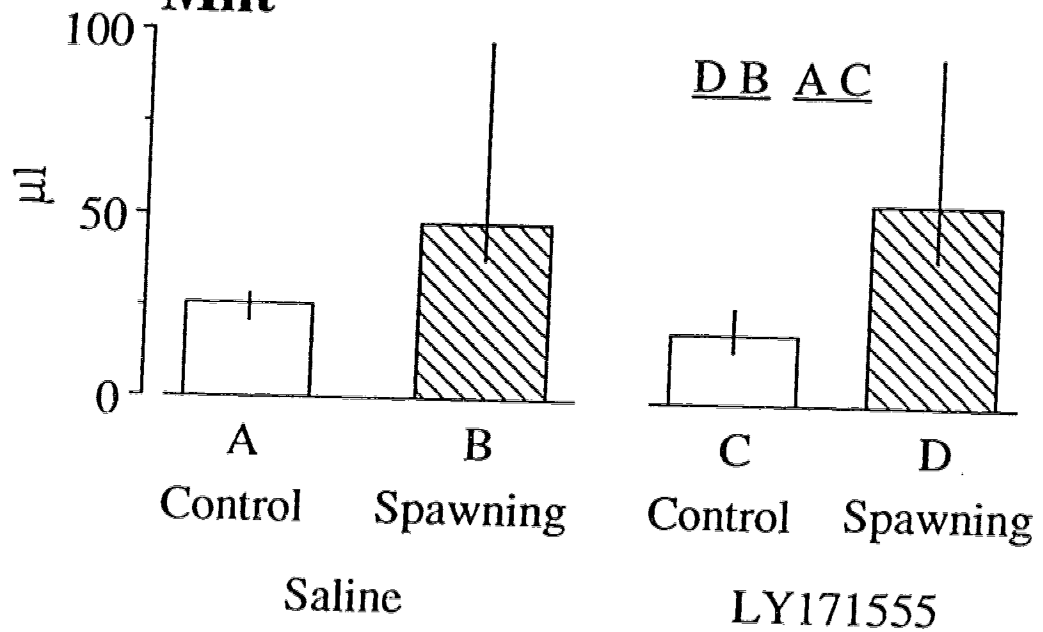

Milt
100
50
0
µl
D B A C
A
B
C
D
Control
Spawning
Control
Spawning
Saline
LY171555

Figure 3.7. Inhibitory effect of a GnRH antagonist (analog E) on 17,20ß-P-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Fish were anesthetized, stripped of initial milt and injected with either analog E ([Ac-Δ^3-Pro^1, 4FD-Phe^2, D-$Trp^{3,6}$]-mGnRH; 1 µg/g) or Pg-saline at 0 h, exposed to 5×10^{-10} M 17,20ß-P or EtOH at 1 h, bled and again stripped of milt at 11 h. Letters not joined by underlining indicate groups that differ significantly. n = 10 in all groups.

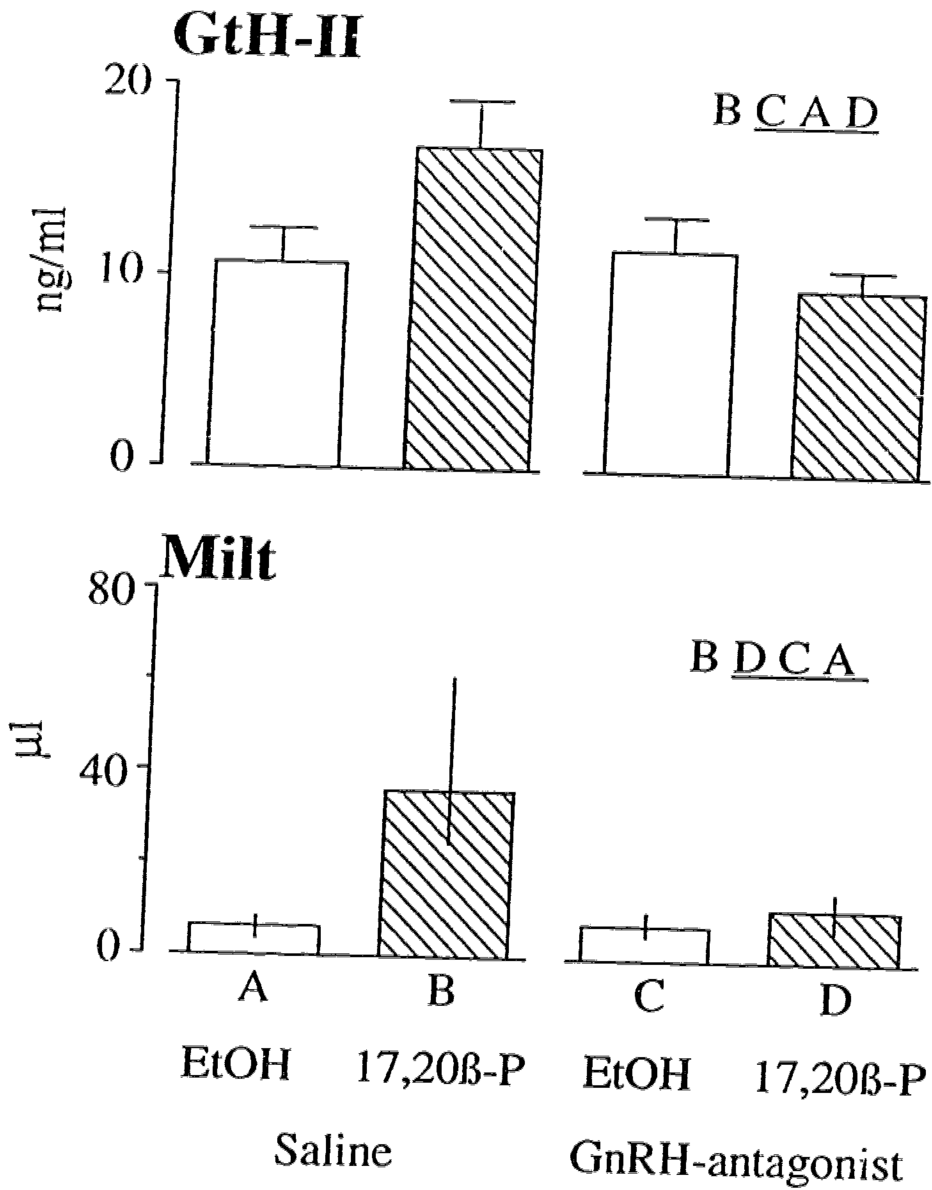

GtH-II
20
10
0
ng/ml
B C A D
Milt
80
40
0
µl
B D C A
A
B
C
D
EtOH
17,20ß-P
EtOH
17,20ß-P
Saline
GnRH-antagonist

Figure 3.8. Effect of analog E on spawning-induced increases in GtH-II (mean + s.e.m.) and milt volume (median ± quartile ranges). Spermiating males were anesthetized, stripped of milt and injected with either 1 μg/g analog E ([Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH) or Pg-saline at 0 h. Spawning males were placed with $PGF_{2\alpha}$-injected females at 1 h and control males were untreated. At 3 h, males were bled and again stripped of milt. Letters not joined by underlining indicate groups that differ significantly. n = 10 in all groups.

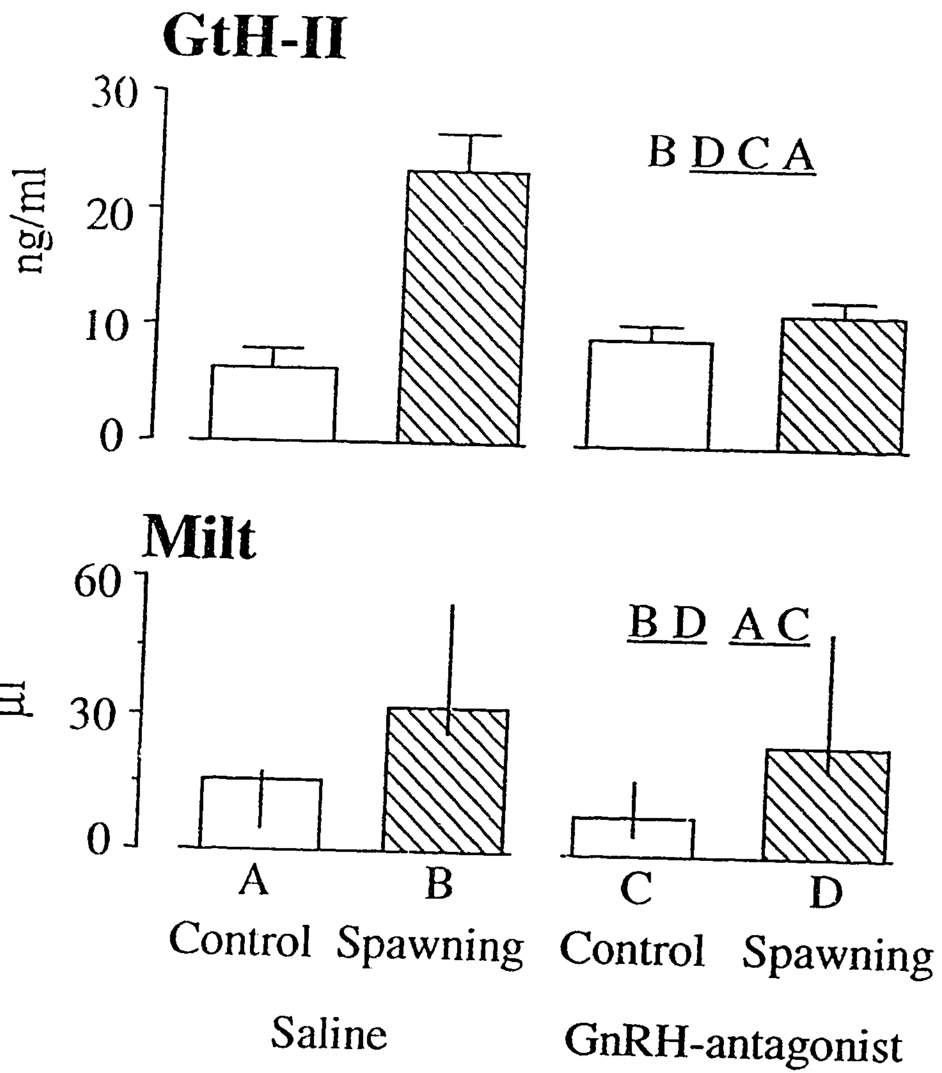
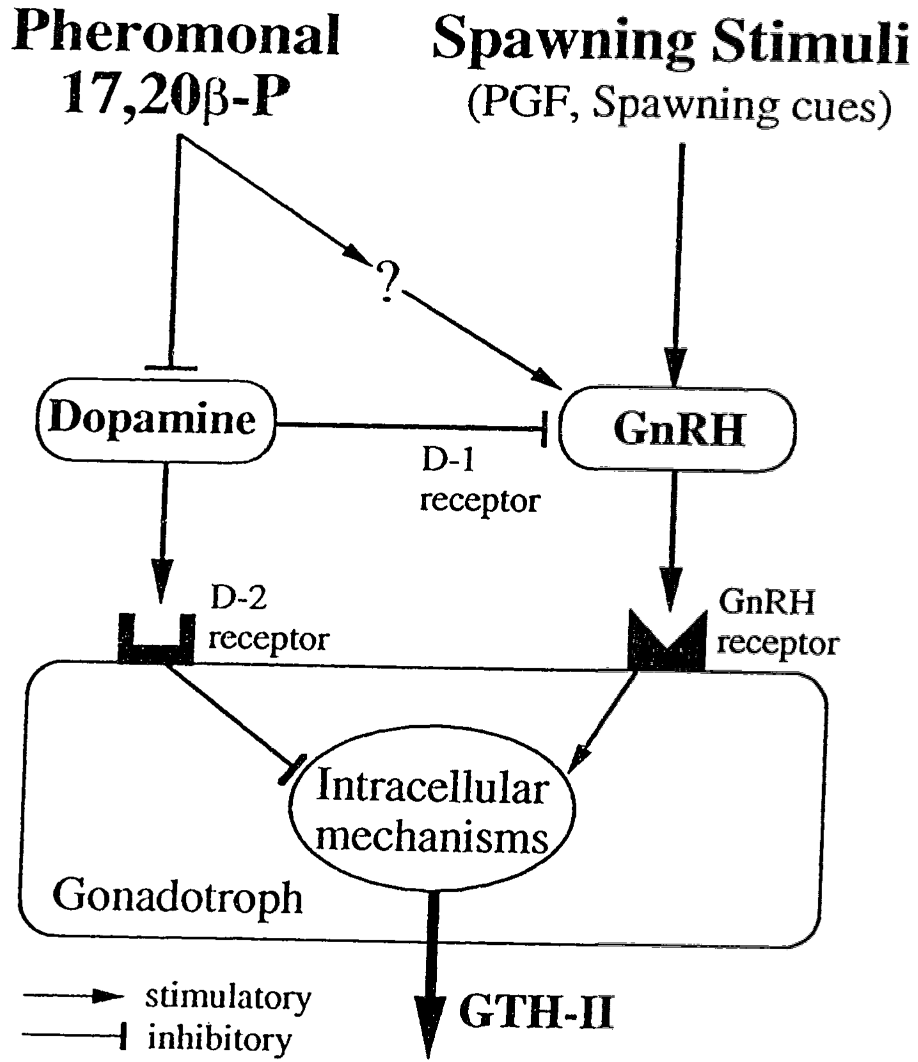

