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FISH SEX PHEROMONES: CURRENT STATUS AND POTENTIAL APPLICATIONS

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N. E. Stacey, P. W. Sorensen, J. G. Dulka, J. R. Cardwell and A. S. Irvine (1991) Fish sex pheromones: current status and potential applications. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 189-227. Research during the last decade demonstrates that a number of fish species use released hormones as sex pheromones that have potent effects on both reproductive physiology and behavior. Hormonal pheromones of fish appear to be among the best characterized vertebrate sex pheromones, and offer exciting possibilities for both basic and applied research. This paper briefly reviews the current literature on the identities and functions of proposed fish hormonal pheromones, and suggests areas in which additional research will both increase our understanding of these reproductive chemosignals, and facilitate application of this information to problems associated with the controlled reproduction of cultured species.

Key words: Pheromones, Olfaction, Spawning, Prostaglandins, Steroids.

In many species of teleost fishes, it has been demonstrated that conspecific odors induce a variety of reproductive responses including changes in the rate of gonadal development, induction of ovulation and spermiation, synchronization of courtship and spawning behaviors, and mediation of parent-young interactions (Colombo *et al.*, 1982; Liley, 1982; Liley and Stacey, 1983; Stacey *et al.*, 1986, 1987a; Van Weerd, 1990; Sorensen, 1991; Stacey and Sorensen, 1991). These biologically active odors generally have been termed pheromones, and have been described as exerting either *releaser* effects (rapid behavioral responses) or *primer*

effects (slower physiological responses). However, despite the potentially ubiquitous nature of fish reproductive pheromones, and the obvious diversity and importance of their functions, pheromonal techniques have not been incorporate into the technologies of controlled reproduction in cultured fish. This lack of impact of fundamental pheromone research on applied aspects of fish reproduction can be attributed primarily to the fact that chemical identification of a fish sex pheromone—a major prerequisite for determining feasibility of most pheromonal applications—has been achieved only recently.

Some of the initial attempts to chemically characterize fish reproductive pheromones used behavioral bioassay to evaluate the relative effectiveness of crude and semi-purified odors from conspecifics in different reproductive states (Stacey *et al.*, 1986). These studies suggested a diversity of chemicals might serve as fish sex pheromones: *e.g.* a proteinaceous releaser pheromone from the gonads of pondsmelt, *Hypomesus olidus* (Okada *et al.*, 1978); water-ether soluble basic releaser pheromones from ovaries of ayu, *Plecoglossus altivelis* (Honda, 1979), and rainbow trout, *Oncorhynchus mykiss* (Honda, 1980a). In other cases, the fact that pheromonal activity often was present in extracts of the gonads or genital cavity fluids, and could appear rapidly with a change in reproductive condition (*e. g.* during the periovulatory period), focussed attention on gonadal hormones as pheromonal candidates. These studies provided the first proposed identities of fish sex pheromones: *e.g.* etiocholanolone glucuronide as a releaser pheromone from the testes of the black goby, *Gobius jozo* (Colombo *et al.*, 1980, 1982); glucuronides of testosterone and 17β-estradiol as a releaser pheromone from the ovaries of the zebrafish, *Brachydanio rerio* (Van Den Hurk and Lambert, 1983). The importance of these initial findings inevitably stimulated further research indicating that several types of gonadal hormones and their metabolites and conjugates have pheromonal functions in a variety of fishes. This paper first briefly reviews our current understanding of fish hormonal pheromones, and then suggests what research might now be conducted to evaluate the applicability of pheromonal techniques to fish culture.

HORMONAL PHEROMONES

Although convincing evidence for

fish hormonal pheromones is rather recent, the idea that released hormones can serve pheromonal functions is neither new nor restricted to fish. Indeed, the impetus for early speculations about hormonal pheromones in fish (Doving, 1976; Colombo *et al.*, 1982) evidently arose from invertebrate studies (Kittredge *et al.*, 1971) indicating that the crustacean moulting hormone, crustecdysone, has potent releaser effects in some crab species. These findings (which unfortunately have not been confirmed; Gleeson *et al.*, 1984) led Kittredge and Takahashi (1972) to propose that the evolution of hormonal pheromones might occur readily in aquatic animals because their naturally-released hormones in effect are pre-adapted as water-borne chemical signals, being already soluble in the medium in which they are dispersed and detected, and synthesized and released in synchrony with discrete reproductive events. With the potential signal (released hormone) already in place, chemical interaction between conspecifics might arise through mutations which externalize the hormone receptors on appropriate chemosensory membranes. Although both hormonal (Nagahama, 1990) and olfactory receptors (Rosenblum *et al.*, 1991) for one fish sex pheromone appear to be membrane-bound, it is not known if these two receptors are chemically and genetically related.

In spite of recent and dramatic progress characterizing hormonal sex pheromone identity and functions in several teleosts (Lambert *et al.*, 1986; Stacey *et al.*, 1987a; Sorensen and Stacey, 1990), there are several reasons why equally rapid success should not be expected when these findings are applied to other species of aquacultural significance. First, progress in identifying hormonal pheromones has been greatest in those species for which there already was considerable information on reproductive biology and endocrinology foundation. Because many important cultured fish (eels, many marine species) are not so well understood, progress in understanding their hormonal pheromones may be correspondingly slower. Second, we expect that, unlike the conservative endocrine systems from which they are derived, teleost hormonal pheromone systems are quite diverse. Aspects of this diversity which migh have negative impact on aquacultural applications are (1) that for the regulation of any specific reproductive function (*e.g.* male attraction

to female) only some species will use a hormonal pheromone, (2) that only some species will have evolve pheromonal responsiveness to any specific released hormone, (3) that different fish species will have evolved different responeses to the same released hormone, and (4) that, for any particular hormone (*e. g.* testosterone), species differences in metabolism prior to release will have enabled the evolution of numerous, chemically distinct hormonal pheromones. The rationale for these predictions is that, whereas fish hormonal systems can be regarded as homologous regulators of a standard set of reproductive functions (gamete growth and maturation, spawning and related behaviors), hormonal pheromone systems undoubtedly have arisen repeatedly in unrelated groups, and often undergone later evolutionary specialization as components of a species' reproductive isolating mechanisms. For these reasons, we suspect that unlike research on reproductive hormones, in which specific findings in one species usually are of widespread relevance, hormonal pheromone research is much more likely to be species-oriented, findings in one species perhaps being of no direct relevance to any other.

In spite of these reservations about the immediate practicality of using hormonal pheromones to manipulate reproduction in cultured, we feel further studies in this direction are warranted by the tremendous potential. Hormonal pheromones offer the opportunity to easily and specifically alter reproductive development and activity with relatively little effort and minimal if any stress to the fish. Hormonal pheromone techniques, because they involve non-invasive use of natural products to trigger natural endogenous events in responsive individuals, would not present some of the problems associated with hormonal injection techniques: *i. e.* the need for prior hand selection of fish, the cost of treating unresponsive fish and producing immature gametes, and the implications of applying drug therapies to food fish. Expansion of the presently small research effort on hormonal pheromones need not require the development of entirely new research programs, but could occur simply by reorienting a small portion of the research currently ongoing in a variety of established fish reproduction laboratories.

CURRENT STATUS OF FISH HORMONAL PHEROMONES

Considering the tremendous diversity of teleost fishes, evidence for hormonal pheromones comes from disturbingly few species. The most thoroughly studied of these is the goldfish (*Carasius auratus*) which we will use as a model species to illustrate the complexity of hormonal pheromone systems.

Hormonal Pheromones in Goldfish

In temperate climates, goldfish are spring spawners in which the combined influence of increasing temperature and aquatic vegetation (spawning substrate) trigger a mid-photophase preovulatory gonadotropin (GtH) surge inducing ovulation and spawning the following morning (Stacey *et al.*, 1979). In the laboratory, goldfish ovulate and spawn readily, exhibiting an irregular ovulatory cycle which appears to be controlled by positive feedback effects of testosterone and/or estradiol (Kobayashi *et al.*, 1988, 1989).

