The Journal of Experimental Biology 200, 2833–2840 (1997)
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JEB1102

THE STEROID PHEROMONE 4-PREGNEN-17α,20β-DIOL-3-ONE INCREASES FERTILITY AND PATERNITY IN GOLDFISH

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Accepted 21 August 1997

Summary

Previous studies in goldfish (*Carassius auratus*) showed that the oocyte maturation-inducing steroid 4-pregnen-17α,20β-diol-3-one (17,20βP) functions after release as a pheromone that increases male serum gonadotropin II (GtH II) concentration, milt (sperm and seminal fluid) volume and sexual activity, effects hypothesized to increase male reproductive success in the sperm competition of multi-male spawnings. The present study tested this hypothesis by determining whether overnight exposure to 17,20βP increases fertility. In pair spawnings, 17,20βP-exposed males fertilized a greater percentage of eggs than did control males, apparently because 17,20βP-exposed males had more releasable sperm at the onset of spawning. Microsatellite DNA paternity analysis showed that 17,20βP-exposed males also fertilized more eggs in competitive spawnings involving one control male and one 17,20βP-exposed male. This effect of 17,20βP on competitive fertility could be due to demonstrated increases in spawning activity, milt volume, duration of sperm motility and proportion of motile sperm. However, it appears that a change in sperm quality is a major component of the pheromonal effect because, in competitive *in vitro* fertilizations, sperm from 17,20βP-exposed males fertilized more eggs than did sperm from control males. The results indicate that the response to pheromonal 17,20βP is a major determinant of reproductive success in male goldfish.

Key words: 4-pregnen-17α,20β-diol-3-one, pheromone, paternity, goldfish, *Carassius auratus*, sperm, microsatellite DNA fingerprinting, fertility.

Introduction

Since Colombo *et al.* (1982) first provided evidence that an androgen metabolite (etiocholanolone glucuronide) is a sex pheromone in a goby (*Gobius jozo*), many studies have shown that a diversity of fish use released hormones (steroids, prostaglandins and their metabolites) as hormonal pheromones (for reviews, see Sorensen, 1992*a*,*b*; Stacey and Sorensen, 1991; Stacey *et al.* 1994*a*; Stacey and Cardwell, 1995). Hormonal pheromone function is best understood in the goldfish (*Carassius auratus*), in which the periovulatory female sequentially releases a preovulatory steroidal pheromone that increases milt (sperm and seminal fluid) volume prior to spawning and a postovulatory prostaglandin pheromone that triggers male sexual behaviour (Sorensen, 1992*b*; Stacey *et al.* 1994*a*). The importance of the postovulatory prostaglandin pheromone to male reproductive success has been demonstrated by studies showing that males normally commence sexual activity in response to F prostaglandins released by ovulated females, but exhibit almost no sexual response if they are rendered anosmic (Partridge *et al.* 1976; Sorensen *et al.* 1988, 1989, 1995*a*; Stacey and Kyle, 1983). In contrast, no study has determined whether exposure to the preovulatory pheromone affects subsequent reproductive success.

During their preovulatory gonadotropin II (GtH II) surge, female goldfish release a resultant surge of three 21-carbon steroids that are major components of the preovulatory pheromone (Scott and Sorensen, 1994; Sorensen *et al.* 1990, 1995*b*; Stacey*et al.* 1989): the maturation-inducing steroid 4-pregnen-17α,20β-diol-3-one (17,20βP) and two of its conjugated metabolites, 17,20βP-20β-glucuronide (17,20βP-20G) and 17,20βP-20β-sulphate (17,20βP-20S). Electro-olfactogram studies (Sorensen *et al.* 1987, 1990, 1995*b*) show that 17,20βP and 17,20βP-20S are the most potent of the three (detection thresholds between 10^{-12} and 10^{-11} mol l^{-1}) and indicate they act *via* two distinct olfactory receptor mechanisms, the mechanism detecting 17,20βP also responding to 17,20βP-20G. Although male goldfish increase serum GtH II concentration in response to each of the three steroids, only the mechanisms mediating responses to 17,20βP have been investigated. 17,20βP-induced sensory activity projects centrally *via* the medial olfactory tracts (Sorensen *et al.* 1991) and reduces dopaminergic inhibition of GtH II

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secretion (Dulka *et al.* 1992; Zheng and Stacey, 1997), resulting in an increase in serum GtH II concentration within 15 min, an increase in GtH-II-stimulated stripped milt volume within 6 h and an increase in sperm motility the following morning (DeFraipont and Sorensen, 1993; Dulka *et al.* 1987; Zheng and Stacey, 1996). In addition to these neuroendocrine and gonadal effects, pheromonal 17,20βP also increases swimming and spawning activity (Bjerselius *et al.* 1995*a*; DeFraipont and Sorensen, 1993).