GtH-II
30
20
10
0
ng/ml
B D C A
Milt
60
30
0
µl
B D A C
A
B
C
D
Control
Spawning
Control
Spawning
Saline
GnRH-antagonist

Figure 3.9. A proposed model for different neuroendocrine mechanisms mediating the effects of pheromonal 17,20ß-P and PGF/spawning on GtH-II release. Pheromonal 17,20ß-P acts on specific olfactory receptors, resulting in an increase of GtH-II concentration by decreasing tonic DA inhibition of D-2 receptors on gonadotrophs as well as D-1 receptors on GnRH terminals. 17,20ß-P may also stimulate GtH-II release by directly increasing GnRH release (question mark). Pheromonal PGF acts on other olfactory receptors different from those detecting 17,20ß-P, eliciting sexual activity in the males. The sensory inputs from pheromonal PGF and/or other spawning cues stimulate GnRH release, which increases GtH-II release.


Pheromonal
17,20β-P
Spawning Stimuli
(PGF, Spawning cues)
?
Dopamine
GnRH
D-1
receptor
D-2
receptor
GnRH
receptor
Intracellular
mechanisms
Gonadotroph
stimulatory
inhibitory
GTH-II

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CHAPTER 4

EFFECT OF PHEROMONAL 17,20ß-P ON FERTILITY IN MALE GOLDFISH: MICROSATELLITE DNA FINGERPRINTING STUDIES

INTRODUCTION

It is well established that sex pheromones are involved in a variety of reproductive functions in fish (Liley and Stacey, 1983; Stacey, 1987). In goldfish (*Carassius auratus*), considerable evidence suggests that pheromones derived from released hormones (steroids, prostaglandins and their metabolites) play an important role in synchronizing spawning by affecting male reproductive behavior and gonadal physiology (Stacey *et al.*, 1994). During natural spawning, the final oocyte maturation-inducing steroid hormone, 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) and its glucuronated and sulfated metabolites (Scott and Sorensen, 1994; Sorensen and Scott, 1994; Sorensen *et al.*, 1995a) are released into the water by females for approximately 10 hours before ovulation. Male goldfish detecting the water-borne 17,20ß-P respond not only with a rapid increase of blood gonadotropin-II (GtH-II) within 15 minutes and milt (sperm and seminal fluid) volume within 4-6 hours (Dulka *et al.*, 1987), but also with increased swimming activity (Bjerselius *et al.*, 1995), sperm motility and spawning activity when encountering sexually receptive females (Defraipont and Sorensen, 1993). In addition, in pair (one male-one female) spawning, male goldfish previously exposed to pheromonal 17,20ß-P achieve significantly higher fertilization rates than males have not been exposed previously (Zheng *et al.*, 1995a). However, despite the convincing evidence that

exposure to pheromonal 17,20ß-P affects male hormones and increases milt volume, no study has yet determined whether these male responses result in increased fertilization rates in the multi-male spawning competitions that are typical of goldfish. Such information about 17,20ß-P effects on paternity in such a mating system should offer insight into the factors favoring evolution of such hormonal pheromone systems.

Conventional fingerprinting techniques based on multilocus minisatellites or allozymes have been used for population and paternity analysis in fish such as the sailfin molly, *Poecilia latipinna* (Laughlin and Turner, 1994) and bluegill sunfish, *Lepomis macrochirus* (Philipp and Gross, 1994). However, the application of these techniques to paternity studies is difficult when only small amounts of tissue are available for analysis. For paternity analysis in non-parental species like goldfish, which usually have high larval mortality rates, it is essential to collect larval samples from the early developmental stage for paternity analysis because collecting samples at later stages could alter paternity estimates if the offspring of different fathers have different mortality rates. Because the relatively small amount of goldfish larval tissue (< 1 mg) would require a polymerase chain reaction (PCR) based DNA fingerprinting technique for paternity studies, I have recently isolated and characterized five microsatellite loci in goldfish (Zheng *et al.*, 1995b). The aim of this study was to investigate whether males exposed to pheromonal 17,20ß-P would increase their proportion of fertilized eggs both in competitive spawning and in competitive *in vitro* fertilization using this microsatellite DNA fingerprinting.

MATERIALS AND METHODS

Experimental animals

Goldfish were purchased from Ozark Fisheries Co., Inc. (Stoutland, Missouri) in spring, 1995. Mature male goldfish (identified by the presence of expressible milt and tubercles on the pectoral fins or the opercula), and gravid females (identified by a soft distended abdomen), were separated by sex in groups of four in 65-liter flowing-water aquaria at 16-18 °C under a 16L: 8D photoperiod (light on at 0800 h) and fed at least once daily with commercial fish food (Nutrafin). Fish held in the same tank were identified by pigmentation. At least one month before experiments, blood (approximately 20 µl) was taken from the caudal vasculature of each fish during anesthetization with 2-phenoxyethanol (Syndel, Vancouver; 0.05%) for parental genotype identification.

Microsatellite analysis

DNA was extracted from blood of experimental fish and whole larval tissue using the QIAamp Tissue Kit (QIAGEN). Experimental fish and larvae samples were genotyped for goldfish locus 1 (GF1) using the established method (Zheng *et al.*, 1995b). Briefly, primers for GF1 were synthesized using DNA synthesizer (forward primer ATG AAG GGT AGG AAA AGT GTG A was labeled with the fluorescent dye TET, and reverse primer CAG GTT AGG GAG AAG AAG GAA T was not labeled). Microsatellite DNA was amplified by PCR in a 25 µl volume containing 100 ng of genomic DNA, 120 µM dNTP, 0.16 µM of each primer, 1 x *Taq* buffer, and 0.5 units of *Taq* DNA polymerase. PCR condition was similar to that previously described (Zheng *et al*, 1995b): 1 min at 94 °C followed by 2 cycles of 30 s at 94 °C, 20 s at 58 °C, and 1 s at 72 °C, and then 35 cycles of 15 s at 94°C, 20s at 58°C and 1 s at 72 °C, followed by 30 s

at 72 °C. PCR products were then resolved by electrophoresis using polyacrylamide sequencing gel. Bands were automatically sized using Genescan 672 software (ABI).

Milt volume

Milt was collected and its volume was measured as described by Kyle *et al.* (1985) and Stacey and Sorensen (1986). Briefly, fish were anesthetized with 2-phenoxyethanol (Syndel, Vancouver; 0.05%) and milt was collected using pre-weighed hematocrit tubes by applying a gentle pressure to the abdomen. Contamination of milt with urine, which affects sperm quality (Perchec *et al.*, 1995), was carefully avoided by pressing the posterior abdomen where the urinary bladder is located, and wiping the cloaca with a piece of tissue before milt sampling. Milt density is assumed to be 1.0 and milt data are expressed as volumes (µl) rather than as weight.

GtH-II assay

Serum GtH-II was measured by a validated carp GtH-II specific radioimmunoassay (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992). Blood samples (approximately 300 µl) were taken from the caudal vasculature of anesthetized fish with a 25-gauge needle. Samples were put on ice to clot for about four hours. After 10 minutes of centrifugation at 10,000 x *g*, serum was separated and stored at minus 20 °C. All samples were measured for GtH-II concentration within two months.

Experiment 1: 17,20β-P and fertility during competitive spawning

On day 1, two experimental males with distinct genotypes were chosen and placed in different 65-liter aquaria at 18 °C. To avoid the milt increase that can occur in isolated male goldfish (Stacey, 1991), two additional mature males were added to each aquarium.

On the evening of day 2, all fish were exposed to either 5×10^{-10} M 17,20ß-P (Sigma; added in 100 µl ethanol, Dulka *et al.*, 1987a) or an equivalent concentration of ethanol. At the same time, one female was injected with Ovaprim (combination of GnRH analog and dopamine antagonist; Syndel, Vancouver; 0.5 ml/kg intraperitoneally to induce ovulation) and transferred to an 18 °C 100-liter spawning tank with floating vegetation as a spawning substrate. In the early morning of day 3, the two males were transferred to the female holding aquarium after checking ovulation by gently squeezing a few ovulated eggs from the female. The entire spawning sequence was observed and the number of spawning acts performed by each male were recorded.

Goldfish spawning is accomplished by the males following the female into vegetation, flipping forward with the female and at the same time releasing sperm as she releases eggs. In trio (2 males and one female) spawning, there are three levels of male participation: 1) only one male spawns with the female; 2) both males spawn with the female, and are equally close to the female during the spawning act; 3) both males spawn with the female, but one male is closer to the female. For data analysis, the spawning male was scored 4 and the non-spawning male scored 0 in the first condition. In the second condition, both males were scored 2 and in the third condition, the male closer to the female was scored 3 and the other male scored 1.

All fish were removed when the female had stopped spawning. All fertilized eggs from the spawning tank were incubated until larvae had hatched (4-5 days) and a random sample of 72 larvae was used for paternity analysis. Such trio spawnings were conducted four times, using different males and females for each replicate. As well, two of the tested male pairs were allowed to recover for one month and then given reversed treatments, i.e., the male that was previously exposed to pheromonal 17,20ß-P served as control fish and the previous control male was exposed to 17,20ß-P.

Experiment 2: 17,20ß-P and fertility in competitive in vitro *fertilization*

Because pheromonal 17,20ß-P increases not only milt volume (Dulka *et al.*, 1987), but also male sexual activity and sperm motility (Defraipont and Sorensen, 1993), it is not clear whether the effect of 17,20ß-P on male fertility during spawning (Experiment 1) is simply due to the increased amount and/or timing of sperm release, or due to changes in sperm function. This question was addressed by determining the effects of pheromonal 17,20ß-P on male fertility in competitive *in vitro* fertilization.

Selection, handling, and pheromone exposure of males, and induced ovulation of females, were as described above. Because control males often have little strippable milt, both control and pheromone-exposed males were taken early on the morning of day 3 and placed with prostaglandin F2α (PGF)-injected females for 30 min. PGF-injected females become sexually active (Stacey, 1987) and release PGF and PGF metabolites that act as a sex pheromone triggering male sexual activity and a rapid increase in milt volume (Kyle *et al.*, 1985; Sorensen *et al.*, 1988, 1989, 1995b). Control and pheromone-exposed males were then anesthetized, urine was voided, and milt stripped by aspirating the milt into hematocrit tubes. A 50 µl aliquot of milt from each male was then mixed gently on ice, and the milt mixture was added to approximately 300 eggs in a plastic Petri dish. Fertilization was initiated by directly adding 20 ml tank water to the Petri dish. The Petri dish was topped with tank water 2 min later and the eggs incubated at room temperature. Water was changed every few hours until all fertilized eggs hatched. A sample of 72 hatched larvae was collected for paternity analysis. This experiment was conducted four times, using different sperm and egg donors for each replicate.