Goldfish spawning behavior involves no evident male aggression, and neither sex is territorial or parental. During the hour or so required to release her ovulated eggs, the spawning female is pursued by a group of males which compete for position beside the female as she enters the vegetation for oviposition. In such a spawning situation, a male's reproductive success (number of eggs fertilized) is expected to be determined not only by his own spawning activity—the number and quality of sperm he can release during each spawning act, and the distance between eggs and sperm at the time of oviposition—but also by the spawning activities of male competitors—the numbers of sperm they release, and their positions relative to the female at oviposition. This promiscuous mating system, appears similar to that exhibited by many cyprinid fishes. Indeed, using Balon's (1975) catagories of fish reproductive guilds, the non-guarding, open-substrate spawning of goldfish is typical of the majority of cultured teleosts (Reay, 1984), the salmonids, which hide their eggs but also engage in sprem competition (Gross, 1984), being notable exceptions.

Virtually all of our studies of hormonal pheromones in goldfish have focussed on male responses to the female. Our current understanding of these phenomena is that during the periovulatory period, male reproductive physiology and behavior change rapidly in response to two distinct female hormonal pheromones:

a preovulatory primer pheromone which increases the volume of milt (sperm and seminal fluid) in the sperm ducts, and a postovulatory releaser pheromone which triggers male courtship behavior (Fig. 1). The principal component of the prevulatory primer pheromone appears to be the maturation-inducing steroid, 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20β-P) (Stacey and Sorensen, 1986; Stacey *et al.*, 1989). Blood 17, 20β-P increases within several hours of the GtH surge onset, reaches peak levels during early scotophase, and decreases dramatically by 1 hr after ovulation (Stacey *et al.*, 1989); concurrent with this periovulatory surge of blood 17, 20β-P is an equally dramatic and discrete

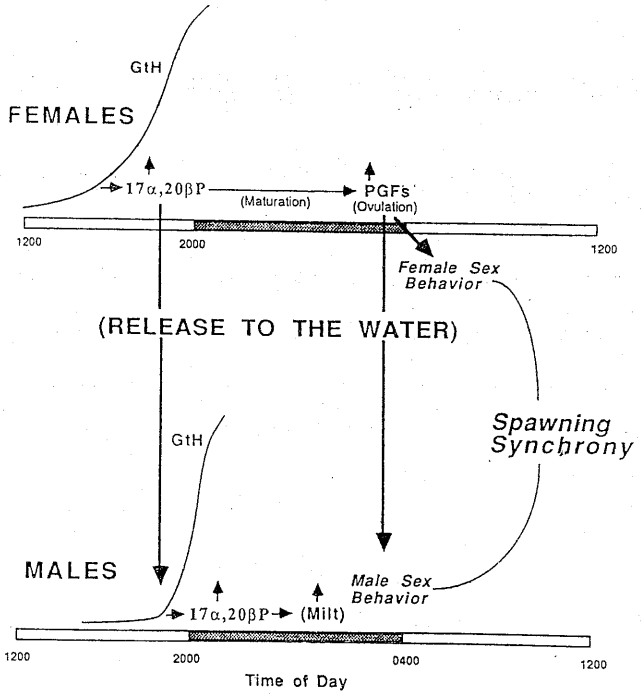

Fig. 1. Model of hormonal pheromone system of female goldfish proposed by Sorensen *et al.* (1988). An ovulatory GtH surge, triggered by environmental cues, stimulates synthesis of ovarian 17, 20β-P which in turn induces oocyte final maturation. 17, 20β-P released during the preovulatory period exerts a primer pheromone effect on males, acting *via* the male olfactory system to increase blood GtH, which in turn stimulates testicular 17, 20β-P synthesis and increases milt (sperm and seminal fluid) volume. At ovulation, eggs in the reproductive tract stimulate synthesis of PGF_{2a} which acts as a hormone stimulating female spawning behavior. During the time that ovulated eggs are in the reproductive tract, PGF_{2a} is metabolized and released to the water where it acts as a releaser pheromone triggerring male sexual arousal.

surge of 17, 20β-P release to the water. Blood GtH increases in males which either are held in direct contact with ovulatory females, or are exposed to their water-borne odors (Kobayashi *et al.*, 1986a, b; Stacey *et al.*, 1989). That this GtH increase in males is due at least in part to 17, 20β-P released by females is strongly indicated by the fact that males exposed to synthetic, water-borne 17, 20β-P exhibit both olfactory responses (Sorensen *et al.*, 1987a, 1990a) and GtH increase (Dulka *et al.*, 1987a, 1990a).

Olfactory response to water-borne 17, 20β-P is determined by electro-olfactogram (EOG) recording, a technique in which an extracellular recording electrode placed near the surface of the olfactory epithelium measures voltage gradients believed to reflect multi-unit generator potentials of the olfactory receptors (Ottoson, 1971; Van As *et al.*, 1985). Although the physiological basis of the EOG is poorly understood, and some have questioned whether it may on occasion reflect non-specific irritationl responses (Erickson and Caprio, 1984), we have consistently been able to confirm the validity of EOC responses to hormones and metabolites using whole animal bioassays (Sorensen *et al.*, 1988, 1990a) and electrical recording from the central nervous system (Sorensen *et al.*, 1989b). The EOG technique has made invaluable contributions to our pheromone studies not only because it indicates what general classes of hormones might have pheromonal functions, but also because it enables determinations of olfactory sensitivity and specificity. EOG recordings demonstrate that the goldfish olfactory epithelium is remarkably sensitive to water-borne 17, 20β-P (detection threshold 0.1-1.0 pM; Sorensen *et al.* 1987a). Indeed based on cumulative release of 17, 20β-P from periovulatory females (Stacey *et al.*, 1989), this sensitivity is sufficient to allow detection in the immediate vicinity of the female even if the release is constant (Sorensen and Stacey, 1990). Furthermore, by using EOG to compare olfactory detection thresholds for a variety of 17, 20β-P precursors and metabolites (Sorensen *et al.*, 1990b), it is clear the olfactory system is most sensitive to 17, 20β-P.

We believe that the major functional significance of the GtH increase in male goldfish exposed to the 17, 20β-P primer pheromone is to increase the number of sperm which are moved to the sperm duct for release. This response, which we

expect increases a male's fertility during sperm competition, is detectable within 2-4 hrs of 17,20 β-P exposure and increases for several additional hours (Dulka *et al.*, 1987a). The short latency of this gonadal effect indicates that a male encountering a female early in the preovulatory GtH/17,20β-P surge will be able to increase releasable sperm stores in time for ovulation and spawning. Exposure to 17,20β-P also appears to influence male sexual activity, increasing their competitive success in gaining access and spawning with females (Defraipont and Sorensen, unpublished results). It is unclear whether this behavioral effect, which is likely to be quite significant, is actually an indirect consequence of heightened endocrine activity.

The principal component of the postovulatory female releaser pheromone (Fig. 1) appears to be comprised of metabolites of prostaglandin F_{2a} (PGF) (Sorensen *et al.*, 1988), although much less is known of this pheromone than is the case with 17,20β-P. Both the hormonal and pheromonal effects of endogenous PGF can easily be demonstrated in nonovulated goldfish; females (or males; Stacey, 1987) in any stage of gonadal development become attractive to males within minutes of PGF injection, and soon thereafter commence spawning behavior, albeit without releasing eggs. However, neither the source of the postovulatory PGF source nor the chemical identity of the released pheromone are known. There is considerable indirect evidence that, at ovulation, an interaction between the ovulated eggs and the reproductive tract triggers synthesis of PGF which enters the circulation and acts within the brain to trigger female spawning behavior (Stacey and Goetz, 1982; Stacey, 1987). PGF synthesis remains elevated until ovulated eggs are released, at which time synthesis is reduced, circulating PGF is rapidly metabolized and/or cleared from the circulation, and spawning behavior ceases (Fig. 2). Just as the preovulatory profile of released 17,20β-P serves as an accurate indicator of endogenous 17,20β-P levels (and hence impending ovulation), we propose that the postovulatory increase in endogenous PGF is indicated by an equally discrete pattern of pheromone release indicating the female's sexually receptive condition.