In the present study, we determine whether exposure to 17,20βP increases male fertility, both in pair spawning (one male, one female) and in situations where sperm from control and pheromone-exposed males compete for access to eggs.

Materials and methods

Study fish and maintenance

Goldfish *Carassius auratus* were purchased from Ozark Fisheries Co., Inc., Stoutland, Missouri, USA, and kept for various times in 1000 l or 3400 l flow-through fibreglass stock aquaria at 17–20 °C (males) or 12–15 °C (females). In the stock tanks and experimental aquaria described below, fish were held under a 16 h:8 h L:D photoperiod (lights on at 08:00 h) and fed at least once per day with commercial flake food (Nutrafin, R. C. Hagen Ltd, Montreal, Canada).

Pheromone exposure

Male goldfish (28–55 g) were exposed to water-borne 17,20βP using procedures described previously (Zheng and Stacey, 1996, 1997). Mature males (identified by the presence of expressible milt and tubercles on the pectoral fins or opercula) were removed from stock tanks on day 1, anaesthetized by immersion in 0.05 % 2-phenoxyethanol (Syndel), and weighed. After assigning fish to treatment groups that would not differ in mean body mass, fish were placed in trios in 60 l, flow-through glass experimental aquaria at 20 °C. At 22:00 h on day 3, the water was shut off and each aquarium received either 100 µl of ethanol vehicle (control) or 17,20βP (Sigma, St Louis, Missouri) in 100 µl ethanol to create a water concentration of 5×10^{-10} mol l^{-1}.

Ovulation induction and spawning tests

At 21:00 h on day 2, 12 h before spawning tests, postvitellogenic female goldfish (with soft, distended abdomens) were injected intraperitoneally with Ovaprim (Syndel, Vancouver, British Columbia, Canada; 0.5 ml kg^{-1}), to induce ovulation (Peter *et al.* 1988), and placed in 100 l flow-through (20 °C) glass spawning aquaria containing a floating spawning substratum (green acrylic yarn). If ovulation occurred the following morning (determined by gently squeezing the abdomen to release eggs from the ovipore), one (non-competitive pair spawning) or two males (competitive trio spawning) were added to the spawning aquarium. Typically, newly introduced males begin to follow and chase an ovulated female within several minutes and continued to do this throughout the duration of spawning. Spawning acts occur when the female (followed by the male) enters the floating spawning substratum and the pair (with the male behind and below) turn on their sides and press briefly together to release gametes. Depending on the number of eggs ovulated, females will perform up to 100 spawning acts before all the ovulated eggs are shed. At the termination of spawning, the spawning substratum was removed, and the adhering eggs were counted and incubated to the eyed stage or hatching.

To determine how prior exposure to pheromonal 17,20βP affects fertility over the course of non-competitive pair spawning, the fertility of control and 17,20βP-exposed males was determined on the basis of the number of eggs fertilized during two spawning acts, performed either without prior exposure to spawning females or after 1 or 2 h of spawning activity immediately preceding the test. For males tested without prior spawning, one control or 17,20βP-exposed male was placed with an ovulated female and, when two spawning acts had been performed, the male and spawning substratum (with adhering eggs) were removed and immediately replaced with a second male (17,20βP-exposed or control) and fresh substratum. This procedure (alternately allowing control and 17,20βP-exposed males to perform two spawning acts) was continued with the same female until six males (from one control trio and one 17,20βP-exposed trio) had been tested; it was repeated for three additional females (N=12 males each for control and 17,20βP-exposed groups). On alternate days, the same procedure was used to compare the fertility of control and 17,20βP-exposed trios (N=6 males each for control and 17,20βP-exposed groups) that had been spawning for 1 or 2 h with a pair of non-ovulated females induced to spawn by intramuscular injection of approximately 200 ng g^{-1} prostaglandin $F_{2\alpha}$ (Upjohn, Kalamazoo, Michigan, USA) (Zheng and Stacey, 1996, 1997). For all treatment groups, eggs adhering to the removed floating substrata after removal from the aquarium were incubated for 1 day to determine the proportion fertilized.