Experiment 3: 17,20β-P and fertility in pair spawning (one male-one female)

This experiment was conducted to determine whether 17,20ß-P exposure will increase male fertilization rates in the absence of sperm competition. Mature male goldfish were placed in two 65-liter aquaria (four fish each) on day 1. In the evening of day 3, water was turned off and the 4 fish in one tank were exposed to 5×10^{-10} M 17,20ß-P while the other 4 fish in the other tank were exposed to ethanol control. At the same time, eight gravid females held in two 65-liter aquaria were injected with 0.5 ml/kg Ovaprim to induce ovulation. On day 4, each male was allowed to spawn with one ovulated female in a 100-liter aquarium with floating vegetation. Fish were removed from the tanks when they finished spawning. Eggs on the vegetation were incubated overnight and fertilization rates were calculated by counting the proportion of eggs developed to eye-stage.

Experiment 4: 17,20β-P and fertility in pair spawning (one male-one female) during the course of spawning

Because male fertilizing ability may decrease during prolonged spawning as found in the lemon tetra *Hyphessobrycon pulchripinnus* (Nakatsuru and Kramer, 1982), this experiment was conducted to compare fertilization rates of 17,20ß-P-exposed and control males during spawning. Three groups of six mature male goldfish were placed in 65-liter tanks with 3 per tank two days before the experiment. On day 1, fish from each group were exposed to either 5×10^{-10} M 17,20ß-P ($n = 3$) or ethanol control ($n = 3$) at 22:00. At the same time, gravid females were injected with 0.5 ml/kg Ovaprim and separated into two 100-liter tanks. All tanks contained removable artificial vegetation. Next morning at

08:00, for the first group, one male from either 17,20ß-P-exposed treatment or control treatment was allowed to spawn with the ovulated female for two spawning acts; then the male was replaced by another male from the other treatment. Vegetation was also replaced every time a new male was added. The other two groups were first allowed to spawn with PGF-injected females (two females per tank) for one hour and two hours respectively, and then were allowed to spawn with the ovulated female in the same way as the first group. Eggs stuck to the vegetation were collected and incubated overnight, and the proportion developing to the eye-stage were used to determine fertilization rates. This experiment was repeated four times.

Experiment 5: 17,20ß-P and sperm concentration

Since pheromonal 17,20ß-P increases milt volume by increasing blood concentration of GtH-II (Dulka *et al.*, 1987; Zheng and Stacey, in press), it is predicted that 17,20ß-P will also result in testis hydration which is known to be induced by pituitary hormones (Clement and Grant, 1964). This experiment was conducted to determine whether 17,20ß-P affects sperm concentration and the number of total strippable sperm.

Spermiating goldfish were placed in 4 tanks and divided into two groups (three fish per tank; water temperature 18 - 20 °C) on day 1. On day 3, one group was exposed to 5×10^{-10} M 17,20ß-P (n = 6) and the other group was exposed to ethanol (n = 6) at 22:00. Next morning at 08:00, all fish were anesthetized, bled and stripped of milt. After measurement of milt volume, a 5 µl aliquot of milt from each sample was taken and diluted 1:1000 in ISOTON (Coulter Electronics of Canada Ltd., Burlington, Ontario) in which sperm cells are not motile. A 1 ml aliquot of the dilution was then taken and further diluted 1:20 in ISOTON to yield a final dilution of 20,000 x. All samples were

vortexed during dilution. Three 200 µl aliquots of this sperm dilution were then used to measure sperm concentration on a coulter counter (Multi-Sizer, Coulter Electronics Inc., Hialeah, FL). The variability between replicates was less than ± 5% for all samples.

Experiment 6 Effects of 17,20ß-P on sperm motility and duration of motility

This experiment was conducted to determine whether pheromonal 17,20ß-P increases the duration of sperm motility and the percentage of motile sperm, both of which have been used as indicators of fertility (Brown and Gratzed, 1980).

Spermiating males were randomly placed in four 65-liter tanks with three fish per tank (water temperature 18 - 20 °C) on day one. On day 2 at 22:00, fish were exposed to either 5×10^{-10} M 17,20ß-P (n = 9) or ethanol control (n = 9). On Day 3 at 08:00, all fish were anesthetized, bled and stripped of milt. Urine and fecal contamination was carefully avoided by squeezing and wiping the cloaca before milt was stripped. Because spermatozoa of fresh-water cyprinid fish are immobilized due to osmotic pressure in milt (Billard and Cosson, 1989; Morisawa *et al.*, 1983), motility was estimated using a two step dilution technique with a compound microscope (Billard and Cosson, 1989, Cosson *et al.*, 1991). The first dilution was made by diluting 5 µl milt from each sample 1:150 in NaCl saline (150 mM , pH = 7.2) in which sperm are not motile. The second dilution was performed on a glass slide by mixing 1 µl of pre-diluted sperm into 19 µl tank water to activate sperm motility. A stop watch was immediately started to measure the duration of motility. Percentage of motile sperm was determined by counting the non-motile sperm after activation of sperm and counting the total sperm when all sperm stopped moving. Duration of sperm motility was measured by a stop watch from the point when sperm was activated to the point when all sperm stopped moving. Three aliquots of milt

from each sample were taken for measurement of sperm motility and duration of motility. There was no significant difference ($P > 0.05$, t-test) in sperm number observed from the field of view between the treatment group (21.4 ± 4.5; mean ± s.e.m.) and control group (18.5 ± 4.3; mean ± s.e.m.). To obtain an accurate assessment of sperm motility, all milt samples were set on ice immediately after stripping (Cosson *et al.*, 1991; Vizzino *et al.*, 1995), and the amount of time on ice was balanced for all samples.

Statistical analysis

Comparison of GtH-II data, sperm motility, motility duration, sperm concentration, sperm number and individual paternity between different treatment groups were analyzed by unpaired t-test. Because milt volume data were not normally distributed, they were analyzed using Mann-Whitney U-test. Competitive fertility (Experiment 1 and 2) was analyzed by X^2 with null hypothesis that 17,20ß-P treated and control males each fertilize 50% of the eggs. The relationship between spawning and fertility in experiment 1 was assessed by Spearman Rank Correlation.

RESULTS

Experiment 1: 17,20ß-P and fertility during competitive spawning

In all of the six replicates, larvae hatched within 4 days after fertilization. The total number of hatched larvae was not counted but was estimated to be more than 2000 for each of the six replicates. Although 72 larval samples from each experiment were extracted for DNA, PCR amplification of the DNA was sometimes unsuccessful due to

loss of DNA during extraction, resulting in sample sizes ranging from 42 to 72. In all four male spawning pairs tested, the paternity achieved by 17,20ß-P-exposed males was higher than control males, even when the treatments were reversed for two of the pairs ($P = 0.0001$; Table 4.1).

Males exposed to 17,20ß had numerically greater spawning scores than control males in five of the six spawning tests although only in three if these pairs (pair 2, 4 and 2R) was the difference striking (Table 4.2). Spawning scores (percentage of total score achieved by the male pairs; Table 4.2) and fertilization rates achieved by the 17,20ß-P-exposed males were significantly correlated ($r = 0.943$, $P = 0.0167$; Fig. 4.1).

Experiment 2: 17,20ß-P and fertility in competitive in vitro *fertilization*

In the competitive *in vitro* fertilization experiment, all four 17,20ß-P-exposed males achieved higher paternity than control males ($P < 0.0001$; Table 4.3).

Experiment 3: 17,20ß-P and fertility in pair spawning (one male-one female)

Males exposed to 17,20ß-P achieved a small but significantly higher fertilization rate than the control males ($P < 0.05$, *t*-test; Fig. 4.2).

Experiment 4: 17,20ß-P and fertility in pair spawning (one male-one female) during the course of spawning

17,20ß-P exposed males fertilized more eggs than control males during initial spawnings and after 2 hours spawning with PGF-injected females ($P < 0.05$, *t*-test).

After spawning for one hour with PGF-injected females, fertility in both 17,20ß-P-exposed male and control male was equivalent ($P > 0.05$; Fig. 4.3).

Experiment 5 Effect of 17,20β-P on sperm concentration and total sperm number

17,20ß-P significantly increased serum GtH-II ($P < 0.05$, *t*-test) and milt volume ($P < 0.05$, *U*-test) in male goldfish (Fig. 4.4). Although sperm concentration in the 17,20ß-P-exposed males was significantly ($P < 0.05$, *t*-test) lower than in control males, the total sperm number in stripped milt was significantly higher in the 17,20ß-P exposed males ($P < 0.05$; Fig. 4.4) due to the increased milt volume.

Experiment 6 Effects of 17,20β-P on sperm motility and duration of motility

Pheromonal 17,20ß-P significantly increased serum GtH-II and milt volume in male goldfish ($P < 0.05$; Fig. 4.5). Exposure to 17,20ß-P significantly increased both the percentage of motile sperm ($P < 0.05$) and the duration of sperm motility ($P < 0.05$) in the experimental males (Fig. 4.5).

DISCUSSION

This study has provided the direct evidence that a sex pheromone enhances male reproductive success in fish. The results clearly indicate that 17,20ß-P exposure increases male fertility both in one male-one female pair spawning and in two-male

competitive spawning. More importantly, sperm from 17,20ß-P-exposed males fertilize more eggs in competition with sperm from control males during *in vitro* fertilization, indicating pheromonal 17,20ß-P increases sperm fertilizing capacity.

The effect of pheromonal 17,20ß-P on fertility could operate through multiple levels. First, the effect on behavior could increase the chance of males to find an ovulated female or enable the male to obtain a spawning position that increases the chance of sperm reaching released eggs at the time of spawning. Secondly, the pheromonal effect on increasing milt volume at the time of spawning could also increase the male's fertility by increasing the number of releasable sperm. Finally, the pheromonal effect on sperm quality could be the most important aspect in enhancing fertility during sperm competition.

Results from this study show that pheromonal 17,20ß-P affects male spawning behavior (Table 4.2), consistent with earlier behavioral studies showing that male goldfish exposed to 17,20ß-P became more active (Bjerselius *et al.*, 1995; Defraipont and Sorensen, 1993) and more successful in performing spawning with receptive females than control males (Defraipont & Sorensen 1993). Indeed, the relationship between spawning scores and fertility in 17,20ß-P-exposed males (Fig. 4.1) suggests that 17,20ß-P-exposed males could achieve higher fertility during spawning simply by increasing spawning participation. Similarly, the increase of swimming activity and swimming speed in response to 17,20ß-P may increase the male's chance to encounter ovulated females as suggested by Bjerselius *et al.* (1995).

The effect of 17,20ß-P on increasing milt volume (Dulka *et al.*, 1987; Zheng and Stacey, in press) appears to play an important role in increasing male fertility, at least by increasing the number of releasable sperm. In multi-male breeding systems, numerical superiority has been suggested to be an adaptive strategy for sperm competition (Simmoms, 1987; Moller, 1988; Svard and Wiklund, 1989). Therefore, males with

more releasable sperm at the time of spawning should be able to fertilize more eggs. Indeed, male goldfish exposed to 17,20ß-P prior to spawning achieved higher fertilization rates at the onset of pair spawning (Fig. 4.3). Because the number of released sperm was not measured, it is still possible that the increased fertilization rates in 17,20ß-P-exposed males were entirely due to the increase of sperm quality. However, the observation that control males significantly increased fertilization rates (from less than 35% at the onset of spawning activity to the same level as 17,20ß-P-exposed male; Fig. 4.3) within 1 h of spawning activity suggests that the increased milt volume measured by stripping results in more sperm actually released at spawning.

This study provides evidence that pheromonal 17,20ß-P increases sperm fertilizing capacity. The finding that sperm from 17,20ß-P-exposed males fertilize more eggs in competition with an equal volume of milt from control males (Table 4.3) indicates that the increase of fertility during competitive spawning is at least partly due to an increase of sperm quality. Although pheromonal 17,20ß-P has been found to increase sperm motility (Defraipont and Sorensen, 1993; this study), there is no evidence that this increased sperm motility is functionally related to higher fertility. 17,20ß-P also might change other properties of sperm such as sperm membrane antigens (Naz *et al.*, 1983; Saling *et al*, 1985) to increase their fertilizing ability.

Pheromonal 17,20ß-P appears to benefit male goldfish throughout the whole spawning period from the beginning of spawning. Unlike pair-spawning species which show a decline in fertilization rates during spawning (Nakatsuru and Kramer, 1982), male goldfish in pair spawning are able to achieve high fertility within one hour of performing sexual activity and show no obvious decline in fertility over 2 hours of continuous spawning (Fig. 4.3). Pheromonal 17,20ß-P appears not only to increase male fertility at the initiation of spawning (Fig 4.3) but also to increase sperm quality to ensure high fertility during competitive spawning (Table 4.1).