Interestingly, the endocrine/gonadal responses to pheromonal PGF differ from those to 17,20β-P, in that they are not direct effects of exposure which can be observed in

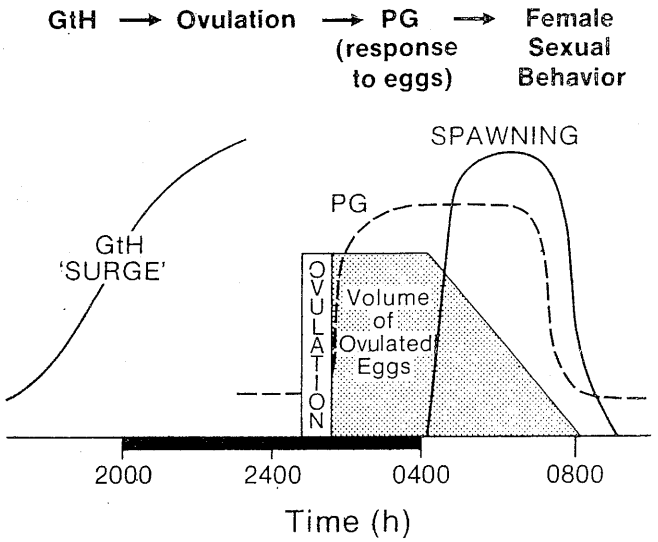

Fig. 2. Model of the regulation of female spawning behavior in goldfish proposed by Stacey (1987). Spawning is synchronized with photoperiod by the timing of the preovulatory GtH surge. Spawning is synchronized with the presence of eggs by the hormonal action of PGF_{2a} (PG). As spawning depletes the store of ovulated eggs, PGF_{2a} synthesis decreases, PGF_{2a} is metabolized and released to the water, and spawning behavior is terminated. It is proposed that male goldfish have evolved a pheromonal response to released PGF_{2a} metabolites because these accurately reflect the behavioral status of the female.

isolated individuals, but rather are the indirect result of pheromone-induced courtship interactions (Stacey and Sorensen, 1986; Sorensen *et al.*, 1989a). Whether the milt response to pheromonal PGF is mediated by GtH increase is not clear. The observed GtH increases should be capable of increasing milt volume through stimulation of testicular 17,20β-P synthesis (Ueda *et al.*, 1985). However, because milt volume is increased more rapidly by sexual interation than by hormonal stimulation (Dulka, 1989; Sorensen *et al.*, 1989a), and because milt increase during spawning can occur without evident GtH change (Kyle *et al.*, 1985), it seems that any milt increase which occurs early in spawning must be stimulated by a non-endocrine mechanism such as contraction of testis and/or sperm duct muscles (Dulka and Demski, 1986).

In contrast to the situation with 17,20β-P, in which hormonal and pheromonal functions appear to be performed by a single compound, PGF evidently is converted to an

unknown metabolite which is then released as a pheromone. The first evidence for this was the finding (Sorensen *et al.*, 1986) that male courtship is stimulated if holding water from females injected with PGF is added to their aquaria, but not if the same amount of PGF is added directly. Subsequent EOG studies showed that of a variety of PG compounds tested, the most potent was 15-keto-prostaglandin F_{2a} (15-K-PGF) (Sorensen *et al.*, 1988), a mammalian PGF metabolite not yet identified in the blood of fish. These results are consistent with the findings that PGF has a more potent hormonal effect on female spawning behavior than does 15-K-PGF (Sorensen *et al.*, 1987b), and that water-borne 15-K-PGF has a more potent pheromonal effect on male courtship behavior than does water-borne PGF (Sorensen *et al.*, 1989a). Unfortunately, more recent work shows that although female goldfish release several radiolabelled olfactory stimulants following injection with labelled PGF, none of these is PGF or 15-K-PGF (Sorensen and Goetz, unpublished results).

Although our proposed model of hormonal phermones of goldfish (Fig. 1) might appear to be unexpectedly complex, there are several reasons for believing that it is in fact a great oversimplification. In particular, preliminary evidence indicates that the model omits three potentially important aspects of hormonal pheromone function in goldfish: (1) female response to 17,20β-P, (2) release of 17,20β-P by males, and (3) pheromonal effects of androstenedione-like steroids (hereafter 'androgens').

Water-borne 17,20β-P increases the rate of 'spontaneous ovulation' (*i.e.* ovulation not induced by exogenous GtH) in goldfish (Sorencen and Stacey, 1987), a finding consistent with reports of synchronous ovulation in small groups under laboratory conditions (Kobayashi *et al.*, 1988) and with the fact that male and female goldfish exhibitit equivalent EOG responses to water-borne 17,20β-P (Sorensen *et al.*, 1987a). Determining the adaptive function of 17,20β-P-induced ovulatory synchrony likely will require studies under natural conditions in which the fish can form groups of appropriate size and sex ratio. However, two potential benefits to females might be predator swamping by increasing the numbers of spawners and their offspring, and reducing the injury and interference to females which we observe in laboratory spawnings where the ratio of female spawners

is low. Indeed, it is possible that the original primer function of 17,20β-P was as a synchronizing signal among females, and that males later evolved their milt response because the signal indicated that many spawning partners would be available.

At present, there are two pieces of circumstantial evidence that male goldfish release pheromonal 17,20β-P. First, the male's response to water-borne 17,20β-P includes increased blood levels and presumably increased release, of 17,20β-P (Dulka *et al.*, 1987a); however, blood 17,20β-P levels in 17,20β-P-exposed males are only about 10% of the levels in ovulatory females (Stacey *et al.*, 1989). Second, our recent unpublished studies show that if one male of a group is injected with hCG, a treatment which increases blood 17,20β-P levels (Kobayashi *et al.*, 1986c), milt volume increases in uninjected group members. It is not surprising that males under intense sperm competition would exhibit equivalent milt responses to 17,20β-P released by males or females, since 17,20β-P release by either sex presumably indicates imminent spawning opportunities.

Evidence that *androgens* might have a pheromonal function in goldfish was first provided by EOG studies (Sorensen *et al.*, 1990a, b) intended to examine the specificity of the olfactory response to 17,20β-P. Here, the cross-adaptation technique, in which the olfactory epithelium is adapted to one odor before being exposed to a second, confirmed our expectations that a variety of 17,20β-P-like steroids with low olfactory potency act *via* the same olfactory mechanism as 17,20β-P. EOG responses to the androgens androstenedione and testosterone were only marginally (and not significantly) reduced by adaptation to 17,20β-P, suggesting that responses to these steroids are mediated by an independent receptor mechanism (Sorensen *et al.*, 1990a and unpublished results). Unexpectedly, however, EOG responses to these androgen were not affected by prior adaptation to 17,20β-P, indicating they act *via* a separate olfactory mechanism. Because water-borne androgens do not stimulate milt production (Stacey and Sorensen, 1986), we examined the possibility that they might have some inhibitory function. Support for this hypothesis is the finding that the milt increase induced by water-borne 17,20β-P is inhibited by simultaneous exposure to androgen (Fig. 3). It is not known

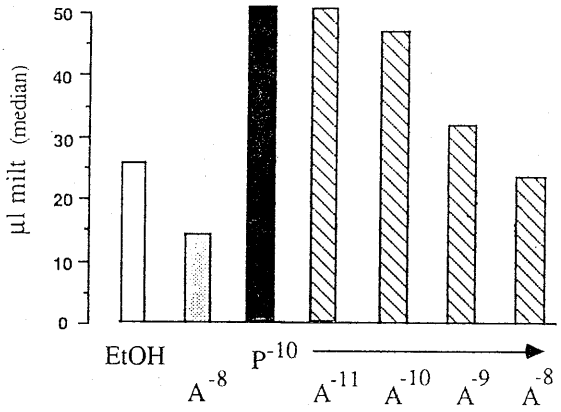

Fig. 3. Effect of water-borne 17,20 β-P (P) and androsteneione (A) on milt volume in the goldfish. Groups of 3 males (n=12–15 per treatment) in 80 liter aquaria were exposed to 200 µl ethanol (Etoh) or steroids and stripped of milt the following morning. Milt volume was increased significantly ($p<0.05$) by exposure to 10^{-10} M 17,20 β-P alone (black bar). Males exposed simultaneously to 10^{-10} M 17,20 β-P and 10^{-8} M androstenedione (hatched bar) had significantly ($p<0.05$) lower milt volumes than males exposed to 17,20 β-P alone.

whether androgen acts by blocking 17,20β-P-induced GtH increase, and also unclear whether the androgen inhibition is likely to occur at the olfactory epithelium or at higher levels. Although the results of EOG cross-adaptation (Sorensen *et al.*, 1990a) indicate that androgens and 17,20β-P act *via* separate olfactory mechanisms, androgens do displace 17,20β-P from putative 17,20β-P olfactory receptors when applied at 50 times the concentration of 17,20β-P (Rosenblum *et al.*, 1991); however, specific binding of androgens to olfactory tissues, and the ability of 17,20β-P to displace androgens, have yet to be demonstrated.