The effect of prior spawning activity on fertility was analyzed separately for control and 17,20βP-exposed groups using Kruskal–Wallis nonparametric analysis of variance (ANOVA) and Dunn's multiple-comparisons tests. Differences between control and 17,20βP-exposed groups at each of the three treatment levels (0, 1 and 2 h of prior spawning activity) were analyzed using Mann–Whitney U-tests. These and all other statistical procedures in this study employed two-tailed probabilities with α set at 0.05.

Microsatellite DNA fingerprinting

We used microsatellite DNA fingerprinting (Zheng *et al.* 1995) to determine whether prior exposure to pheromonal 17,20βP increases paternity in competitive trio spawning, in which one control male and one 17,20βP-exposed male compete for the opportunity to fertilize the eggs of one ovulated female. Prior to behavioural testing, DNA was extracted (QIAamp Tissue Kit; QIAGEN Inc., Santa Clara, California, USA) from the blood of potential experimental fish and genotyped for goldfish locus 1 (GF1) (Zheng *et al.* 1995)

to enable the selection of spawning trios that would yield undisputed paternity of the hatched offspring. On the morning of day 3, one control and one 17,20βP-exposed male (selected to have equivalent body masses) were placed with each of four ovulated females, and each spawning trio was observed continuously throughout the duration of spawning to determine the relative spawning activities of both males (see below). When each trio had completed spawning, the floating substratum and adhering eggs were removed, the eggs were incubated to hatching, and a random sample of 72 larvae was taken for genotyping (Zheng *et al.* 1995). After the first spawning test, males from two of the four trios were held without further treatment for 1 month. The two previous control males were then exposed to 17,20βP, the two previous 17,20βP-exposed males were exposed to ethanol vehicle, and a second competitive spawning test was conducted using new females. All other procedures were the same as those used in the first four competitive spawnings. The effect of 17,20βP exposure on the proportion of eggs fertilized was analyzed separately for each competitive spawning using χ^2-tests.

From the behavioural observations made during the spawning of each trio, each male was assigned a spawning score on the basis of its participation and position in each spawning act, the assumption being that the male closer to the female during the spawning act would have the greater opportunity to fertilize eggs. A spawning score (the sum of a male's performance during all spawning acts) was then calculated using the following behavioral categories: 1 point, the male participated but was separated from the female by the other male; 2 points, both males participated equally, one on each side of the female; 3 points, the male was between the female and the other male during the spawning act; 4 points, only one male participated in the spawning act. Between males of each pair, differences in spawning scores, and in the number of spawning acts performed (regardless of the category of participation), were analyzed using Wilcoxon matched-pairs test. Because the numbers of spawning acts differed among the male pairs, the relationship between spawning activity and fertility was examined (Spearman rank correlation) using two forms of normalized behavioural data: *spawning ratios* (the total number of spawning acts of the 17,20βP-exposed male expressed as a proportion of the total number of spawning acts performed by both males of the pair) and *normalized spawning scores* (the spawning score of each 17,20βP-exposed male expressed as a proportion of the combined spawning scores of both males in the pair).

Microsatellite DNA fingerprinting (Zheng *et al.* 1995) was also used to determine whether exposing males to 17,20βP affects the fertility of their sperm in competitive *in vitro* fertilization. At 09:00 h on day 3, control and 17,20βP-exposed males were allowed to spawn with non-ovulated, prostaglandin $F_{2\alpha}$-injected females for 1 h, to ensure that control males had sufficient milt for stripping (Zheng and Stacey, 1996, 1997). Male pairs (one control and one 17,20βP-exposed male previously genotyped and selected to give undisputed paternity with the ovulated female) were then anaesthetized, inverted in a moist, slotted foam pad, and voided of urine by applying gentle pressure to the cloacal area. Milt was then stripped by applying gentle pressure to the abdomen, aspirated into haematocrit tubes, and held on ice in polypropylene tubes. A 100 µl milt mixture (50 µl from each male) was then added to approximately 300 ovulated eggs in a plastic dish, and fertilization was initiated by adding aquarium water. Eggs were incubated to hatching, and a random sample of 72 larvae was taken for paternity analysis (Zheng *et al.* 1995). Competitive *in vitro* fertilizations were conducted on four male pairs, each tested with eggs from different females. As with competitive spawning, the effect of 17,20βP exposure on paternity was analyzed for each competitive *in vitro* fertilization using χ^2-tests.