It is unclear from the present study how pheromonal 17,20ß-P increases sperm quality in goldfish. One possibility is through hormonal regulation. In goldfish, pheromonal 17,20ß-P increases the circulating concentrations of GtH-II and 17,20ß-P in the males (Dulka *et al.*, 1987). Notably, the increase of milt volume induced by pheromonal 17,20ß-P is dependent on the endogenous GtH-II surge (Zheng and Stacey, in press). GtH-II acts not only on the sperm duct to regulate ion concentration (Marshall *et al.*, 1989), but also on the testis to stimulate 17,20ß-P synthesis (Ueda *et al.*, 1985). Whether the change of ion concentration in milt or the increase of testicular 17,20ß-P have effects on sperm fertilizing capacity in goldfish is not known. However, in salmonids, hormonal 17,20ß-P was found to increase sperm motility by increasing semen pH and cyclic AMP (Morisawa and Morisawa, 1986; Miura *et al.*, 1992). In mammalian species, there is also evidence that steroids such as progesterone and 17α-hydroxyprogesterone can act through a rapid non-genomic mechanism to increase sperm intracellular calcium and stimulate hyperactivated motility (Baldi *et al.*, 1994). Perhaps such steroid effects on mammalian sperm are analogous to the mechanism mediating pheromone-induced sperm fertility changes in goldfish.

In summary, this study demonstrates that pheromonal 17,20ß-P increases male fertility both *in vivo* and *in vitro*. Together with the earlier finding (Zheng and Stacey, in press) that 17,20ß-P-induced increase in milt volume is GtH-II-dependent , it is concluded that besides its effects on sexual activity, pheromonal 17,20ß-P increases male fertility also by stimulating endogenous GtH-II release that stimulates the increase of releasable sperm and sperm quality.

Table 4.1. Parental and larval genotypes, number of larvae, and paternity ratio from six trio-spawning tests.

	Genotype[a]						
	C-male	P-male	Female	Larvae of C-male	Larvae of P-male	Numbers of Larvae	Paternity ratio P-male / C-male
Pair 1	313/313	301/307	301/301	301/313	301/301 301/307	72	1.9 : 1
Pair 2	301/301	313/313	301/311	301/301 301/311	301/313 311/313	72	3.8 : 1
Pair 3	313/313	307/311	307/311	307/313 311/313	307/307 307/311 311/311	61	1.8 : 1
Pair 4	301/301	311/311	301/313	301/301 301/313	301/311 311/313	58	18.3 : 1
Pair 1R	301/307	313/313	301/301	301/301 301/307	301/313	72	2.0 : 1
Pair 2R	313/313	301/301	301/301	301/313	301/301	42	6.0 : 1
						Median Ratio	3.0 : 1

[a] The two numbers separated by "/" represent the sizes of the two alleles at locus GF1

C-male: control male;
P-male: 17,20ß-P-exposed male
Pair 1R: same males of pair 1 with treatment reversed
Pair 2R: same males of pair 2 with treatment reversed

Table 4.2. Spawning acts performed in different condition and spawning scores of the six trio-spawning tests from Table 4.1.

	Spawning acts in different condition					Spawning Score		
	C	C/p	CP	c/P	P	C-male	P-male	% total (P-male)
Pair 1	7	8	15	9	6	91	89	49.4
Pair 2	5	6	2	7	23	49	123	71.5
Pair 3	0	0	7	1	0	15	17	53.1
Pair 4	0	0	3	2	7	6	40	87.0
Pair 1R	5	1	4	2	7	33	43	56.6
Pair 2R	3	1	7	3	11	32	68	68.0
						(mean ± s.e.m.)		64.3 ± 5.7

C-male: control male; P-male 17,20ß-P-exposed male; C: only C-male spawns; C/p: both males spawn but C-male is closer to the female; CP: both males spawn and are equally close to the female; c/P: both males spawn but the P-male is closer to the female; P: only P-male spawns

Table 4.3. Parental and larval genotypes numbers of larvae, and paternity ratio from four *in vitro* fertilization pairs.

	Genotype					Numbers of Larvae	Paternity ratio P-male / C-male
	C-male	P-male	Female	Larvae of C-male	Larvae of P-male		
Pair 1	313/313	301/307	301/301	301/313	301/301 301/307	65	7.1 : 1
Pair 2	305/311	313/313	301/313	301/305 301/311 305/313 311/313	301/313 313/313	59	4.4 : 1
Pair 3	305/311	301/313	313/313	305/313 311/313	301/313 313/313	72	17.0 : 1
Pair 4	307/311	301/313	313/313	307/313 311/313	301/313 313/313	59	1.7 : 1
						Median Ratio	5.7 : 1

[a] The two numbers separated by "/" represent the sizes of the two alleles at locus GF1
C-male: control male
P-male: 17,20ß-P-exposed male

Figure 4.1. Relationship between % total spawning score and % fertility in 17,20ß-P-exposed males engaged in competitive spawning. $r = 0.943$.

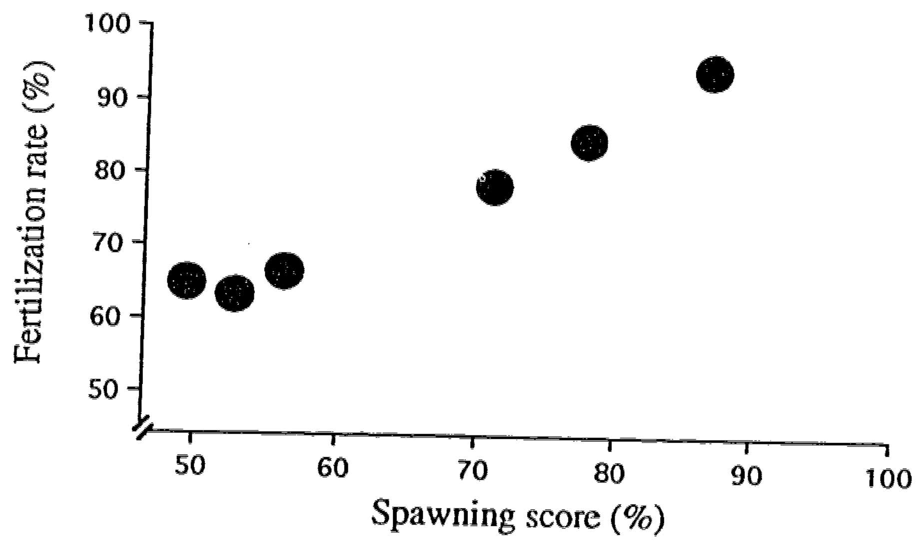

100
90
80
70
60
50
Fertilization rate (%)
50
60
70
80
90
100
Spawning score (%)

Figure 4.2. Effect of pheromonal 17,20ß-P on male fertilization rates in one male-one female spawning. Spermiating males were exposed to either 17,20ß-P or ethanol control overnight, and allowed to spawn with one ovulated female. All eggs were incubated to eye-stage to determine fertilization rates. *= $P < 0.05$; *t*-test. n = 4.

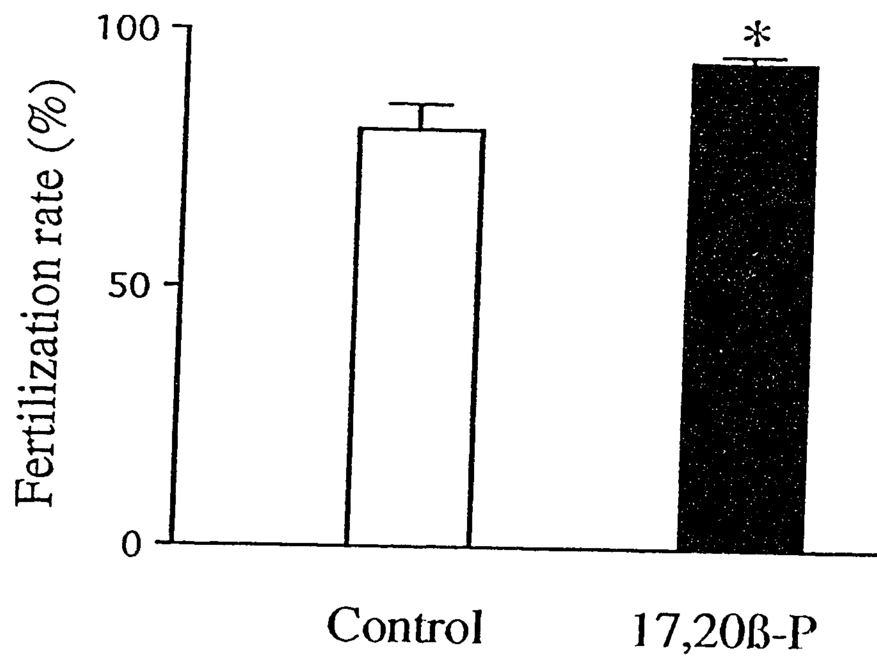

100
50
0
Fertilization rate (%)
*
Control
17,20ß-P

Figure 4.3. Effect of pheromonal 17,20ß-P on male fertilization rates during the course of spawning. Spermiating males were exposed to either 17,20ß-P (black bars) or ethanol control (clear bars) overnight, and allowed to perform two spawning acts with ovulated females at either 0 h, 1 h or 2 h after spawning with PGF-injected females. All eggs were incubated to eye-stage to determine fertilization rates. * = $P < 0.05$; *t*-test. n = 12 in each group.

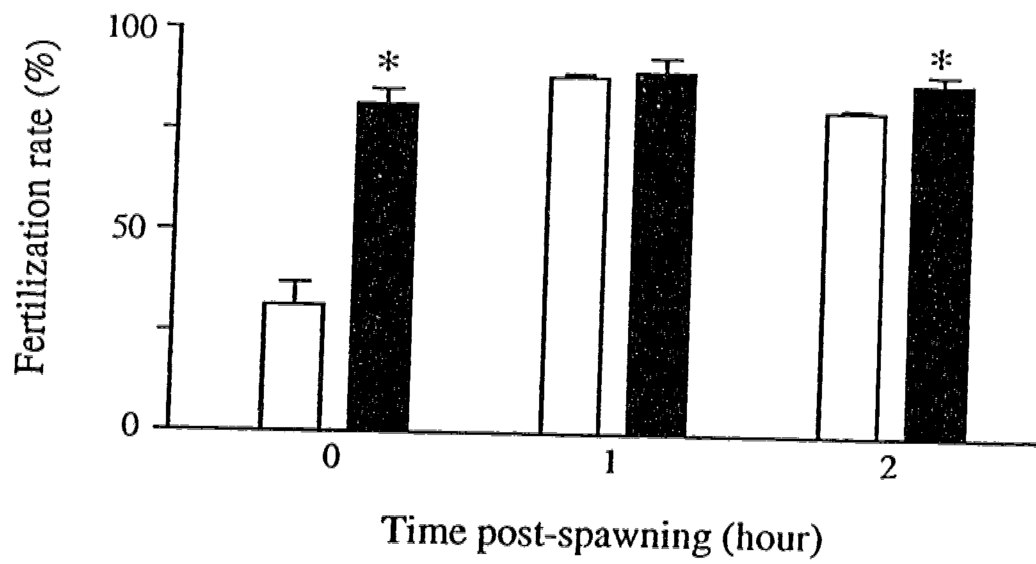
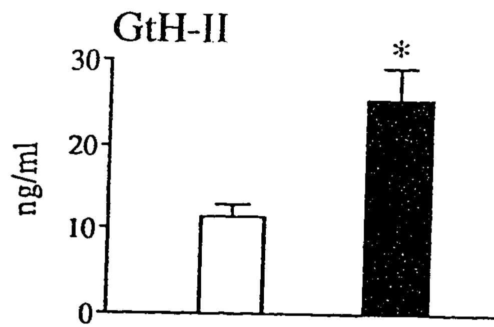
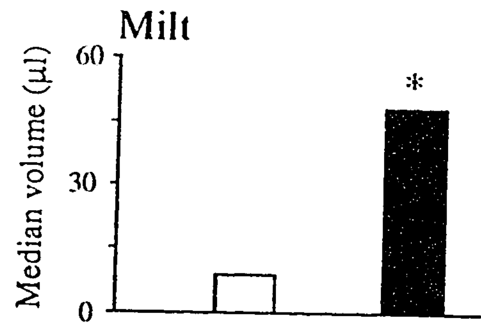
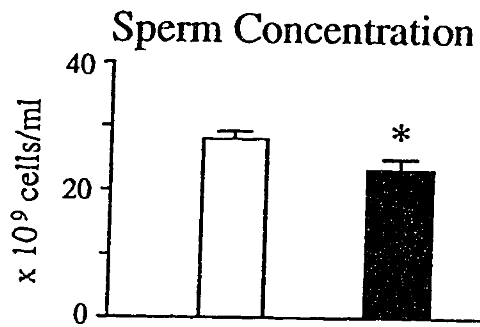

100
50
0
Fertilization rate (%)
*
*
0
1
2
Time post-spawning (hour)

Figure 4.4. Effect of pheromonal 17,20ß-P on serum GtH-II, milt volume, sperm concentration and sperm number. Spermiating males were exposed to either 17,20ß-P (black bars) or ethanol control (clear bars) overnight, bled and stripped of milt to determine sperm concentration and sperm numbers. * = $P < 0.05$; *t*-test. n = 6.