Whereas the milt response to pheromonal 17,20β-P appears to be an obvious male reproductive tactic for achieving enhanced fertilization success during sperm competition, it is not clear what benefit results from having the milt response also inhibited by androgen. Our present hypothesis is that all goldfish normally release progestins and androgens, and that an ovulatory primer pheromone is produced not simply by increasing the rate of progestin release, but also by increasing the progestin:androgen ratio. Our reasoning is that, because goldfish social interactions involve direct and frequent naso-genital contact, males would run the risk of being stimulated by non-ovulatory females (and

Table 1
Identified hormonal pheromones in teleosts

Species	Donor sex	Proposed pheromone	Response	Evidence[1] Res.[2]	Syn.[3]	Rel.[4]	Olf.[5]	Research group
goldfish (*Carassius auratus*)	F	17*o*, 20 β-dihydroxy-4-pregnen-3-one (17, 20 β-P)	increases milt volume and induces ovulation	+	+	+	+	Stacey, Sorensen, Dulka
	F	prostaglandin F_{2o} metabolites	stimulates male courtship	+	+	+	+	Stacey, Sorensen, Dulka
	F/M	Δ_4-3-keto-C19 steroids (*e.g.* androstenedione)	antagonizes effects of 17, 20 β-P	+	+	−	+	Stacey, Sorensen, Dulka
zebrafish (*Brachydanio rerio*)	F	testosterone and estradiol glucuronides	attract male and stimulates male courtship	+	+	−	−	Van Den Hurk, Lambert
	M	steroid glucuronides	induces ovulation	+	+	−	−	Van Den Hurk, Lambert
loach (*Misgurnus anguillicaudatus*)	F	prostaglandin F_{2a} metabolites	stimulates male courtship	−	(+)	(+)	+	Kitamura, Ogata
African catfish (*Clarias gariepinus*)	M	5β-pregnane-3*o*, 17*o*-diol-20-one-3*o*-glucuronide	attracts ovulated female	+	+	−	+	Resink *et al.*
black goby (*Gobius jozo*)	M	etiocholanolone glucuronide	female is attracted and stimulated to oviposit	+	+	−	−	Colombo *et al.*
Baikal sculpin (*Cottocomephorus grewingki*)	M	11β-hydroxytestosterone	induces ovulation	+	+	U	−	Dmitrieva *et al.*
	M	Δ_4-3-keto-C19 steroids (*e.g.* testosterone)	induces female behavior	+	+	U	−	Dmitrieva *et al.*

[1] Evidence relating to the isolated pheromone; [2] Response demonstrated by exposure to pure pheromone; [3] Synthesis of proposed pheromone demonstrated; [4] Release of proposed pheromone demonstrated; [5] Olfactory responses to proposed pheromone demonstrated. +=published evidence; −=no evidence available; U=in urine; PC=personal communication.

thus potentially wasting sperm) if they were to make their milt response contingent simply on the detection of 17, 20β-P. Indeed, making the assumption that goldfish release 17, 20β-P only in pulses of urine, we calculate that, in the absence of inhibitory andogens, even the low levels of 17, 20β-P released by non-ovulatory females (Stacey *et al.*, 1989) would be detectable in their immediate vicinity (Sorensen and Stacey, 1990).

If the function of the goldfish ovulatory primer pheromone does involve changing the ratio of stimulatory progestins and inhibitory androgens, then a male's milt production might normally be inhibited by interaction with unstimulated conspecifics (*i. e.*, those releasing low progestin:androgen ratios). Indeed, preliminary information suggests this is the case. If males are isolated from conspecifics overnight, they exhibit an increase in milt volume which can be inhibited by waterborne androstenedione. However, it remains to be determined whether either of these effects are mediated by changing GtH levels.

Our initial model of goldfish hormonal pheromones (Fig. 1) also is likely to be simplistic in that it focusses on the actions of single compound, whereas it now seems more likely that mixtures of compounds are involved. Goldfish are known to release (Van Der Kraak *et al.*, 1989) and detect (Sorense *et al.*, 1990a) a number of 17, 20β-P-like steroids, and there is good evidence that they release several PGF metabolites and have at least two classes of olfactory receptors for PGF metabolites (Sorensen *et al.*, 1988 and unpublished results). Also, electrical recordings from the goldfish medial olfactory tract, which is known to be necessary for pheromonal responsiveness in goldfish and some other teleost species (Kyle *et al.*, 1987), demonstrate responses to a broad range of non-hormonal olfactory stimulants (Sorensen *et al.*, 1989b and unpublished results) which may have a social function of some kind, perhaps supplementing the effects of the hormonal components and providing information about species or individual identity (Bryant and Atema, 1987). Circumstantial evidence for a non-hormonal releaser sex pheromone in goldfish is Yamazaki's (1990) finding that if females are injected with estradiol (which elicits no EOG response in either free or glucuronated form; Sorensen *et al.*, 1987a, 1990a), their urine is more effective in stimulating

male courtship behavior.

Van Den Hurk and Lambert (1983) suggest that a sex pheromone of zebrafish, *Brachydanio rerio*, is composed of a mixture of compounds, and this concept is widespread in the mammalian pheromone literature (MacDonald *et al.*, 1990). Although a hindrance to researchers interested in pheromone identities, pheromonal mixtures likely benefit the animal using them both because they should carry more information than single compounds, and because they should facilitate orientation if specific components can be distinguished from each other and have different thresholds and dose-response relationships (Sorensen *et al.*, 1988). It could be that pheromones we have identified in goldfish are key triggerring components, but not the entire pheromonal signal. Although we are only beginning to understand the functions of hormonal pheromones in goldfish, it seems likely that what we originally perceived as simple mechanisms enabling male goldfish to detect ovulation and sexual receptivity in females, are in fact complex mechanisms enabling reciprocal intersexual and intrasexual detection of endocrine changes related to spawning.

Hormonal pheromones in other teleosts

There is good evidence that pheromonal functions for released hormones have evolved in a variety of teleost species, some of which employ the egg scattering spawning strategy seen in the goldfish, and some of which do not.

In the zebrafish, spawning appears comparable to that of goldfish in that it is promiscuous, and involves neither territoriality or post-spawning care of the scattered eggs. Van Den Hurk and co-workers have provided evidence that hormonal pheromones both stimulate ovulation and attract males to ovulated females. When held in mixed-sex groups in the laboratory, females exhibit a short (4-5 day) ovulatory cycle. This cycle is disrupted if females are isolated from males, but resumes after exposure to male holding water, providing the female has not been made anosmic (Van Den Hurk *et al.*, 1987a). Isolated females can be induced to ovulate by exposure to either a testis homogenate or a testis fraction containing steroid glucuronides; however, treating the fraction with β-glucuronidase abolishes its effect on ovulation (Van Den Hurk *et al.*, 1987a). Although *in vitro* testis

incubations produce a variety of steroid glucuronides, one of which has been identified in male holding water, exposing isolated females to known synthetic glucuronides does not induce ovulation (Van Den Hurk *et al.*, 1987a, b).

As with males of many teleost species (Stacey *et al.*, 1986), male zebrafish discriminate ovulated from nonovulated females, and do not court ovulated females if they are made anosmic (Van Den Hurk and Lambert, 1983). Studies measuring the attraction of males to an odor source in an aquarium indicate that ovarian steroid glucuronides might be responsible for these male responses. Aqueous 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 releaser pheromone.

In the cobitid loach, *Misgurnus anguillicaudatus*, a cyprinid which scatters adhesive eggs in submerged vegetation, there is good evidence that F prostaglandins from ovulated females have a releaser effect on male courtship behavior (Kitamura and Ogata, 1990). Males prefer the odor of ovulated females to that of nonovulated females (Honda, 1980b), and will court nonovulated females injected either wither with PGF or two of its metabolites (15-K-PGF and 13, 14-dihydro-15K-PGF) (Kitamura and Ogata, 1990). Although the nature of the stimulant released by ovulated and FG-treated females is not known, EOG recordings demonstrate that, as in goldfish (Sorensen *et al.*, 1988), male *Misgurnus* are extremely sensitive to 15-K-PGF (threshold 10^{-13} M) and less sensitive to PGF (threshold 10^{-10} M). However, 13, 14-dihydro-15-keto-PGF, which is a relatively ineffective olfactory stimulant in goldfish (Sorensen *et al.*, 1988), is as effective as 15-K-PGF in *Misgurnus* (Kitamura and Ogata, 1990).