Effects of 17,20βP on GtH II and sperm parameters

To determine how 17,20βP might enhance fertility during competitive spawning or competitive *in vitro* fertilization, we examined, in two groups of males, the effects of 17,20βP exposure on serum GtH II concentration, milt volume and the concentration, number and motility of sperm. Both groups were exposed to either 17,20βP or ethanol vehicle from 22:00 h on day 2, and anaesthetized at 08:00 h on day 3 to strip milt and to remove a blood sample from the caudal vasculature for determination of serum GtH II concentration (Van Der Kraak *et al.* 1992).

Milt from the first male group was used to determine the effect of exposure to 17,20βP on the number and concentration of sperm in each male's sample. After vortexing the stripped milt sample, a 5 µl sample was diluted 1:1000 in Isoton (Coulter Electronics, Burlington, Ontario, Canada), and a 1 ml sample of the resulting vortexed dilution was further diluted 1:20 in Isoton to yield a final dilution of 1:20 000. Three 200 µl samples of this dilution were again diluted 1:100 in Isoton and used to measure sperm concentration (mean of three replicates for each male) in a Coulter counter (Multi-Sizer, Coulter Electronics). The total volume of each milt sample was then used to calculate the number of sperm removed by stripping.

Milt from the second male group was used to examine the effect of exposure to 17,20βP on sperm motility. Stripped milt samples were set on ice until all males had been sampled. Then, working alternately with samples from control and 17,20βP-exposed fish (to ensure that milt samples from control and experimental fish were on ice for equivalent times), a 5 µl sample from each milt sample was diluted 1:150 in 150 mmol l^{-1} NaCl (pH 7.2), in which sperm are immotile. To activate sperm motility, three 1 µl samples from each saline dilution were further diluted 1:20 in aquarium water at 20 °C on a glass slide. Viewing by eye a 0.44 mm diameter field at 400× magnification, the duration of motility (mean of three replicates for each male) was determined as the time from activation until the last sperm stopped moving. In the same activated samples, the percentage of motile sperm was determined by counting both the sperm that were non-motile when the sample was first activated and the total number of sperm in the sample when all motion had ceased.

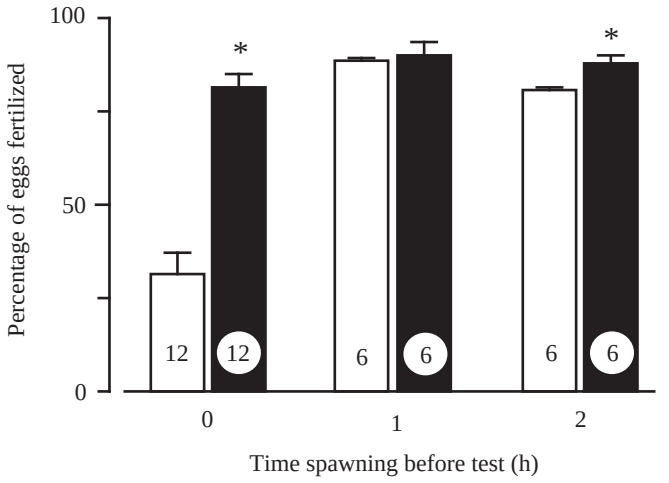

Fig. 1. Fertilization rate (mean + S.E.M.) during non-competitive spawning (one male, one female) is increased by immediate prior spawning activity and by overnight exposure to pheromonal 17,20βP. *P<0.05, 17,20βP *versus* control. 17,20βP-exposed males (filled bars); ethanol-exposed control males (open bars). Values of N are given within columns.

The effects of 17,20βP exposure on serum GtH II concentration, sperm concentration, sperm number, the duration of sperm motility and the proportion of motile sperm were analyzed using unpaired *t*-tests. The effects on milt volume were analyzed using Mann–Whitney *U*-tests.

Results

Fertility in non-competitive pair spawning

Over the total course of pair spawning, males exposed overnight to 17,20βP fertilized a significantly greater percentage of eggs (94±1.5 %; N=4) than did control males (81±4.8 %; N=4) (P<0.05; *t*-test).