GtH-II
ng/ml
30
20
10
0
*


Milt
Median volume (µl)
60
30
0
*


Sperm Concentration
x 10⁹ cells/ml
40
20
0
*

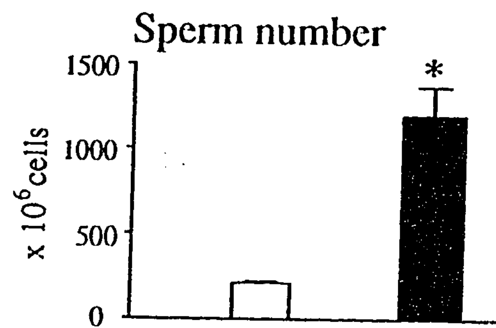
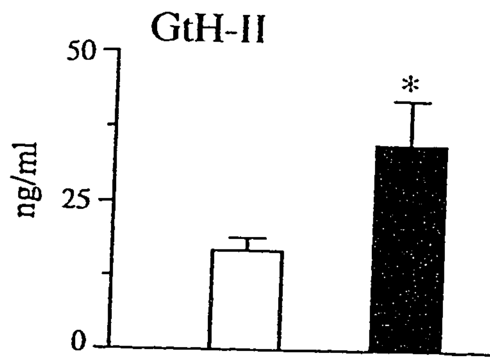
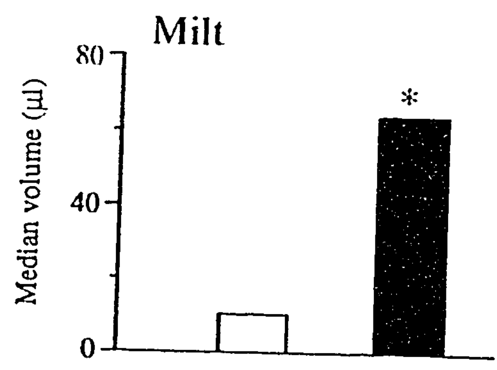
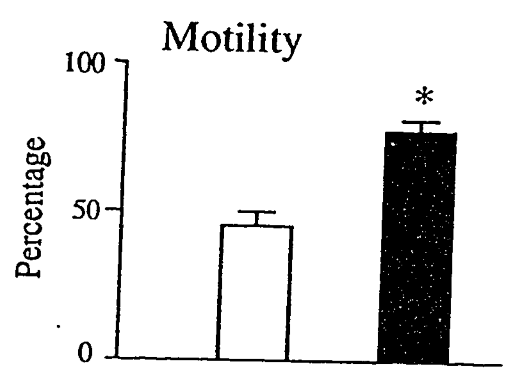
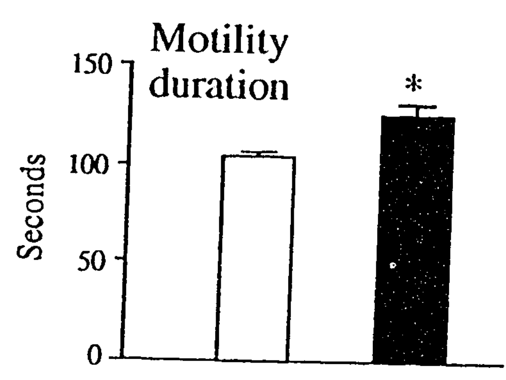

Sperm number
x 10⁶cells
1500
1000
500
0
*

Figure 4.5. Effect of pheromonal 17,20ß-P on serum GtH-II, milt volume, sperm motility and duration of motility. Spermiating males were exposed to either 17,20ß-P (black bars) or ethanol control (clear bars) overnight, bled and stripped of milt to determine milt volume, sperm motility and motility duration. * = $P < 0.05$; t-test. n = 9.


GtH-II
50
25
0
ng/ml
*


Milt
80
40
0
Median volume (µl)
*


Motility
100
50
0
Percentage
*


Motility
duration
150
100
50
0
Seconds
*

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CHAPTER 5

GENERAL DISCUSSION

This study has demonstrated that the two sex pheromones (17,20ß-P and PGF) act through distinct neuroendocrine mechanisms to affect fertility in the male goldfish. Studies in Chapter 2 indicated that 17,20ß-P and PGF stimulate an increase in milt volume through different neuroendocrine pathways. Results in Chapter 3 further demonstrated that 17,20ß-P and PGF act via different neuroendocrine mechanisms to increase endogenous GtH-II and milt volume. Chapter 4 presented data on the effect of pheromonal 17,20ß-P on male fertility in both spawning competition and *in vitro* sperm competition. This Chapter summarizes the major findings of this thesis and discusses our current understanding about the biological function of the goldfish sex pheromone system. A final model based on this study is proposed for future studies on pheromonal effects on male fertility in goldfish and other species in general.

Goldfish hormonal pheromones

Studies on the goldfish pheromone system during the past 15 years have provided valuable information about the chemical nature and the physiological and behavioral effects of sex pheromones. As its hormonal pheromone system is the best understood in vertebrates, goldfish undoubtedly provide an excellent animal model for pheromone studies. Like many other fish, goldfish use released hormones (steroids, prostaglandins and their metabolites) as sex pheromones. Two classes of pheromones have been identified in the goldfish: the final oocyte maturation-inducing hormone, 17,20ß-P, is released in late evening approximately 10 hours before ovulation (Stacey *et al.*, 1989).

Males respond to the water-borne 17,20ß-P and its metabolites, 17,20ß-P-glucuronide (17,20ß-P-G) and 17,20ß-P-sulfate (17,20ß-P-S), with a rapid elevation of blood GtH-II concentration within 15 minutes and milt volume within 4-6 hours (Stacey *et al.*, 1989; Dulka *et al.*, 1987; Sorensen *et al.*, 1995b), resulting in a greater quantity of releasable sperm at the time of spawning. The second pheromone (PGF) is a mixture of $PGF_{2\alpha}$ (which is produced in the oviduct, and acts as a hormone in the females to stimulate sexual arousal) and its metabolites (Sorensen *et al.*, 1995a). PGF is released into the water in early morning from the time of ovulation after the release of 17,20ß-P ceases (Sorensen *et al.*, 1988, 1989; Stacey *et al.*, 1989), and acts as a pheromone in the males to stimulate sexual arousal. Notably, male goldfish that detect PGF and perform spawning behavior show an increase in blood GtH-II and milt volume within 30 minutes (Kyle *et al.*, 1985).

As mentioned in Chapter 1, goldfish also have a third receptor mechanism detecting androstenedione (AD) which inhibits the increase in milt volume induced by 17,20ß-P (Stacey *et al.*, 1991). Although the neuroendocrine mechanism for AD inhibition is unclear, it is believed to act by inhibiting GtH-II release (Stacey *et al.*, 1991). Because the 17,20ß-P : AD ratio changes in the females as the ovulatory GtH-II surge progresses (Scott and Sorensen, 1994), it is suggested that the biological significance of the inhibitory AD is to restrict male responses to odors from ovulatory females in which the 17,20ß-P : AD ratio is high, and therefore avoid sperm wastage (Stacey, 1991; Stacey *et al.*, 1994).

The functional significance of both GtH-II release and milt production induced by both 17,20ß-P and PGF likely is to maximize releasable sperm during spawning. Because male goldfish typically compete with other males when spawning with ovulatory females (Stacey *et al.*, 1991), males with more sperm at the time of spawning will have greater advantage for sperm competition. For the males, 17,20ß-P serves as a signal that

spawning will occur in a few hours whereas PGF indicates immediate spawning opportunity. Because it has been estimated that a male goldfish must stay within a few body lengths to detect the pheromonal 17,20ß-P (Sorensen and Stacey, 1990), it would not be surprising that some males may not have an opportunity to detect 17,20ß-P during the female preovulatory period. Selection therefore should have favored those males who are able to rapidly increase their sperm competition for the immediate spawning opportunity. It is therefore reasonable to suggest that males have evolved a mechanism independent of the pituitary-gonadal system for rapid milt increase in response to spawning stimuli.

Different mechanisms for pheromone-induced sperm production

Although there is some evidence suggesting 17,20ß-P and PGF increase milt volume differently (Stacey *et al.*, 1987), the present study is the first to have provided strong evidence demonstrating the different neuroendocrine mechanisms mediating the effects of 17,20ß-P and PGF on milt increase. Pheromonal 17,20ß-P evidently acts through endogenous GtH-II to stimulate an increase in milt volume whereas PGF acts through a GtH-II-independent mechanism by stimulating spawning behavior. Hypophysectomy blocked 17,20ß-P-induced increase in milt volume, but did not block the behaviorally-induced increase in milt volume. In addition, injection of hCG swamped the effect of 17,20ß-P on milt volume, but did not affect the milt response to spawning behavior. Furthermore, the increase of milt volume in response to 17,20ß-P exposure and hCG injection had similar latency (4-6 h) and were similarly delayed by low temperature, whereas increase of milt volume induced by spawning behavior occurred much more rapidly (within 0.5-2 h) and was less affected by low temperature (Chapter 2). Although the effect of PGF on milt increase appears to act through spawning

behavior and to be independent of GtH-II, the exact mechanism is still unknown. Future work should investigate the possible mechanism of sperm duct contraction (Dulka and Demski, 1986) and the possible involvement of neuropeptides such as oxytocin or related peptides which facilitate sperm transport in mammals (Bore *et al.*, 1983; Sharaf *et al.*, 1993; Nicholson and Pickering, 1993).

The demonstration of two distinct mechanisms for the increase of milt volume in this study has provided an excellent working model for hormonal pheromone studies of other fish species. Recent EOG studies showing that a variety of cyprinid species detect PGF and steroids (Stacey *et al.*, 1994) suggest their hormonal pheromones system may be similar to the one in goldfish. According to the proposed model (Chapter 2, Fig. 6), elevation of endogenous GtH-II is the key mechanism for 17,20ß-P-induced increase in milt volume. On the other hand, stimuli from spawning activity can also increase milt volume through a GtH-II-independent mechanism. Although the involvement of pituitary hormones in stimulation of milt production has been established in many fish species (Billard *et al.*, 1982), pheromonal stimulation of milt production by a GtH-independent mechanism has not been reported in other species. However, the involvement of spawning behavior in the elevation in milt volume and circulating concentrations of hormones is not limited to goldfish. In rainbow trout (*Oncorhynchus mykiss*), for example, interaction with spawning females is an essential stimulus for the increases in milt volume and blood hormones (Rouger and Liley, 1993). Male snapper (*Sparus aurata*) injected with GnRH do not produce as much milt as natural spawning males (Pankhurst, N.W., personal communication), indicating a possible spawning stimuli-dependent mechanism for the increase in milt volume.

The demonstration of two mechanisms for the increase in milt volume also provides valuable information for aquaculture. When hormone-injection is performed to obtain sperm from a species with similar pheromonal system, one may expect greater milt

production if the males are exposed to spawning stimuli to activate both mechanisms for milt production.