Resink and co-workers have conducted a substantial series of elegant experiments demonstrating that released hormones perform pheromonal functions in the African catfish, *Clarias gariepinus*, a species with a spawning strategy similar to the goldfish. In *Clarias*, factors associated with flooding appear to trigger spawning by inducing synchronous ovulation. Males then compete for

access to a spawning partner, and the resulting pair enters submerged vegetation where the eggs are scatterred, fertilized, and abandoned (Lambert *et al.*, 1986; Van Oordt *et al.*, 1987). Although there is evidence for a *Clarias* primer pheromone stimulating ovulation (Resink *et al.*, 1989b), almost all hormonal pheromone research in *Clarias* has focussed on a male releaser pheromone produced by the seminal vesicle, an accessory sex gland demonstrated to synthesize and store a variety of free and conjugated steroids (Lambert *et al.*, 1986; Schoonen *et al.*, 1988).

If given a choice between two odor sources, ovulated *Clarias* prefer male odor to female odor, whereas ovulated and anosmic females show no preference (Resink *et al.*, 1987); further choice tests clearly indicate that the seminal vesicle is the source of the male attractant (Resink *et al.*, 1989a). 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.*, 1989a). EOG studies (Resink *et al.*, 1989c) provide further evidence that steroid glucuronides are the active releaser components of seminal vesicle fluid. The olfactory potency of male odor is reduced by seminal vesicle removal, and increased by castration, which causes seminal vesicle hypertrophy. As well, glucuronidase treatment reduces the olfactory potency of fractions of seminal vesicle fluid which contain steroid glucuronides (Resink *et al.*, 1989c).

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.*, 1889a). The low EGO detection thresholds of two of these 7 steroids (5β-pregnan-3α, 17α-diol-20-one-3α-glucuronide; 10^{-11} M: 5β-androstane-3α, 11β-diol-17-one-3α-glucuronide; 10^{-9} M) makes them candidates for active components of seminal vesicle secretions. Unfortunately, it remains to be determined what levels of these steroid glucuronides are released *in vivo*, and what biological response they might effect.

Hormonal pheromones also have been proposed in the black goby, *Gobius jozo* (Colombo *et al.*, 1982) and the yellowfin Baikal sculpin, *Cottocomephorus grewingki* (Dmitrieva *et al.*, 1988), species which display reproductive strategies which are entirely different than those of the

goldfish, zebrafish and African catfish. Males of both *Gobius* and *Cottocomephorus* are territorial and defend a nest-site to which ovulated females are attracted for spawning, a situation favoring the evolution of male signals to attract females.

In *Gobius*, one aspect of male advertisement appears to be etiocholanolone glucuronide (EG) (Colombo *et al.*, 1980, 1982). This conjugated, 5β-reduced androgen, which is the major steroid product of the mesorchial gland, a Leydig cell-rich testicular component common among the gobiids, attracts ovulated females, whereas non-ovulated females are not attracted. Colombo *et al.*, (1982) also find that females are attracted to male urine, but have neither shown that urine contains EG, nor apparently tested the specificity of the female's attraction to steroids.

In *Cottocomephorus*, results of a variety of studies (Dmitrieva and Ostroumov, 1986; Dmitrieva *et al.*, 1988, and unpublished results) indicate that sexual interactions are coordinated by two steroidal male pheromones. One of these, tentatively identified as 11β-hydroxy-testosterone, acts as a primer to trigger ovulation, whereas the other (likely a mixture of delta-4-3-keto-steroids such as androstenedione and testosterone), triggers spawning of ovulated females at the nest-site. The fact that both steroids have been identified in urine, and the capacity of the male bladder increases approximately five-fold during the breeding season, suggests males may actively signal their territorial location.

In addition to species in which there is substantial evidence for hormonal pheromone function, there are others in which the evidence is indirect, or in which work is only beginning. In the fathead minnow, *Pimephales promelas*, males exhibit courtship responses when exposed to the odor of PGF_{2a}-injected females (Cole and Smith, 1987), whereas in milkfish, *Chanos chanos*, males become sexually aroused when exposed to water-borne PGF_{2a} (Kelley, Tamaru, and Lee, personal communication). Of particular interest are recent EOG studies of two salmonids. In Arctic char, *Salvelinus alpinus*, sexually active individuals are extremely sensitive to PGF_{2a} (10^{-11} M threshold) and related compounds (Sveinsson and Hara, 1990). In Atlantic salmon, *Salmo salar*, precocious male parr are extremely sensitive to testosterone, but not to testosterone glucuronide or a variety of other steroids (Moore and Scott, 1991). Of great

importance is the finding that this sensitivity is seen only in the precocious parr, and then only for a brief period at the onset of spawning, suggesting a rapid onset and termination of testosterone olfactory receptor function. These maturity-related changes in EOG function of *Salmo* appear to be completely different from the situation in both goldfish (Sorensen *et al.*, 1987a) and common carp, *Cyprinus carpio* (Irvine and Sorensen, unpublished results), where juvenile, recrudescent, and mature fish of both sexes exhibit equivalent EOG responses to a several hormones and hormone metabolites. In Pacific herring, *Clupea harengus pallasi*, a releaser pheromone in milt, which immediately triggers spawning in both sexes (Stacey and Hourston, 1982), has been the object of intensive investigations (Sherwood *et al.*, 1991; Scott *et al.*, 1991); although the chemical identity remains unknown, chemical properties are similar to those of a polar steroid or prostaglandin (Sherwood *et al.*, 1991). The herring milt pheromone is of theoretical interest, because it illustrates a dramatic bisexual releasing effect not currently under investigation in other fish, and of practical interest, because it may provide a simple technique for controlling oviposition in the "roe-on-kelp" industry.

PRESENT CONCERNS IN HORMONAL PHEROMONE RESEARCH

The evidence that a variety of teleosts exhibit specific and evidently adaptive reproductive responses to water-borne hormones and hormone metabolites released by conspecifics offers realistic promise of novel approaches for controlled reproduction in fish culture. Already, it is possible to envisage specific cases where hormonal pheromones can be fruitfully applied (*e.g.* sperm production and ovulation in cyprinids; spawning in herring). However, it also is certain that the potential applications for hormonal pheromones will be neither understood nor developed without dramatically increased effort in basic research. Presently, no aspect of hormonal pheromone function is well understood for even one species, and several important aspects have not been investigated in any species. If we are to seriously consider the application of hormonal pheromone techniques to aquaculture, it is necessary to first appreciate the very real shortcomings in our present

understanding. The following are issues we consider of major importance to a comprehensive approach to this problem.

Sex pheromones: concepts and definitions

Although the synonymous use of the terms *pheromone* and *chemical communication* is widespread in the literature, it is important to appreciate that this is a complex issue which is central to the concept of what we perceive a pheromone to be, and consequently how we believe it should function.

The word pheromone was derived from the Greek terms *pherin* (to transfer) an *hormon* (to excite) by Karlsson and Luscher (1959) who defined pheromones 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." In addition, Karlsson and Luscher included in their definition the more contentious statement that "the substance is... secreted... outside the body '(to) serve *communication* between individuals." The problem here revolves around the concept of communication, which has been discussed at length elsewhere (Burghardt, 1975; Wilson, 1975; Liley, 1982; Stacy and Sorensen, 1991). Briefly, the consensus appears to be that for chemical communication to occur, it is not sufficient that a receiver respond in some adaptive way to conspecific odor, but that there also is some "evolutionary development or specialization of the stimulus making it a more effective signal." (Liley, 1982). Because the selective pressure for signal specialization is the benefit which the signaller receives from the receiver's response (an example of intersexual selection in the case of an intersexual pheromone), the implication is that, historically, this communicative interaction has passed through a stage in which the receiver responded, but in which signal specialization had not yet begun (*i.e.* only natural selection pressures on the receiver were involved). Indeed, it is just such a pre-communicative stage which has been inferred by other workers (Kittrede and Takahashi, 1972; Doving, 1975; Colombo *et al.*, 1982) who have proposed the likelihood of hormonal pheromone evolution.