When fertility during pair spawning was determined on the basis of the number of eggs fertilized during two spawning acts, either at the onset of spawning or after 1 or 2 h of prior spawning activity, males exposed to 17,20βP fertilized an equivalent percentage of eggs (P>0.05) at all three sample times (Fig. 1). In contrast, the percentage of eggs fertilized by control males was significantly lower (P<0.0001) at the onset of spawning than after prior spawning activity and was also significantly lower than that of 17,20βP-exposed males both at the onset of spawning (P<0.0001) and after 2 h of prior spawning activity (P<0.01) (Fig. 1). On the basis of the number of eggs used to compare the fertility of control and 17,20βP-exposed males (i.e. those eggs adhering to the floating substratum), females (N=8) released equivalent numbers of eggs (P>0.05; Wilcoxon test) when spawning with control males (median 556 eggs released during six spawning acts) and 17,20βP-exposed males (median 678 eggs).

Fertility in competitive trio spawning and in vitro *fertilization*

In each of the four competitive spawnings in which males were being tested for the first time, males exposed overnight to 17,20βP fertilized significantly more eggs (P<0.05–0.001) than did control males (Table 1). Furthermore, when two of the male pairs (1R and 2R in Table 1) received reversed treatments 1 month after the initial spawning test, they exhibited corresponding reversals of fertility, with the 17,20βP-exposed males again achieving significantly more fertilizations (P<0.01, 0.001) than the control males.

In the six competitive pair spawning tests, the 17,20βP-

Table 1. *Effect of 17,20βP on male fertility during competitive spawning*

	Genotypes[a]								No. of larvae[b]	Paternity ratio (17,20βP male: control male)	χ^2
	Control male	17,20βP male	Female	Larvae of control male (number)		Larvae of 17,20βP male (number)					
Pair 1	313/313 [38.2][c]	301/307 [37.4][c]	301/301	301/313 (25)		301/301 (18)	301/307 (29)		72	1.9:1	P<0.01
Pair 1R[d]	301/307	313/313	301/301	301/301 (10)	301/307 (14)	301/313 (48)			72	2.0:1	P<0.01
Pair 2	301/301 [32.5]	313/313 [34.6]	301/311	301/301 (7)	301/311 (8)	301/313 (29)	311/313 (28)		72	3.8:1	P<0.001
Pair 2R[d]	313/313	301/301	301/301	301/313 (6)		301/301 (36)			42	6.0:1	P<0.001
Pair 3	313/313 [36.8]	307/311 [39.1]	307/311	307/313 (12)	311/313 (10)	307/307 (8)	307/311 (18)	311/311 (13)	61	1.8:1	P<0.05
Pair 4	301/301 [42.3]	311/311 [39.8]	301/313	301/301 (2)	301/313 (1)	301/311 (28)	311/313 (27)		58	18.3:1	P<0.001

[a]Genotype numbers represent the sizes of the alleles at GF1; [b]some of the 72 samples were lost during processing; [c]body mass (g); [d]males received reversed treatments 1 month after the first test.

exposed male performed more spawning acts than the control male in four tests, the same number of acts in one test, and one less than the control male in the sixth test; similarly, 17,20βP-exposed males had a higher spawning score than the control male in five tests. Although there was no significant difference between either the number of spawning acts (P=0.12) or the spawning scores (P=0.062) of 17,20βP-exposed and control males, the trend for greater spawning activity in 17,20βP-exposed fish is clear from the distributions of spawning ratios and spawning scores in Fig. 2. Both spawning ratios (Fig. 2A) and normalized spawning scores (Fig. 2B) were correlated significantly (P=0.033) with the percentage of eggs fertilized.

In each of the four *in vitro* fertilizations, 17,20βP-exposed males fertilized significantly more eggs than did control males (P<0.05–0.001; Table 2).

Effects of 17,20βP on GtH II levels and sperm parameters

Exposure to 17,20βP significantly increased serum GtH II concentration (P<0.05) and stripped milt volume (P<0.05) in both groups of males (Fig. 3A,B). In the first male group, the sperm concentration in milt from fish exposed to 17,20βP was significantly lower than in milt from control fish (P<0.05); however, owing to the larger stripped milt volumes of fish exposed to 17,20βP, the numbers of sperm stripped from this group were significantly greater than those from control fish (P<0.05; Fig. 3A). Compared with control males in the second male group, males exposed to 17,20βP had a significantly greater proportion of motile sperm (P<0.05), and their sperm remained motile for longer (P<0.05) (Fig. 3B).