As discussed in Chapter 2, spawning stimuli can also increase GtH-II concentration which in turn could replenish milt stores depleted during spawning. This mechanism appears to be important for males to maintain sufficient sperm during prolonged spawning because 17,20ß-P-induced increase in GtH-II will quickly decline to basal levels after pheromonal 17,20ß-P is removed (Stacey *et al.*, 1991). Since females stop releasing 17,20ß-P by the time of ovulation, males would have not been able to maintain high GtH-II concentration for spermiation if they had not evolved another mechanism other than 17,20ß-P for GtH-II release. Indeed, males respond to spawning stimuli with a rapid increase in GtH-II concentration (Kyle *et al.*, 1985). This observation led to the investigation of whether 17,20ß-P and spawning stimuli increase GtH-II concentration via the same endocrine mechanism (Chapter 3).

Mechanisms for pheromone-induced increase in GtH-II concentrations

Unlike the mammalian brain-pituitary system in which the gonadotrophs are under the endocrine control of GnRH, the release of GtH-II in goldfish is under the direct neural control of stimulatory GnRH terminals and inhibitory dopamine (DA) terminals (Peter *et al.*, 1991a, 1991b) operating through DA type 2 (D2) receptors (Chang *et al.*, 1984a, 1984b, 1991). Pheromonal 17,20ß-P evidently stimulates GtH-II release by decreasing DA inhibition because D2 agonists block 17,20ß-P-induced GtH-II release, but do not affect the release of GtH-II induced by spawning stimuli (Chapter 3). Whether 17,20ß-P has a direct effect on GnRH release remains to be examined. Since the effect of 17,20ß-P on the release of GtH-II can be blocked by GnRH antagonist (Murthy *et al.*, 1994; Chapter 3), it is possible that 17,20ß-P has a direct effect on the release of GnRH.

It is also possible that 17,20ß-P only decreases DA inhibition to increase GtH-II release. The blocking effect of GnRH antagonist on GtH-II release may be due to the inhibition of GnRH action which is required for GtH-II release regardless the concentration of DA.

As discussed in Chapter 3, it is possible that different populations of GnRH are involved in the regulation of GtH-II release induced by pheromonal 17,20ß-P and spawning stimuli. However, whether sGnRH and cGnRH-II differentially mediate the responses to these two stimuli remains to be determined. Although both sGnRH and cGnRH-II immunoreactive fibers are located widely in the goldfish brain area and the pituitary (Kah *et al.*, 1986; Kim *et al.*, 1995), future studies using *in situ* hybridization of specific GnRH mRNA may provide answers to this question.

The demonstration that 17,20ß-P and PGF increase GtH-II and milt volume through different pathways (Chapter 3) has provided an important working model for studying neuroendocrine mechanisms of sex pheromones in goldfish. There are several other identified hormonal pheromones which also have physiological effects on male goldfish. For example, 17,20ß-P-G and 17,20ß-P-S are potent stimulants to the olfactory system of male goldfish (Sorensen *et al.*, 1987, 1990, 1991) and can increase GtH-II and milt volume (Sorensen *et al.*, 1995b). Because 17,20ß-P-G stimulates the same class of olfactory receptors as 17,20ß-P (Sorensen *et al.*, 1987,1990, 1991), its effect on increasing milt volume is expected to be the same as 17,20ß-P and to be dependent on GtH-II. Also, the similarity in the effects of 17,20ß-P-S and 17,20ß-P on GtH-II and milt volume (Sorensen *et al.*, 1995b) suggests 17,20ß-P-S acts via similar endocrine mechanisms. However, the mechanism for the interaction of 17,20ß-P-S and 17,20ß-P in stimulating GtH-II release remains to be examined because 17,20ß-P-S and 17,20ß-P have different classes of olfactory receptors (Sorensen *et al.*, 1990, 1995b)

Answers to these questions may also explain the mechanism for an inhibitory pheromone, androstenedione (AD) which uses a different class of olfactory receptors than

17,20ß-P or PGF (Sorensen, *et al.*, 1991). Because AD inhibits 17,20ß-P-induced increase in milt volume and isolation-induced increase of GtH-II and milt in goldfish (Stacey *et al.*, 1991), it is suggested that AD exerts its effect by inhibiting the increase in GtH-II. Considering that 17,20ß-P increases GtH-II by decreasing dopamine inhibition (Chapter 3), it is possible that AD acts by decreasing GnRH release or by increasing dopamine release. Although it is unknown how AD acts on GnRH or dopamine neurons, several neurotransmitters and neuropeptide (norepinephrine, serotonin, γ-aminobutyric acid, neuropeptide Y) may mediate the effect of AD because they are known to modulate GnRH and dopamine actions (Peter *et al.*, 1991b). If this is true, it may explain how male goldfish can process the information from all the different pheromones which act through different olfactory receptors.

Finally, this model (Chapter 3, Fig.3.9) may also provide a basis for studies on neuroendocrine sexual dimorphism. Because 17,20ß-P is a bisexual pheromone in goldfish (Sorensen and Stacey, 1987), it is suggested that female goldfish have similar neuroendocrine mechanisms regulating GtH-II release. Similarly, in species which show sexually dimorphic responses to pheromones such as domestic pig (Dorries, *et al.*, 1991, 1995), a difference of neuroendocrine mechanisms between sexes may be expected.

In summary, the goldfish pheromone system offers a model for neuroendocrine regulation of pituitary hormones. The complexity of brain-pituitary system regulating the release of GtH-II in goldfish seems to reflect a functional specificity of each specific peptide in response to specific stimuli.

Pheromones and male fertility

A major contribution of this thesis is the development of a microsatellite DNA fingerprinting technique for the goldfish (Appendix) which has enabled me to determine one biological function of pheromone exposure on male fertility (Chapter 4). Although a number of genetic techniques such as minisatellite DNA fingerprinting and allozymes have been used for paternity and population analysis (Laughlin and Turner, 1994; Philipp and Gross, 1994), only the microsatellite technique makes it feasible to conduct paternity analysis in goldfish because it is PCR based and requires a small amount of larval tissue. Growing larvae not only is time consuming, but also could alter paternity analysis because of the high mortality rates of larvae. The scientific value of the developed primers for the sequenced microsatellite loci can also be extended to some other cyprinid species (Zheng *et al*., 1995).

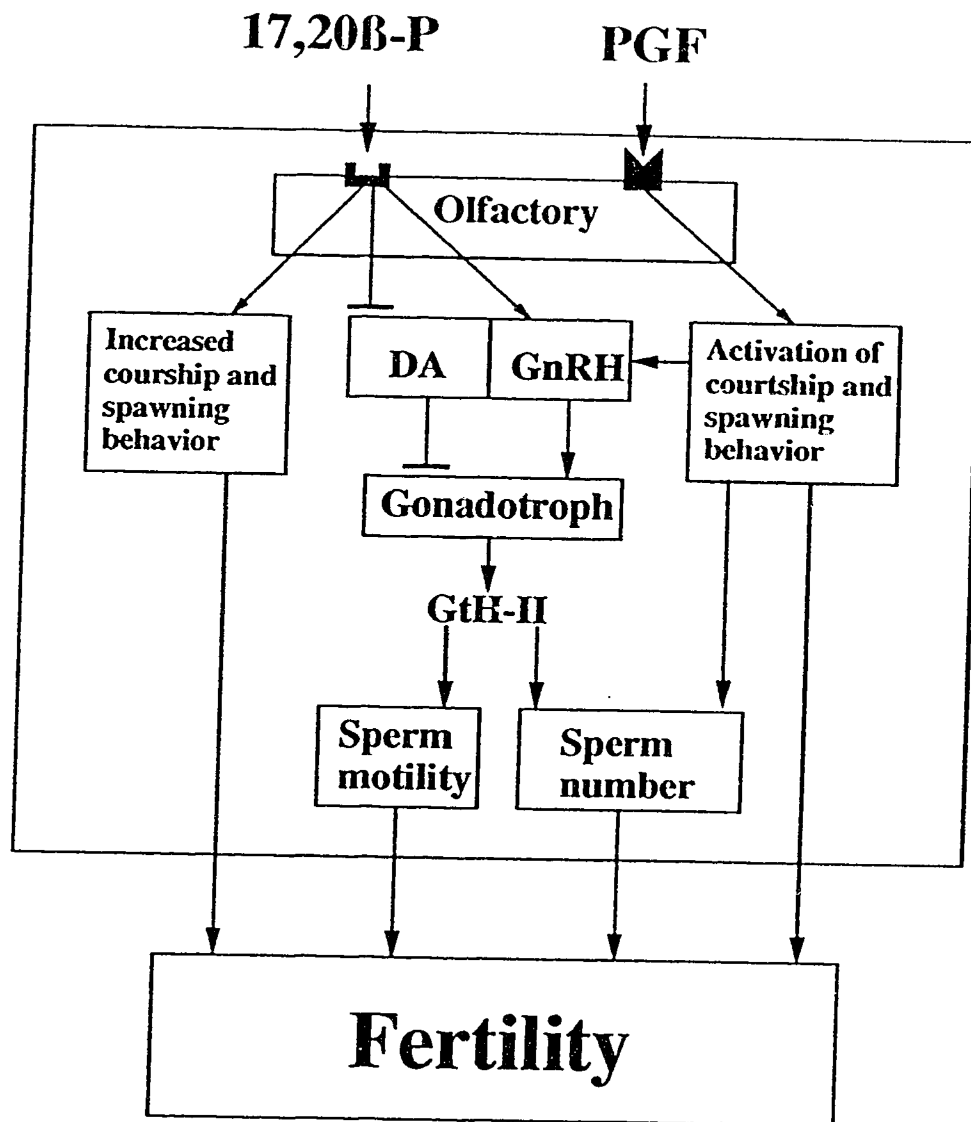
One of the most important contributions of this thesis is the demonstration that pheromonal 17,20ß-P enhances male fertility during both spawning and *in vitro* fertilization. This is the first study in vertebrates to have directly demonstrated that sex pheromones increase fertility by improving sperm quality. Despite earlier evidence that 17,20ß-P increases male spawning success with PGF-injected females (Defraipont and Sorensen, 1993), this study is the first to demonstrate that 17,20ß-P increases male fertility with ovulated females (Chapter 4). Specifically, this study has demonstrated that 17,20ß-P increased male fertility both during pair spawning (one male-one female) and competitive spawning (two males-one female). The present study also demonstrated that the effect of 17,20ß-P on increased fertility is through increasing sperm quality because sperm from 17,20ß-P-exposed males sired more offspring than sperm from control males (Chapter 4).

It is not clear from the current study how pheromonal 17,20ß-P changes sperm quality. Fish sperm appear to have a mechanism for increasing motility rapidly. One example is the finding that common carp (*Cyprinus carpio*) sperm with low motility can increase motility within 30 minutes of *in vitro* incubation in KCl saline (Cosson *et al.*, 1991). It has also been reported that the motility duration of rosy barb (*Barbus conchonius*) sperm increase from 2 minutes to 23 minutes when milt is exposed to ovarian fluid (Amanze, 1994). In salmonids, Miura *et al.* (1992) have shown that pituitary hormone acts through testicular 17,20ß-P to increase sperm motility. In goldfish, it is possible that GtH-II stimulates testicular 17,20ß-P synthesis (Ueda *et al.*, 1985; Dulka *et al.*, 1987) which in turn increases sperm motility. However, it remains to be determined whether this pheromone-induced increase in sperm fertility is analogous to the steroid effect in mammalian systems, where sperm motility is increased by progesterone and 17α-progesterone produced by ovarian follicles (Baldi *et al.*, 1994).

In conclusion, the major findings of this thesis are incorporated into a comprehensive model (Fig. 5.1). This model summarizes the neuroendocrine pathways mediating the effects of sex pheromones (17,20ß-P and PGF) on reproductive behavior, hormonal regulation, gonadal response and fertility in male goldfish. According to this model, during natural spawning, 17,20ß-P is released by the female goldfish several hours before ovulation and acts on specific receptors on the male olfactory epithelium (Rosenblum *et al.*, 1991). One of the effects of 17,20ß-P is to increase the males' activity of swimming and chasing other females. It also increases males' spawning activity when encountering receptive females. The endocrine effect of 17,20ß-P is to stimulate GtH-II release by decreasing DA inhibition and increasing GnRH stimulation. The increase of endogenous GtH-II then stimulates milt production by regulating sperm duct ion concentrations and by stimulating testicular 17,20ß-P synthesis. The increase of testicular 17,20ß-P may act through an unknown mechanism to increase sperm motility.