We feel the important question of whether communication is a

prerequisite for pheromonal interactions is put into proper perspective by considering how chemical communication might evolve. In the specific cases of hormonal pheromones we have proposed (Stacey and Sorensen, 1991) this evolution likely involves three distinct stages (Fig. 4). In the initial stage, a hormone or metabolite is released but is not detected by conspecifics. An intermediate stage of *chemical spying* could be reached if mutation enables a conspecific to derive unilateral reproductive benefit by detecting and responding to the released hormone. At this point, the chemical spying stage could proceed to the *chemical*

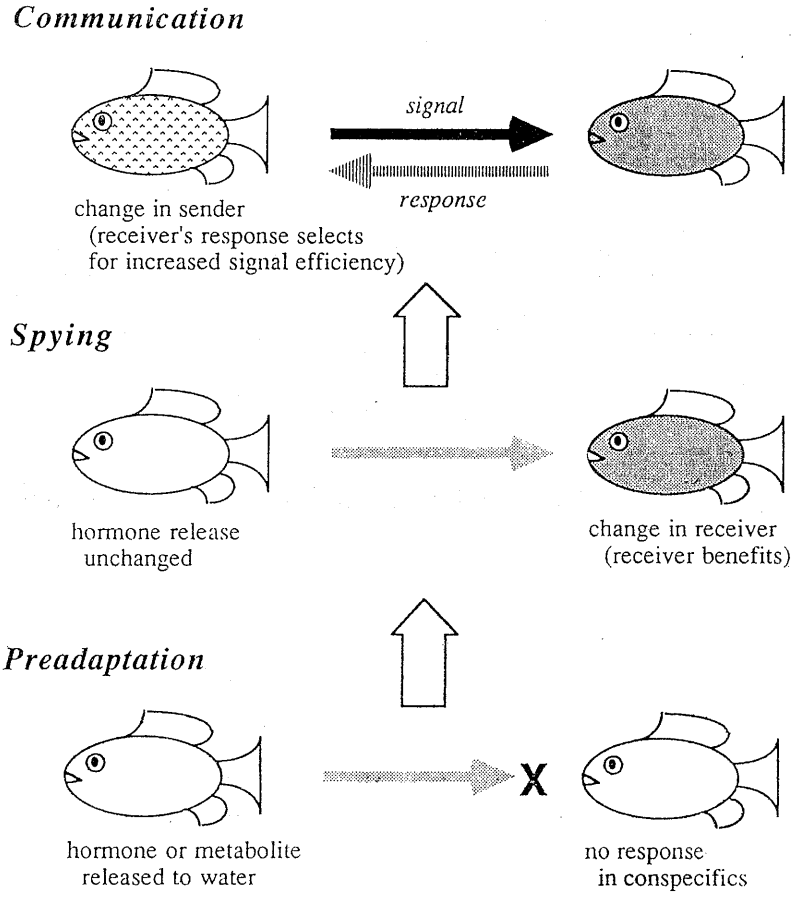

Fig. 4. Proposed stages in the evolution of hormonal pheromones (Stacey and Sorensen, 1991). Hormones are preadapted for pheromonal function because they are released to the water in synchrony with specific reproductive events. The first stage in evolution pheromonal function likely involves the acquisition of responsiveness to a released hormone or metabolite. The result may simply be a unilateral benefit to the received (chemical spying). If the receiver's response can benefit the individual releasing the hormonal pheromone, chemical communication may evolve.

communication stage if the originally unilateral benefit to the receiver results in increased reproductive success of the sender; here the receiver's response functions as a selective pressure for signal specialization in the sender.

To cite specific examples, we propose that the endocrine response of male goldfish to 17, 20β-P released by females is an example of chemical spying. Although increased male fertility generally would be considered to increase female reproductive success, we suspect that in this case the competitive nature of male reproduction is such that sperm availability does not limit female fertility, and consequently increased male fertility in respone to released 17, 20β-P has not been a selective pressure for specialization of the 17, 20β-P signal. In *Gobius jozo*, on the other hand, the proposed attraction of ovulated females to male etiocholanolone glucuronide (Colombo *et al.*, 1982) is an excellent example of how the female response could select for signal specialization by increasing the reproductive success of males which produce the strongest steroid signal.

The role of communication in hormonal pheromone function is not a trivial one because study of hormonal pheromones is the study of interactions between individuals. A situation in which one individual receives chemical information passively released by another can be fundamentally different from a situation in which one individual is specialized in some way to transmit the information, particularly if behavioral displays are normally involved with, and therefore might contribute to the effectiveness of, pheromone delivery. A hormonal pheromone with such a complex communicative function would likely be much more difficult to apply in an aquacultural situation than would a hormonal pheromone which functions in chemical spying.

Species-specificity of hormonal pheromones

A commonly espoused concept is that sex pheromones should be species-specific, a feature suggested necessary if costly inter-specific responses are to be avoided and reproductive isolation maintained. This concept, however, seems completely at odds with our proposal that fish have commonly evolved pheromonal functions for released hormones, because the limited chemical diversity of the known hormonal pheromone types (steroids,

prostaglandins) would be expected to have led to the evolution of the same (or very similar) hormonal pheromones in many species. The solution, of course, is that the stated functions of species-specific pheromones are restricted to those situations in which one species normally comes within the active space of another species' pheromone. Thus, experimental demonstrations that one species either does (Ingersoll and Lee, 1980), or does not (Honda, 1982b) respond to heterospecific odor are not in themselves particularly instructive, unless, as has been done by McKinnon and Liley (1987), the demonstrated presence or absence of species specificity is coupled with information about the nature of the species' reproductives sympatry. Indeed, to borrow a quote from McKinnon and Liely (1987), "pheromones are not necessarily species-specific, nor are they expected to be so—species that are allopatric, live in different habitats, or have different breeding seasons may well use the same pheromone, or react in experiments to the pheromones of other species" (Dobzhansky, 1970, p. 321).

From a functional perspective, the issue of species-specific pheromones revolves around two major factors; the size of the active space, and the discriminatory ability of the fish olfactory system. Although the active space of any hormonal pheromone has yet to determined, we have calculated (Sorensen and Stacey, 1990) that if a male goldfish is to detect pheromonal 17, 20β-P (the only hormonal pheromone for which both olfactory threshold and release are known), he must be within several body lengths of an ovulatory female. As for olfactory discrimination, EOG studies in goldfish have demonstrated extraordinary for pheromonal 17, 20β-P (Sorensen *et al.*, 1990a), suggesting even minor changes in pheromone structure could provide species specificity. If these examples from the goldfish are representative, the chemical diversity of released hormones may well be sufficient to enable species specificity.

From an evolutionary perspective, the issue of species-specific pheromones is complex, and intimately related to the issue of chemical communication. As discussed in more detail elsewhere (Stacey and Sorensen, 1991), functional species specificity could arise by active or passive mechanisms. Passive specificity would describe sympatric species that initially release different forms of a hormone (*e.g.* unmodified, metabolized or conjugated), and then

independently evolve pheromonal responses to the conspecific form. Active specificity would describe sympatric species that previously used the same hormonal pheromone. In this case, any reduced reproductive success resulting from responses to heterospecific odor could select for specificity, although the nature of this selection would depend on whether the chemical interaction involved spying or communication. In spying, natural selection would operate only on the pheromone receiver, whereas in communication sexual selection would also operate on the signaller.

Mode of release and patterns of exposure

Before we can claim full understanding of a hormonal pheromonal function, we must have some idea of how the response is influenced by the pattern of exposure. In the enclosed space of the circulatory system, the concentration of a hormone provides a reasonable estimate of target organ exposure. However, when the hormone and its modified forms released to the potentially infinite external space, assessing an individual's exposure to the resulting hormonal pheromone becomes more difficult, because the effective exposure will be simultaneously determined at least by the temporal pattern and concentration of released pheromone, movement of the signaller, distance from the signaller, local water turbulence, and configuration of the nares. Although we have measured the periovulatory profiles of released 17, 20β-P and 17, 20β-P-glucuronide in goldfish (Stacey *et al.*, 1989), there is yet no information on the normal pattern of exposure to any hormonal pheromone.

A major factor affecting exposure pattern will be the manner in which a pheromone is released. Two extreme situations are likely: a passive, tonic release across an exposed surface such as the gill, and a controlled, pulsatile release from a storage site such as the urinary bladder or the genital ducts. As discussed more fully by Stacey *et al.* (1986), passive release has not been studied directly, whereas there is evidence for and against urinary release, even in closely related species (*e.g.* Colombo *et al.*, 1982; Tavolga, 1956). It seems likely that urinary contamination of gonadal fluids is responsible for some of the controversy (Stacey *et al.*, 1986), although levels of a number of steroids have recently been measured in both urine and seminal fluid of

several species (Scott *et al.*, 1991; Scott and Canario, 1991).