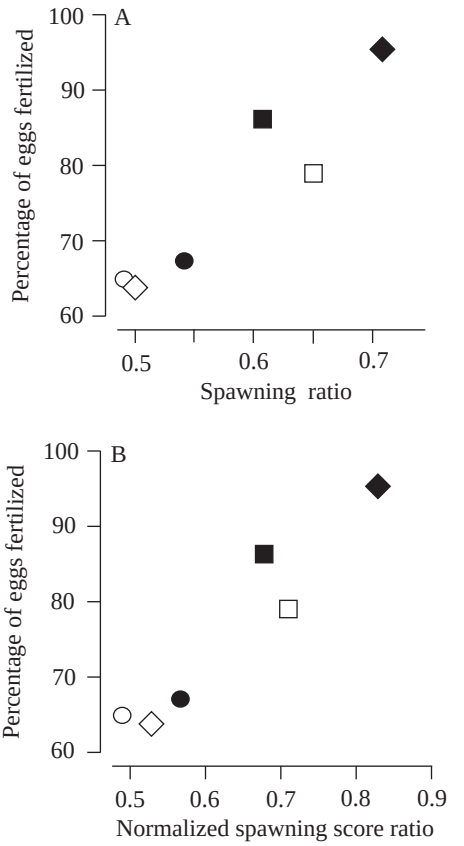

Fig. 2. Relationship between the spawning activity of 17,20βP-exposed males and the proportion of eggs (attached to floating substratum) they fertilized during spawning competition with a control (ethanol-exposed) male. (A) Spawning ratio: the number of spawning acts performed by the 17,20βP-exposed male divided by the total number of spawning acts performed (singly and in competition) by both males. (B) Normalized spawning score ratio: the normalized spawning score (see text) of the 17,20βP-exposed male divided by the summed normalized spawning scores of both males. ○ = pair 1; ● = pair 1 after reversed treatment; □ = pair 2; ■ = pair 2 after reversed treatment; ◇ = pair 3; ◆ = pair 4.

Discussion

The results of this study demonstrate a clear biological function for pheromonal 17,20βP in goldfish by showing that the previously demonstrated effects of the pheromone on male serum GtH II concentration (Dulka *et al.* 1987; Zheng and Stacey, 1997), milt volume (Zheng and Stacey, 1996), sperm

Table 2. *Effect of 17,20βP on male fertility during competitive* in vitro *fertilization*

	Genotypes[a]												
	Control male	17,20βP male	Female	Larvae of control male (number)				Larvae of 17,20βP male (number)		No. of larvae[b]	Paternity ratio (17,20βP male: control male)	χ^2
Pair 1	313/313	301/307	301/301	301/313 (8)				301/301 (30)	301/307 (27)	65	7.1:1	P<0.001
Pair 2	305/311	313/313	301/313	301/305 (3)	301/311 (3)	305/313 (3)	311/313 (2)	301/313 (24)	313/313 (24)	59	4.4:1	P<0.001
Pair 3	305/311	301/313	313/313	305/313 (3)	311/313 (1)			301/313 (32)	313/313 (36)	72	17.0:1	P<0.001
Pair 4	307/311	301/313	313/313	307/313 (13)	311/313 (9)			301/313 (22)	313/313 (15)	59	1.7:1	P<0.05

[a]Genotype numbers represent the sizes of the alleles at GF1; [b]some samples were lost during processing.