At the time of ovulation, female goldfish start releasing PGF, which stimulates male sexual arousal through olfactory receptors distinct from 17,20ß-P receptors. The stimuli from PGF-induced spawning activity stimulate GnRH release which exerts a stimulatory effect on GtH-II release in addition to the 17,20ß-P effect. The spawning stimuli can also act through a neuromuscular mechanism to increase milt movement from the testes to the sperm ducts for release. As a comprehensive model illustrating neuroendocrine, behavioral and gonadal responses to sex pheromones, this is so far the only pheromonal model in vertebrates demonstrating the effects of sex pheromones on sperm quality and male fertility. In this regard, it can be extended to serve as a working model for future studies on sex pheromone systems in other species.

Figure 5.1. A proposed model for neuroendocrine mechanisms mediating the effects of pheromonal 17,20ß-P and PGF on fertility in male goldfish. During natural spawning, 17,20ß-P released by the female goldfish several hours before ovulation acts on specific receptors on the male olfactory epithelium to increase the males' activity of swimming, chasing an spawning activity. The endocrine effect of 17,20ß-P is to stimulate GtH-II release by decreasing DA inhibition and increasing GnRH stimulation. The increase of endogenous GtH-II then stimulates milt production by unknown mechanisms which might include changes in sperm duct ion concentrations and by stimulating testicular 17,20ß-P synthesis. At the time of ovulation, female goldfish start releasing PGF, which stimulates male sexual arousal through olfactory receptors distinct from 17,20ß-P receptors. The stimuli from PGF-induced spawning activity stimulate GnRH release which exerts a stimulatory effect on GtH-II release in addition to 17,20ß-P. The spawning stimuli can also act through a GtH-II-independent neuromuscular mechanism to increase milt movement from the testes to the sperm ducts for release.


17,20ß-P
PGF
Olfactory
Increased coursship and spawning behavior
DA
GnRH
Activation of courtship and spawning behavior
Gonadotroph
GtH-II
Sperm motility
Sperm number
Fertility

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APPENDIX

DEVELOPMENT OF A MICROSATELLITE DNA FINGERPRINTING TECHNIQUE IN GOLDFISH *Carassius auratus*[1]

INTRODUCTION

Most studies on sperm competition in fish have been focused on milt and/or sperm quantity or on the fertility of individual males. In a "group spawning" species like goldfish, investigation of a male's fertility in a competitive situation can be achieved if and only if a proper genetic marker for paternity analysis is developed. Genetic techniques such as allozyme (Philipp and Gross, 1994) and multilocus minisatellite DNA fingerprinting (Laughlin and Turner, 1994) have been used for paternity analysis in fish. However, in a non-parental care species such as goldfish, paternity analysis must be performed on early stage larva because of the high mortality rate. Thus, a PCR based genetic technique is ideal due to the small amount of larval tissue available for DNA extraction. Microsatellite DNA fingerprinting which detects the variability of single locus microsatellite perfectly fits this requirement.

Microsatellite loci are regions of DNA composed of short ($\leq$ 6 bp) sequences repeated in tandem. Besides being abundant and polymorphic in many mammalian species, microsatellite loci have been suggested to be abundant in all eukaryotic species. Microsatellite analysis has been recently applied to studies of relatedness in wild animals

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[1] A version of this chapter has been published. Molecular Ecology, 1995, 4: 791-792. Sequences of five clones have been deposited in Genbank under accession number 35614-35618

(Amos *et al.* 1993; McDonald and Potts, 1994; Morin *et al.* 1994; Craighead *et al.* 1995) and has been suggested to be an ideal method for studies of parentage and kinship (Queller *et al.* 1993). Development and application of this powerful technique will greatly benefit such ecological research areas as mating tactics and sperm competition (Rico *et al.* 1993). In many aquatic animals, reproductive and behavioral ecology has been limited due to lack of proper genetic markers.

Goldfish exhibit a promiscuous mating system similar to that of many other cyprinid fishes (Stacey *et al.* 1991). Recent progress in endocrine and behavioral research on this species has made it the best understood model for pheromonal systems in vertebrates (Stacey *et al.* 1994). Males that are exposed to a steroidal pheromone (17α,20ß-dihydroxy-4-pregnen-3-one; 17,20ß-P) released by preovulatory females, or that interact with ovulated or prostaglandin-injected females, rapidly increase the volume of milt in the sperm duct (Dulka *et al.*, 1987; 1986; Kyle *et al.*, Stacey and Sorensen, 1985), suggesting that, during spawning competition with other males, they are able to release more sperm. However, little is known about the extent or nature of sperm competition in this species. Development of a microsatellite technique will provide valuable tools for paternity study in this species and, therefore, increase our understanding about the evolutionary significance of pheromonal systems in fish reproduction. This study was designed to isolate and characterize microsatellites in the goldfish. Obtained clones will also be examined in nine other cyprinids and four salmonids to explore the potential application of this technique to other important cultured and wild cyprinids.

MATERIALS AND METHODS

Isolation of microsatellite loci

Genomic DNA isolated from blood of a female goldfish was digested with BamHI (Pharmacia) and electrophoresed in 1% agarose gel. To eliminate the highly repetitive DNA, DNA fragments in the size range 4-10 kb were excised from the gel and isolated by electroelution (IBI). The resulting DNA was then digested with Sau3AI (GIBCO BRL) and fragments ranging from 300-500 bp were again collected by electroelution and dephosphorylated using calf intestinal phosphatase. A genomic library was constructed by ligating the dephosphorylated DNA into the vector M13 mp18, followed by transfection into *E. coli* DH5α F' by electroporation (*E. Coli Pulser*, Bio-Rad).

Recombinant clones were screened for microsatellites using a biotinylated $(GT)_{11}$ oligonucleotide probe (systhesized by Dr. Strobeck, Department of Biological Sciences, University of Alberta). A total of 108 of the strongest positive clones were collected and 12 clones were randomly selected and sequenced (Table 1). These clones were amplified by PCR using universal forward and reverse sequencing primers (4 min at 94 °C followed by 30 cycles of 15 s at 94 °C, 15 s at 56 °C, 75 s at 72 °C, followed by 8 min at 72 °C performed on a Perkin Elmer Cetus 9600 thermal cycler). The PCR products were sequenced on an Applied Biosystems Inc. (ABI) 373A automated DNA Sequencer using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (ABI).

Characterization of microsatellite loci

Primers were designed using OLIGO (National Biosciences Inc., Version 4.0) software for eight clones (clone 21, clone 23, clone 26 and clone 32 were rejected because the repeats were too close to one end, had too few repeats or contained a

complicated set of repeats) and synthesized on a DNA Synthesizer (ABI model 391) with one primer from each pair labeled with the fluorescent dye TET (ABI).

Microsatellite PCR amplification was conducted in a 25 µl volume containing 100 ng genomic DNA, 120 µM dNTP, 0.16 µm of each primer, 1x *Taq* buffer, and 0.5 unit of *Taq* DNA polymerase (3 min at 94 °C followed by 2 cycles of 30 s at 94 °C, 20 s at 58 °C, and 1 s at 72 °C, and then 35 cycles of 15 s at 94 °C, 20 s at 58 °C and 1 s at 72 °C, followed by 30 s at 72 °C). PCR products were resolved by electrophoresis using polyacrylamide sequencing gel run on the DNA Sequencer. Data were collected and analyzed on an Apple Macintosh computer using GENESCAN 672 software (ABI).

Cross-species examination of goldfish microsatellite loci

Amplification of the goldfish loci was also investigated in two individuals from each of nine other cyprinids and four salmonids. DNA was extracted from blood samples. PCR conditions were the same as above and the products were resolved as before.

RESULTS

Isolation of microsatellite loci

Approximately 20,000 recombinant clones were screened for microsatellites using a biotinylated $(GT)_{11}$ oligonucleotide probe. More than 500 positive clones were detected. A total of 108 positive clones were collected and 12 of these clones were selected and sequenced. All sequenced microsatellite clones contained GT repeats ranging from 6 to 14 (Table 1).

Characterization of microsatellite loci

Allelic variability of these loci was examined using DNA isolated from blood samples from 20 goldfish purchased from Ozark Fisheries, Stoutland, MO (12 females and 8 males). Only five of these eight primer sets gave clean results. All PCR products from these 5 loci showed bands of predicted sizes. The number of alleles at each locus and their size range are shown in Table 3. Allele sizes on the first 6 fish at locus 1 are included in this report (Fig. 1).

Cross-species examination of goldfish microsatellite loci

The results of the cross-species amplifications are summarized in Table 3. Under the conditions used, these primers did not work in the four salmonids examined (the lake trout, *Salvelinus namaycush*; the bull trout, *S. confluentus;* the brook trout, *S. fontinalis;* and the rainbow trout, *Oncorhynchus mykiss*). In contrast, they all produced bands of predicted sizes in some of the cyprinids. The PCR products from the species were not sequenced because it is beyond the scope of this study. However, when resolved on polyacrylamide gels, all the bands reported here exhibit static peaks with little non-specific amplification, and a typical microsatellite band pattern.

DISCUSSION

This study is the first to isolate microsatellite loci from goldfish. This technique was used successfully to examine the effect of 17,20ß-P on paternity in competitive spawning and *in vitro* fertilization, and undoubtedly can be used as a powerful tool for further paternity analyses in goldfish.

The finding that all five primer sets developed for the goldfish work in the common carp, *Cyprinus carpio*. is particularly encouraging because the common carp is closer to goldfish than is any of the species used in this study (Winfield and Nelson, 1991). This finding also confirms that microsatellite primers isolated from one species can be used in closely related species (Schlötterer *et al.* 1991). In addition, some loci (GF1 and GF29) readily detect two or three alleles in some of these species. Although it is difficult to assess the level of polymorphism at these loci in these species due to the small sample size, detection of alleles in a similar size range suggests that these five microsatellite loci can be used as valuable genetic markers not only in goldfish but also in some other cyprinids.

TABLE 1. Goldfish microsatellite clone sequences

CLONE 1

GATCCTCCAGGTAAATAACATGGTCTTATGAAGGGTAGGAAAAGTGTGAAGC
TAATGGGTACACACTGCGGTAGAGTAAAGGTT**TGTGTGTGTGTGTGT**
GTGTGTGTGTGATGGCTGCTGAAGTGCTCCAGTAATAAGAGCCGAGAGGG
nTCATGTGTTACTGCTCTGAGTGAGAGGTGTGACAGCCAGGTCTCATGAATAG
TGATGCTAGACAGCTGCTCATTAACAGGAAGCCTGTCAGCCAACAGACTGGA
ACTGCCAAGACACGCAATACACGAACCTCCACAAATAACACACACACAATCTG
TTCACAATTCCTTCTTCTCCCTAACCTGTGGCATGGCCTGAGATGGATC

Clone 11

GATCTCATTCCGTCTAGCATTCTTCTGGTTGGTTC**TGTGTGTG**C**TGTGTG**CT
GCTGAATGGCATTTTAAATCTTGTTTTGCACTTGTTTTCCAGTGCTGCATCCCT
ATAGACACATCCTGGGTCTCTGGCAGCCTGATATCGGGCCCTATGGAGGACT
GCTGAATGTGGTGGTATGATC

Clone 17

GATCATTGAAGGAAATATTCATGTGCGTGTCnGTCTGTACCTATGTAGACTGT
GTGTGGTTGACCnTCGATTTGnAGAGGTATATGCGCGATnATGGGTGTGCCTG
TGGATGTTTACCGCTTCAGGTGCACCCTGGTCTGCTCTGTTCTATTGATACAG
TGCTTTCATAACAGCGTTTAACCCCCTCAACACCTGTGTGGkCCTGTGTGATAT
wTCACAGCTGCGCGTCCAGTATGAATGCTAATCGTGGCTCTGATGGCGTGGG
AACTAGAGCCCACTGACAGTTCACAGCGGAATTTCAnACACTAATTCACACTT
ACAATTTGCGTATGTGTTGTTGTACATAAATCATCTGTGAAAAGGGGAAACGT
GGTATGTGTGAGCATGGGnA**TGTGTGTGTGTGTGT**CTGTTTGTTTATAT
ATGTGTTTGGATATCGTCTCCCAAATGCAGAGAATGTCAATTATAATCGCATTA
TTACATTTGCATTTACATTAGATC