We expect that the relative importance of release mode and exposure pattern in determining response will differ among hormonal pheromones. For a species in which exposure can be controlled by pulsatile pheromone release in urine, it is possible that changing the temporal pattern of release could at least modify the response, if not change the nature of the signal. However, for a species in which a female releaser pheromone attracts the male, it likely will be the male's response, rather than the pattern of release *per se*, which plays the primary role in determining exposure. Finally, for a species in which a pheromone is released passively and does not influence the distance between signaller and receiver, responsiveness may be influenced primarily by factors (light intensity, photoperiod, group size, etc.) that might influence distance between conspecifics.

Understanding how temporal exposure pattern affects pheromone response is of practical importance because the cost of applied hormonal pheromone techniques will depend both on the level and duration of pheromone exposure required. 17,20β-P-induced GtH increase in male goldfish illustrates several aspects of this issue. When 17,20β-P exposure is constant, the GtH response does not appear to be graded by exposure duration, since the GtH levels reach within 15 min (the shortest exposure period examined; Dulka *et al.*, 1987a) are maintained for at least 8 hours (Figs. 5a and 5c; Dulka, 1989). Under similar constant exposue conditions, the response also does not seem to be graded by dosage (Dulka *et al.*, 1987c), in that exposure to any concentration above the 17,20β-P olfactory threshold (Sorensen *et al.*, 1987a) evokes maximal GtH increase (Fig. 5b). On the other hand, evidence for a graded response is seen if 17,20β-P exposure is terminated; GtH levels of males withdrawn from 17,20β-P exposure soon return to basal, whereas those of chronically stimulated males do not change (Fig. 5c). Unfortunately, we have not yet determined whether exposure to repetitive pulses of suprathreshold 17,20β-P (the exposure pattern most likely encountered under natural conditions) is more effective than constant 17,20β-P exposure.

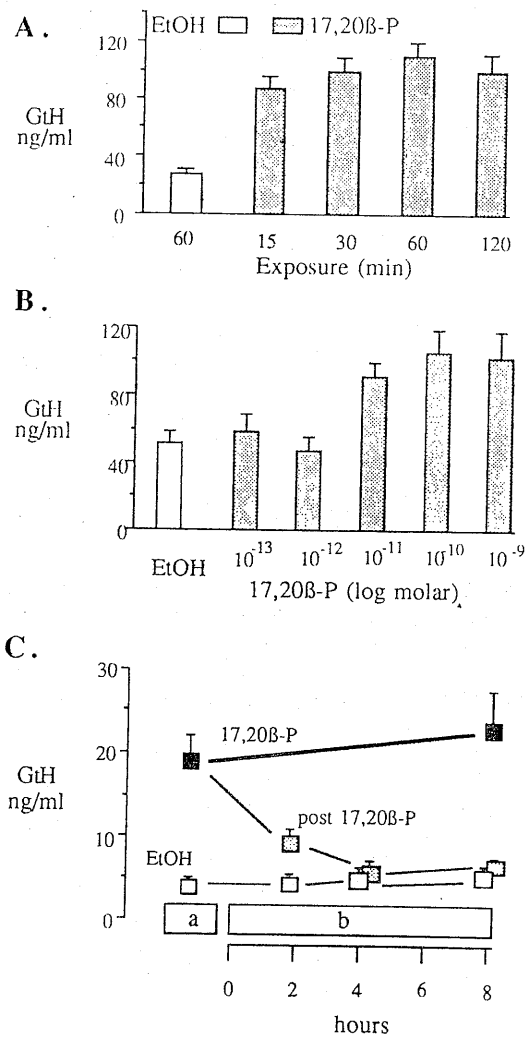

Fig. 5. Effects of time and dosage of exposure to water-borne 17,20 β-P on blood GtH levels in male goldfish. A. Bloof GtH levels (mean±S. E. M.) of males exposed to 5×10^{-10} M 17, 20 β-P are rapidly increased over levels in males exposed to ethanol vehicle (from Dulka *et al.*, 1987a). B. Blood GtH levels (mean±S. E. M.) in males placed in aquaria containing ethanol vehicle (Etoh) or 5 doses of 17, 20 β-P (from Sorensen *et al.*, 1990a). C. Blood GtH levels (mean±S. E. M.) in separate groups of males sampled after exposure to ethanol (clear symbols) or 5×10^{-10} M 17, 20 β-P (black symbols) for 30 min (a), and (b) after transfer to new aquaria containing either ethanol (clear and shaded symbols) or 5×10^{-10} M 17, 20 β-P. Blood GtH of males exposed to 17, 20 β-P returns to control levels within 4 h of removing the 17, 20 β-P stimulus.

Factors influencing responsiveness to pheromones

For any sex pheromone function, it is important to know whether the recipient's responsiveness change with reproductive condition and, if so, what mechanisms are involved. For proposed hormonal pheromones, there is evidence that females of two species respond behaviorally to male releaser pheromones only when they are ovulated (*Gobius jozo*, Colombo *et al.*, 1982; *Clarias gariepinus*, Resink *et al.*, 1987) and that male goldfish exhibit 17,20β-P-induced GtH increase only when the testis is active (Sorensen, unpublished results). Although no study has determined how such changes in pheromonal responsiveness are functionally related to changes in reprodutcive condition, there is circumstantial evidence for two general mechanisms which are not mutually exclusive: (1) hormone changes within the recipient affect the function of the sensory receptors that detect the hormonal pheromone; (2) hormone changes within the recipient alter the central processing of afferent activity stimulated by the hormonal pheromone.

Virtually all studies attempting to identify the sensory modality detecting sex pheromones have implicated the olfactory system (Liley, 1982; Stacey *et al.*, 1986; Sorensen, 1991). There also is weak evidence, not specifically related to reproduction, that taste is involved in some species (Sorensen, 1991); however, recordings from the facial taste system of goldfish have not detected responses to 17,20β-P or PGs (Sorensen, unpublished results). Furthermore, there is speculation (Demski and Northcutt, 1983) that the nervus terminalis (cranial nerve 0), which is anatomically associated with the teleost olfactory system (cranial nerve 1), may function in sex pheromone detection (Dulke *et al.*, 1987c; Kyle *et al.*, 1987). So far, only the olfactory system has been demonstrated to mediate responses to proposed hormonal pheromones. Anosmia, induced either by occulusion of the nares, cautery of the olfactory epithelium, or lesionning of the olfactory tracts, is fully effective in blocking hormonal pheromone responses in several species: *e.g. Brachydanio rerio* (Van Den Hurk and Lambert, 1983); *Clarias gariepinus* (Reink *et al.*, 1987, 1989d); *Carassius auratus* (Stacey and Sorensen, 1991; Dulka and Stacey, 1991). Fish olfactory bulbs connect to the brain by paired olfactory tracts, each of which is divided into medial and

lateral subdivisions (Keyl *et al.*, 1987). Selective lesionning of the lateral and medial subdivisions demonstrate that the medial tract, which contains fibres of the nervus terminalis mediates both behavioral (Stacey and Kyle, 1983; Resink *et al.*, 1987, 1989d) and endocrine (Dulka and Stacey, 1991) responses to hormonal pheromones. Further evidence comes from recordings of goldfish olfactory tract activity in which only the medial tract responded to 17,20β-P and prostaglandins (Sorensen *et al.*, 1989b). Recent studies in goldfish (Fujita *et al.*, 1991) indicate it is the olfactory components of the medial tracts, and not the nervus terminalis components, which are involved in these pheromonal responses; perfusing the olfactory epithelium with 17,20β-P or prostaglandins changed spontaneous activity of olfactory bulb neurons, but had no effect on nervus terminalis neurons.

There is evidence both for and against the proposal that changes in responses to pheromones are due to changes in olfactory receptor function (Sorensen, 1991). EOG recordings in goldfish (Sorensen *et al.*, 1987a) and common carp (Irvine and Sorensen, unpublished results) indicate that there is little if any effect of gender or maturity on either the sensitivity or magnitude of the response to 17,20β-P. In marked contrast, precocious male Atlantic salmon (*Salmo salar*) parr exhibit an extraordinary increase in EOG responsiveness to testosterone (a putative hormonal pheromone) at a time when juvenile male and female parr fail to respond (Moore and Scott, 1991). Although there is yet no direct evidence that a central mechanism is responsible for any change in responsiveness to a hormonal pheromone, the fact that female responsiveness to male odor develops quickly at ovulation (*e.g.* Belontiids; Lee and Ingersoll, 1979) makes it doubtful that an increase in olfactory receptor sensitivity is involved. It also seems unlikely that a circadian rhythm of olfactory receptor activity could explain the finding that the GtH response induced by exposure to PGF-treated females is maximal during scotophase (Dulka *et al.*, 1987b; Hontela and Stacey, 1990).