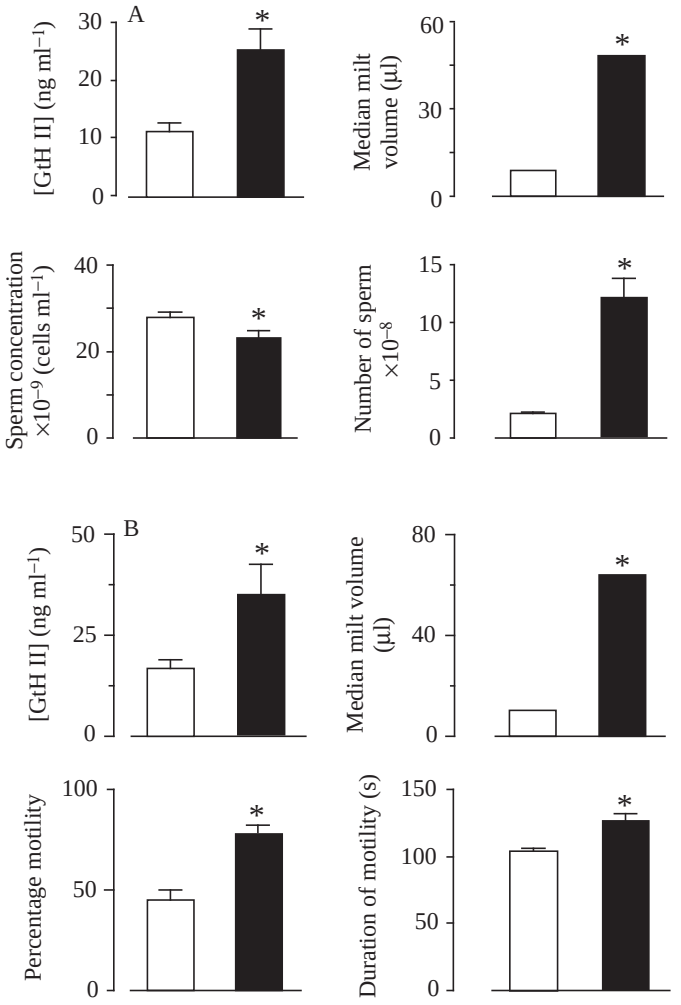

Fig. 3. Effect of pheromonal 17,20βP on serum gonadotropin II (GtH II) level, stripped milt volume and sperm parameters in two groups (A and B; see Materials and methods) of male goldfish. Milt volume data are presented as medians; all other data are presented as mean + S.E.M. **P*<0.05, 17,20βP *versus* control. 17,20βP-exposed males (filled bars); ethanol-exposed control males (open bars). (A) *N*=6; (B) *N*=9.

motility (Defraipont and Sorensen, 1993) and behaviour (Sorensen *et al.* 1989; DeFraipont and Sorensen, 1993; Bjerselius *et al.* 1995*a*) are associated with a dramatic increase in fertility during competitive spawning. The present findings also indicate that the effect of pheromone on fertility during spawning is achieved by changes in both male behaviour and sperm function.

Our finding of increased male spawning behaviour following overnight exposure to 17,20βP, although not statistically significant, is consistent with an earlier study (DeFraipont and Sorensen, 1993) which used non-ovulated, prostaglandin $F_{2\alpha}$-injected females (rather than ovulated females) as spawning partners, and assessed male behaviour using different measurements from those we have employed. Despite these consistent behavioural responses to the pheromone under different experimental conditions, the relative roles of behavioural and testicular responses in pheromone-enhanced fertilization success during spawning is unclear. Certainly, the correlation between male spawning performance and fertilization success (Fig. 2) indicates that the increased spawning activity of 17,20βP-exposed males contributed to their greater fertilization success. However, increased behavioural activity was not solely responsible for the increased fertilization success of 17,20βP-exposed fish, because they achieved significantly more fertilizations in all six competitive spawning tests, including those tests (pairs 1 and 3 in Fig. 2) in which their spawning activity was equivalent to that of their ethanol-exposed competitors.

In competitive spawning, the non-behavioural factors most likely to be responsible for the increased fertility of 17,20βP-exposed males are changes in sperm function and increased numbers of released sperm. Our present finding that sperm from 17,20βP-exposed males are more fertile than sperm from control males in competitive *in vitro* fertilization (Table 2) indicates that a pheromone-induced change in sperm function contributes to the increased fertilization success of 17,20βP-exposed males in competitive spawning (Table 1). Indeed, the relative *in vitro* fertility of sperm from 17,20βP-exposed males is probably even greater than the *in vitro* fertilization data (Table 2) suggest. For example, because milt from 17,20βP-exposed males has a lower sperm concentration (Fig. 3A), our use of equal volumes of milt from control and 17,20βP-exposed males probably resulted in greater numbers of control male sperm in the *in vitro* competitions. Also, it is possible that the difference in *in vitro* fertility between the sperm of control and 17,20βP-exposed males would have been greater had we not employed a 1 h spawning session to ensure that control males would have sufficient milt for stripping; DeFraipont and Sorensen (1993), using semi-quantitative measures of sperm motility and motility duration, report that these measures increase significantly in control males after a similar spawning session.

At present, there is no information on either the numbers of sperm released by spawning goldfish or the possible effects of prior pheromone exposure or male competition on sperm release. The fact that overnight exposure to 17,20βP consistently increases the volume of milt (Dulka *et al.* 1987; Sorensen *et al.* 1989; DeFraipont and Sorensen, 1993; Zheng and Stacey, 1996, 1997) and the number of sperm (Fig. 3) that can be stripped from male goldfish suggests that pheromone-exposed males are capable of releasing more sperm during spawning. If true, lower numbers of sperm released by control males could contribute both to their lower fertilization success in competitive spawning and to their initially low rates of fertilization during non-competitive spawning (Fig. 1), before spawning activity increases their milt volume (Kyle *et al.* 1985; DeFraipont and Sorensen, 1993; Zheng and Stacey, 1996).