Clone 18

GATCCTCCTCTTCATCATTATCTGTACAAAGAGAGAGAGAGAAAGATTGAGAG
TGAGGAGTTGTCTGTGTGGTCAGATGCATGAATATCATA**TGTGTGTGTGTG**
TGTGTGTGAG**TGTGTATGTG**TAACCTTTATGAGAGCTGGAATTGGAATTCT
TCATTATGTCACTTGGGTTCAGGCCTGTAGGGGAAACATCAAGTGACAGnCCA
CTGATTAGATC

Clone 20

GATCACAAGTGTGCAGAGGTGGGTAGAGTACCCAAAAACTTTACTCAAGTAA
AAGTAAAAGTAATTCTCAAAATATTTACTCAAGTAAAAGTAAAAGTACTAGTCT
TGAATAGTTACTTGAGTAAGAGTAAAAGAGTATCCGATAAAAAATCTACTCAA
GTAGTTAGTTACTTTGGGTCATATATACGGAGCCTATTTTTATTTAGATATATA
GATAAAATGTA**TGTGTGTGTGTG**TATAAATGTATATATTTCATCAGCCTTTAC
TCCAATTTATGTAATTTATTATAAAACCCTGTCTGTTTACTTAAGTAACAAATAT
AGGTGTCATGCCATAACATATTTTTAATATGACTGACTTTATATTAAATGTGAA
TTTAACATTGAAAGTTAATGTGATC

Clone 21

GATCTGAATTGCAGGTGTGGGGGAGAGGGGGGGATGCAGGAGGTGAGAAT
GGTGAGAGTGCTTGATTGGAGAGGTGTTCAnGATGGGTTGCAGGAGCTGTTA
ATGGTGTGAACGGTTGTTTGGAGAGGCATTCAGGGCTGGTTGCAGGAGT**TG**
TGAATGG**TGTG**AATGGT**TGTGTG**GGGAGGCATTCAGGGCAGGTGATGGGT
GTTCTTTTAAACCTGCAGTAAAACTCGTTCAGATC

Clone 23

GATCCAGTCGCAGAGTGAAGAATTCAGTCCGAGGTCAATGAGTTTGGAAGCT
AGCTTTATGGGGACTATAGTATTAAAAGCTGAGCTATAGTCAATAAATAGCAG
CCTTACATAGTTCCTGTTATTGCTGTTGA**TGTGTGTG**AGAGAAGAGTGCAGG
ATGTGAGAGATGGCATCATCTGTGGATC

Clone 26

GATCGCCTTAACTGAGAGAGACCGCTGCTGTGTGCGTGTCACATCCACCTAC
AGGCTTTGGCATTTTCCCATGGnTGTCTGTTGTAGTGCATGTGTTCC**TGTGTG**
TGTGTGTGTGTGTGTGTGTGTGTGAC**TGTGTGTGTG**AC**TGTG**TTATGA
AGATC

Clone 27

GATCCAAACTACACACTGCTCCTGCGCTCGGTGTTCTGCAGATAAAGCCATGT
GTGACATTTGAGTATGTTCACATCTTCACCAAGATTTCTGAAGACCTCGTTCAn
ATGCATGATAAAGAAGCATGTTTAGATGAGCATCTGTTCTGAAAAAAT**GTGT**
GTGTGTGTGTGTCTGTGTGTGTGTGTGTGTGTGTGTGTGAGACGGACA
GCTGGTCACCTGTGCTGCTGCTGCTGACCGGTGGGGTCACTCCGCCCGATC

Clone 29

GATCTTGGTGTGCAGAAGCAGAATGTATACTTACACCCACATGCTAGGTGACT
GTT**TGTGTGTG**CA**TGTGTGTGTGTGTGTG**AGTGTAATGAGAGTGTTAGTC
CTTTAGCCTTTAnTCGAGG**TGTGTG**GGAGTTTGTGCTCAGCCCTGTGTATAC**T**
GTGTGTA**TGTGTG**GGTGGGTGGTTTTTCTCAGTGAGACAGCTGCCCTGTGT
GTGAAATTATTAGGAGTGGAGGTGAGATC

Clone 31

GATCCCAnACGCTGACTGGTA**GTGTGTGTGTGTGTGTGTGTGTGT**ATGAG
CCCACTAACATACTAGGCAAGGCAAGTTTATTTATATAGCACATTTCATGCACA
ATGGTAATTCAGAGTGTTTTACATAAAAGAAAGTAAAATAATCATAnAGAAAAA
TnACAAAAATAAAACAAGCAATTTTAAAACTTTTAAAATGATTAAAAAATAAAC
AGAGTAGCGAACATGCTCCGAAGTGCCGATC

Clone 32

GATCATTTACATCTCACACATTACTGGAGATATAGAGCACAGACTCATGTTTAC
ATCAGTTTACATCAGTATCTGATGCACTGTTAGAAACATCTCACCTGCA**GTGT**
GTGTGTGTGTGTGTGTGTGTGAGAGAGAGAGTGAGTGAA**TGTGTGTG**A
GTGTGTGTGTGTGTGTGTGTGTGTGTCACCTGTCGCCCC**TGTGTG**CTGG
CGGTGGGGGGGTTAGTGAnCGAGTGTCGGGGGGATGCTCCGGTCAGACCCC
CGGCGCTCATCAGACTCATGCGTCCGGCTGGAGGAGCTCCTCGGGGGGGCA
TCTGGAAGGGTCTCTGTCCCCGGCGGCTCAAGCCTGCGCTCACAGATC

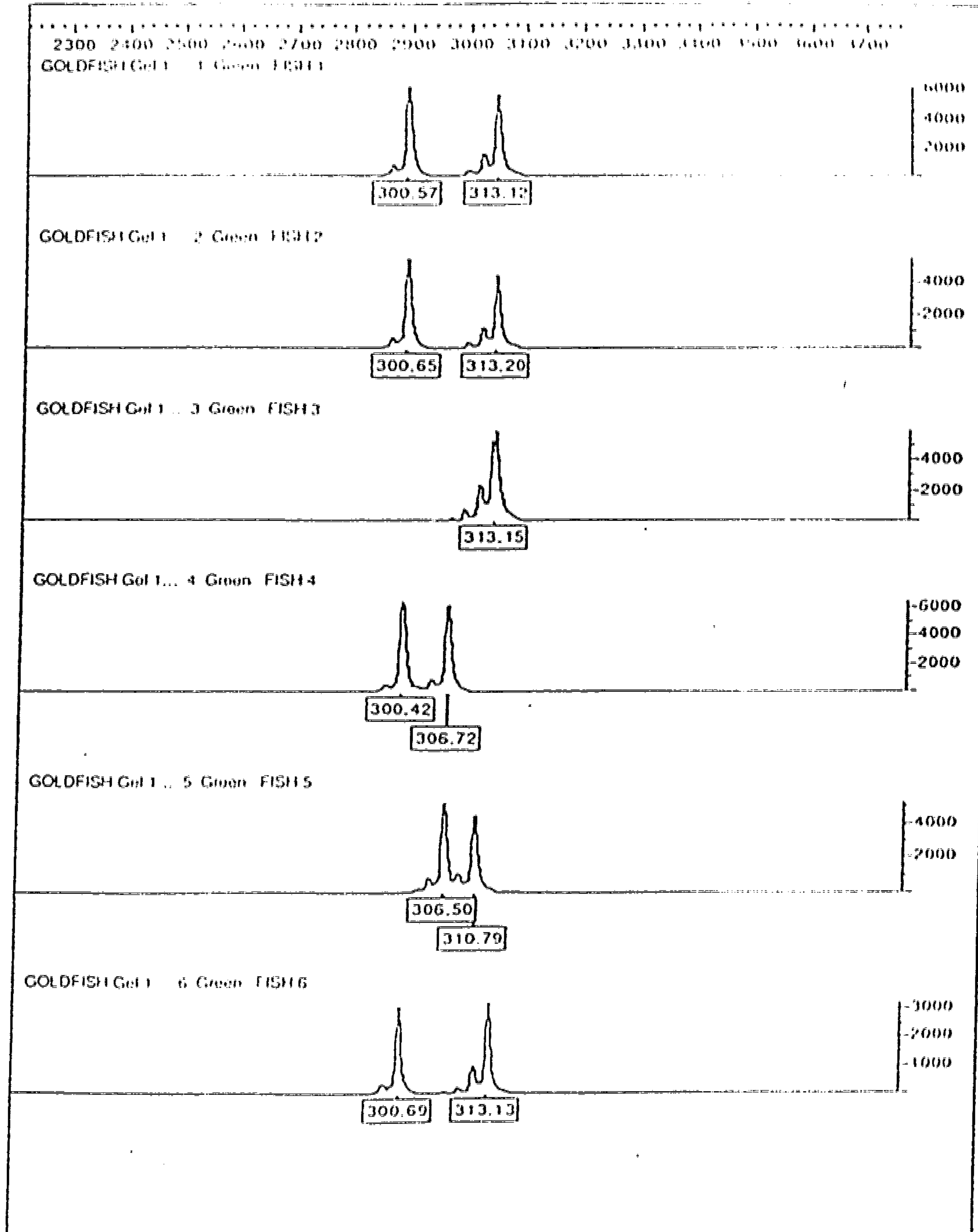
TABLE 2. Primer sequences (5' to 3' orientation) of the goldfish microsatellite loci and the sizes of the observed PCR products. Repeat motif was obtained from the sequenced alleles. Number of alleles was determined from 20 individuals.

Locus	Size Range (bp)	Repeat Motif	Number of alleles	Expected heterozygosity (%)	Primer sequences
GF1	301-313	$(TG)_{14}$	5	65.2	ATG AAG GGT AGG AAA AGT
					GTG A
					CAG GTT AGG GAG AAG AAG
					GAA T
GF11	159	$(TG)_4C(TG)_3$	1	0.0	GCA TTC TTC TGG TTG GTT CT
					ACC ACC ACA TTC AGC AGT CC
GF17	186-210	$(TG)_8$	3	54.1	GGA ACT AGA GCC CAC TGA
					CA
					TGC ATT TGG GAG ACG ATA
GF20	220-238	$(TG)_6$	4	60.0	AAA GTA AAA GTA ATT CTC
					AAA AT
					ATT GGA GTA AAG GCT GAT G
GF29	187-195	$(TG)_4CA(TG)_8$	3	47.1	ATG CTA GGT GAC TGT TTG T
					CAC CTC CAC TCC TAA TAA T

TABLE 3. The sizes (in base pairs) of the PCR products from cross-species amplification with five pairs of goldfish microsatellite primers. Numbers in the table refer to the sizes of the PCR products detected from 2 individuals in each species. (x) denotes too weak PCR amplification or multiband pattern. All species are from the family Cyprinidae.

	Locus				
Species	GF1	GF11	GF17	GF20	GF29
Subfamily Leuciscinae					
Tribe Chondrostomini					
Aristichthys nobilis	291	x	x	x	x
Pimephales promelas	x	x	x	222	190/194
Subfamily Cyprininae					
Tribe Squaliobarbini					
Ctenopharyngodon idella	281/291	x	x	x	x
Mylopharyngodon piceus	291	x	x	x	x
Tribe Cyprinini					
Carassius auratus	301-313	159	186-210	222-232	187-195
Cyprinus carpio	297	160	251	273	249/267/269
Tribe Systomini					
Puntius conchonius	289	x	x	x	184/190
Bardodes gonionotus	307	x	x	123	198/204
Bardodes schwanenfeldii	x	x	x	x	218
Bardodes altus	x	x	x	x	206

Figure 6.1. Electropherogram showing the resolutions of PCR products for goldfish microsatellite locus 1 (GF1) from six individuals.


Applied Biosystems
GF 1
Licensed to Curtis Strobeck, University of Alberta
Genotyper™
2300 2400 2500 2600 2700 2800 2900 3000 3100 3200 3300 3400 3500 3600 3700
GOLDFISH Gel 1 1 Green FISH 1
300.57
313.12
GOLDFISH Gel 1 2 Green FISH 2
300.65
313.20
GOLDFISH Gel 1 ... 3 Green FISH 3
313.15
GOLDFISH Gel 1 ... 4 Green FISH 4
300.42
306.72
GOLDFISH Gel 1 ... 5 Green FISH 5
306.50
310.79
GOLDFISH Gel 1 6 Green FISH 6
300.69
313.13

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