FUTURE PROSPECTS FOR HORMONAL PHEROMONES

The potentially widespread use of hormonal pheromones by teleosts should have considerable impact both on basic and applied aspects of fish

reproduction research. However, it seems likely that the relative impact in these areas will be determined by quite different factors.

In basic research, impact should be high if a large number of fish species have evolved hormonal pheromones, and if that number includes species which presently serve as models for a variety of problems related to reproduction. Because of the relative ease with which production and release of hormonal pheromones can be measured and manipulated, it seems reasonable that hormonal pheromone systems can serve as useful models with which to examine general problems related to the nature and evolution of chemical communication, and to the species-specificity of the chemical signals involved. However, in addition to serving as the focal points of such basic research programs, hormonal pheromones also can serve as valuable tools for basic research into the reproductive processes they normally influence. In goldfish, for example, there has been considerable pharmacological, neuroanatomical and neurochemical evidence that GtH release is normally inhibited by dopaminergic fibres innervating the pituitary, but no direct evidence that dopaminergic activity in the pituitary is alterred during physiologiçal GtH change (Peter *et al.*, 1986). Using pheromonal 17, 20β-P to induce GtH increase in male goldfish, Dulka *et al.* (1991) have shown that dopamine release to the pituitary (assessed by measuring dopamine turnover) is reduced shortly after pheromone exposure.

In applied research and the resulting practical applications, the impact of hormonal pheromones should be determined by three major factors: (1) whether the species of interest uses a hormonal pheromone, (2) whether there is any economic benefit to be gained from manipulating the pheromone's function, and (3) whether such a pheromonal technique is preferable to available alternatives. If the known effects of hormonal pheromones on ovulation (Sorensen and Stacey, 1987; Van Den Hurk *et al.*, 1987a) and sperm production (Dulka *et al.*, 1987a) are indicative of what could be expected in cultured species, then it is clear that some hormonal pheromone functions are worth manipulating. On the other hand, considering the effectiveness of an injection technique (using a combination of GnRH and a dopamine antagonist) for induced ovulation of many cultured teleosts (Peter *et al.*, 1988; Lin and Peter, 1990), it is far

from clear that pheromonal techniques for controlled gamete production can compete either in terms of cost or effectiveness. Factors which would appear to favor pheromonal over pharmacological injection techniques are that the pheromone: (1) can be applied without handling the fish, (2) induces a normal endogenous process, thus eliminating the possibility of alterring future fertility, (3) should only affect fully mature individuals, eliminating the labor and skill required to select suitable individuals for injection therapy.

To gain some insight into the occurrence of hormonal pheromones in fish, we recently have begun to use EOG recording to survey the responses of a variety of fish species to water-borne hormones and hormone metabolites (Table 2). Even from such a limited survey, there are a number of important observations. The first is that EOG responses to water-borne hormones are not resticted to goldfish (Sorensen *et al.*, 1987a, 1988, 1990a), African catfish (Resink *et al.*, 1989c), and salmonids (Sveinsson and Hara, 1989; Moore and Scott, 1991), but are seen in the 'majority of fish examined. However, it remains to be determined whether these EOG responses are indicative of pheromonal functions. The second important observation is that EOG responses to prostaglandins are more common than are responses to steroids. This finding is consistent with our prediction (Sorensen and Stacey, 1990) that, because prostaglandins appear to be the acute hormonal stimulus for female spawning behavior in a variety of fish (Stacey, 1987), released prostaglandins and their metabolites are excellent candidates for female releaser pheromones that trigger male sex behaviors (Fig. 2). A third finding from the EOG survey is that within the cyprinids examined, some (*e.g.* goldfish) respond better to free than to glucuronated 17, 20β-P, whereas other (*e.g.*, *Phoxinus eos*) exhibit the reverse order of responsiveness. Such diverse responses in related fish might provide insight into the nature of species-specific hormonal pheromones. Finally, in keeping with our proposal that hormonal pheromones have potential applications in fish culture, cultured species such as common carp, *Cyprinus carpio* (Irvine, unpublished results) and grass carp, *Ctenopharyngodon idellus*, exhibit sensitive EOG responses to progestins and prostaglandins. These findings should provide strong incentive for further studies of hormonal pheromones in

Table 2
Preliminary survey of EOG responses* 17, 20 β-P and F_2-prostaglandins (PGF) in fish

Order and species	17, 20 β-P		PGF
	free	glucuronated	
Osteoglossiformes			
Hiodon alosoides F	—	—	—
Gadiformes			
Lota lota M	—	—	—
Perciformes			
Perca flavescens F+	—	—	—
Gasteiformes			
Culaea inconstans F+	—	—	—
Scorpaeniformes			
Cottus asper MF	—	—	—
Siluriformes			
Ictalurus punctatus M+	—	—	—
Cypriniformes			
Catostomus commersoni MF	—	—	11
Catostomus catostomus MF	—	—	11
Moxostoma macrolepidotum MF	—	—	10
Brachydanio rerio F	—	—	10
Pimephales promelas F	7	10	9
Phoxinus eos MF	7	11	11
Semotilus margarita M+	9	11	9
Notropis hudsonius M	7	9	10
Campostoma anomalum M+	10	10	11
*Ctenopharyngodon idellus**	10	12	11
Carassius auratus MF	12	10	11

*=electro-olfactogram recordings as in Sorensen *et al.* (1987a, 1990a). All fish exhibited a consistent and measurable response to the standard stimulant, 10^{-5} M Alanine. Data presented indicate minimal dosage (log molar) required to elicit a consistent response. PGF data are for either prostaglandin F_{2a} or 15-keto-prostaglandin F_{2a}. M=only males examined. F=only females examined. MF=both sexes examined. ♯=only sterile triploids examined. +=only one individual examined. (Cardwell and Dulka, unpublished results)

these and related cyprinids of economic importance.

The results of our preliminary survey (Table 2) also illustrate a difficult problem with the EOG technique: how to interpret a lack of response. In some cases, there is sufficient additional information that an absence of EOG response can be regarded as evidence for absence of hormonal pheromone function. In the white sucker

(*Catostomus commersoni*), for example, lack of EOG response to 17,20β-P and related steroids may not be surprising. Because this steroid not only increases in spawning males, but also is elevated both prior to and after ovulation (Scott *et al.*, 1984), it may not convey information of sufficient specificity for pheromonal function. In other cases (*e.g.*, *Lota*, *Perca*) where the appropriate information on hormonal changes at spawning are lacking, it is not clear whether absence of EOG response indicates on pheromonal function, or instead results from a combination of olfactory specificity and inappropriate test substances. In the zebrafish (*Brachydanio rerio*), however, the total lack of EOG responses to a variety of free and glucuronated steroids is particularly puzzling, considering the evidence that steroid glucuronides serve as olfactory stimulants for male sexual behavior (Van Den Hurk and Lambert, 1983) and ovulation (Van Den Hurk *et al.*, 1987a). Finally, in view of Moore and Scott's (1991) findings that EOG responses of *Salmo salar* exhibited dramatic increases near spawning, it is difficult to eliminate the possibility that some negative response were due to inappropriate reproductive state of the test fish.

In general, our preliminary EOG survey indicates a serious shortcoming with this otherwise powerful technique: unless it is known that the test substances include all significant hormones and metabolites released by a species, only positive responses are useful in characterizing the pheromone. The solution, of course, is that EOG recording should not be carried out in isolation, but rather should be an interactive component of multidisciplinary research effort encompassing field studies of a species' reproductive ecology, endocrinological determinations of circulating and released hormones at appropriate reproductive stages, careful analyses of behavioral and physiological responses to both crude conspecific odors and putative hormonal pheromones, and field trials of hormonal pheromones with potential for practical application. Only the work on the African catfish conducted at the University of Utrecht has approached this level of coordinated research effort. Hopefully it will not be long before other laboratories will follow their example.

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