The number of sperm released by spawning goldfish may not be determined simply by the number of sperm in the sperm ducts. For example, in bluehead wrasse (*Thalassoma bifasciatum*) and bucktooth parrotfish (*Sparisoma radians*), the

number of sperm released by spawning males is positively correlated with the number of eggs released by females (Shapiro *et al.* 1994; Marconato and Shapiro, 1996). Evolution of the ability to adjust the numbers of sperm released to the numbers of eggs released (probably by assessing female size) should have been facilitated in these species by the fact that the numbers of eggs ovulated is positively correlated with body size (Shapiro *et al.* 1994) and that females release all their ovulated eggs in a single spawning act (Warner *et al.* 1975; Marconato and Shapiro, 1996). In male goldfish, a similar mechanism to match sperm release to egg release seems unlikely, because female goldfish release their eggs in a variable number of spawning acts.

The physiological mechanisms mediating the effect of 17,20βP exposure on the fertility of male goldfish are unknown. The pheromone-induced increase in milt volume appears to be mediated by increased pituitary release of GtH II, since cutting the medial olfactory tracts (Dulka and Stacey, 1990), removing the pituitary gland (Dulka *et al.* 1987; Zheng and Stacey, 1996) or injecting dopamine D2 receptor agonists (Zheng and Stacey, 1997) blocks not only the pheromone-induced increase in GtH II levels, but also the increase in milt volume. However, it is not known whether pituitary factors other than GtH II are involved or even whether increases in serum GtH II levels and milt volume are necessary for the pheromone to affect fertility. There also is no information about how 17,20βP exposure increases *in vitro* fertility. Milt from 17,20βP-exposed males contains proportionately more motile sperm, with longer durations of motility (Fig. 3B; DeFraipont and Sorensen, 1993), but it remains to be determined whether the effects of pheromonal 17,20βP on *in vitro* fertility are mediated by changes in motility or by changes in other aspects of sperm function.

Assuming that the observed increases in sperm motility in 17,20βP-exposed fish are functionally related to increased *in vitro* fertility, studies of mammals (Baldi *et al.* 1995) and other fish strongly suggest that pheromonal 17,20βP increases the fertility of goldfish sperm through GtH-II-induced steroidogenesis. In masu salmon (*Oncorhynchus masou*), for example, injection of 17,20βP dramatically increases sperm motility within 2 days, evidently by elevating sperm duct pH which, in turn, increases sperm cyclic AMP levels (Miura *et al.* 1992), the latter known to be involved with motility of fish sperm (e.g. Cosson *et al.* 1996). Miura *et al.* (1991) report similar effects of 17,20βP on sperm motility and ductal pH in Japanese eel (*Anguilla japonica*). Rainbow trout (*Oncorhynchus mykiss*) sperm synthesize 17,20βP from 17α-hydroxyprogesterone (Ueda *et al.* 1984), suggesting that the trout sperm stimulate the pH changes and changes in cyclic AMP levels that increase their motility. If this is the case, then the effect of pheromonal 17,20βP on goldfish *in vitro* fertility might be mediated by 4-pregnen-17α,20α-diol-3-one (17,20αP) rather than by 17,20βP, because sperm of the closely related common carp (*Cyprinus carpio*) preferentially synthesize 17,20αP from 17α-hydroxyprogesterone (Asahina *et al.* 1990).

In conclusion, the results of this study not only provide the first evidence that a hormonal pheromone can dramatically influence reproductive success in fish, but also demonstrate that such pheromones can rapidly change sperm function, findings that should broaden perspectives on mechanisms of sperm competition. Although pheromone-enhanced fertility is now documented only for goldfish, we suspect that similar pheromonal mechanisms exist in other fish species, because pheromonal 17,20βP also increases GtH II levels and milt volume in crucian carp (*Carassius carassius*; Bjerselius *et al.* 1995*b*) and common carp (Stacey*et al.* 1994*b*), and because 17,20βP and related steroids are potent olfactory stimulants (and therefore putative pheromones) in many fish species where sperm competition is likely to be an important determinant of male reproductive success (Stacey and Cardwell, 1995).

This work was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada to C.S. and N.S. We thank J. Coffin for assistance with DNA fingerprinting.

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