



Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*

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Oestrogenic endocrine-disrupting chemicals released into the environment have the potential to affect animal behaviour. This study examined the relationship between plasma levels of gonadal steroids and behaviour and the effects of exogenous hormonal perturbation on the behaviour of nesting male three-spined stickleback. Plasma gonadal steroid concentrations were related to levels of nesting and aggressive behaviours: levels of oestradiol were negatively related to courtship behaviours while levels of 11-ketotestosterone were negatively related to nesting behaviours. The behaviour of male three-spined stickleback exposed to environmentally relevant concentrations of an endocrine disrupter differed from the behaviour of control males. Control males increased their aggressive response to a live male conspecific over time, while males exposed to ethinyl oestradiol decreased their aggressive response. This study offers further evidence that low levels of endocrine-disrupting chemicals in the environment may exert subtle yet important effects on animal behaviour.

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Given the important role of hormones in regulating behaviour (Nelson 1995), it is possible that hormones in the environment might affect animal behaviour. Endocrine disrupters are anthropogenic chemicals released into the environment that can affect the vertebrate endocrine system (Crisp et al. 1998; Crews et al. 2000). Acute exposure to oestrogenic endocrine disrupters has caused physiological changes such as the production of vitellogenin, an oestrogen-induced yolk precursor, in male fish (Hansen et al. 1998) and morphological effects such as demasculinization in reptiles (Guillette et al. 1995). Because gonadal steroids influence reproductive and territorial behaviours in many vertebrate species (Bass 1998; Ketterson & Nolan 1999), hormonal perturbations along the hypothalamus-pituitary-gonadal axis by oestrogenic endocrine disrupters could produce deleterious effects on multiple behavioural systems in a variety of taxa. However, only a few studies have looked at the effects of endocrine disrupters on behaviour. Endocrine disrupters have been found to affect sexual behaviour in Japanese quail, *Coturnix japonica* (Halldin et al. 1999), territorial marking behaviour in mice (Parmigiani et al. 1998; Vom Saal et al. 1998) and a male sexual display in guppies (Bayley et al. 1999).

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Male three-spined stickleback are suitable subjects for a study on the effects of endocrine disrupters on reproductive behaviour because their reproductive behaviours have been well studied (Wootton 1976, 1984; Rowland 1989; Bell & Foster 1994) and are influenced by gonadal steroids (Borg & Mayer 1995). For example, castrated males do not display nest-building, territorial or courtship behaviours (Hoar 1962; Wai & Hoar 1963; Baggerman 1966; Borg 1981, 1987; Borg et al. 1993; Borg & Mayer 1995; Bornestaf et al. 1997). In most mammalian species, aromatization of androgens to other hormones, including oestrogens, is essential for androgens to affect behaviour (Borg & Mayer 1995). However, in stickleback, 11-ketotestosterone (11KT), a nonaromatizable androgen, is the most effective androgen in restoring reproductive behaviour in castrated males (Borg 1987). Although it is unclear where naturally occurring oestrogens act along the hypothalamus-pituitary-gonadal axis in stickleback, if naturally occurring oestrogens play a role in regulating male reproductive behaviour in three-spined stickleback, it is more likely to be inhibitory than stimulatory.

The endocrine disrupter that I used in this experiment is ethinyl oestradiol, which was selected for the following reasons. Ethinyl oestradiol is a potent synthetic oestrogen and is the active ingredient of the contraceptive pill and postmenopausal hormone replacement therapy (Sumpter

et al. 1996). Ethinyl oestradiol is pharmacologically similar to oestradiol (Pelissero et al. 1993) and has approximately the same affinity for the oestrogen receptor (Kaspar & Witzel 1985). It has been found in water from sewage effluent at concentrations of 5–200 ng/litre (Tabak et al. 1981; Aherne & Briggs 1989; Stumpf et al. 1996; Desbrow et al. 1998; Snyder et al. 1999), and has induced physiological effects at very low aqueous concentrations (Sheahan et al. 1994). For example, exposure of male trout to 1 ng/litre ethinyl oestradiol stimulated vitellogenin synthesis and produced intersex males (Purdom et al. 1994).

In this study, I asked two related questions. First, I hypothesized that levels of ethinyl oestradiol found in the environment are sufficient to alter the aggressive and courtship behaviours of male three-spined stickleback. Second, in order to answer the first question, I tested the assumption, suggested by castration and implant experiments, that concentrations of plasma gonadal hormones are related to levels of male stickleback reproductive behaviour (Borg & Mayer 1995).

I measured the behaviour of nesting males before and after exposure to water containing 15 ng/litre ethinyl oestradiol or to control water. I evaluated the effects of exposure to exogenous ethinyl oestradiol on males' change in behaviour to two different stimuli: a live male conspecific in reproductive condition and a live gravid female conspecific. There are two advantages to measuring behaviour before and after exposure to ethinyl oestradiol. First, comparing a male's behaviour to his previous behaviour, rather than comparing across individuals, minimizes error variance as a result of individual differences in behaviour. Second, previous studies (Peeke & Veno 1973; Peeke & Figler 1997) have found that repeated interactions with different conspecific males can sensitize a male's aggressive response, prior to eventual habituation. That is, once a male has seen a conspecific male, his subsequent aggressive response to a different male is higher than his first aggressive response. The same is true for the courtship response (Peeke & Figler 1997). Thus a priori we expect that males' courtship and aggressive responses will increase as a function of previous interactions with conspecifics. However, if gonadal hormones affect these behaviours, then the courtship and aggressive responses of hormonally manipulated fish might be more or less sensitized than the responses of control fish.

METHODS

I collected adult freshwater three-spined stickleback from Putah Creek in Yolo County, California, U.S.A., in June 1999. Fish were transported to the laboratory, maintained on a natural photoperiod and temperature ($20 \pm 2^\circ\text{C}$) in holding tanks and fed frozen brine shrimp daily. Male fish that showed nuptial coloration were moved from holding tanks to individual 37.9-litre aquaria with visual access to neighbours. Each aquarium had a substrate of fine gravel, a sponge filter, one anacharis plant and algae from which the males built their nests. Animals in

adjacent aquaria were visually isolated from each other 1 h prior to each behavioural observation.

Because behaviour changes over the course of the nesting cycle, I observed all males at the same nesting stage: after creeping through the nest, a behaviour that marks the transition from the nest-building to courtship phase (Sevenster 1961). Because males varied in the amount of time to nest completion, the time between experimental treatment and behavioural observation varied among males (range 2–20 days, mean \pm SD = 8 ± 5.3 , $N=20$). For the same reason, the duration of exposure, from onset of treatment to killing, also varied among males (range 8–32 days, mean \pm SD = 17.7 ± 10.7 , $N=10$).

I observed males on four occasions, twice with a male conspecific and twice with a female conspecific. First, I presented each focal male with a conspecific male, hereafter referred to as the 'pretreatment male' observation. I filled a clear plastic container ($8 \times 18 \times 15$ cm) with water and placed it at least 15 cm from the focal male's nest. After 5 min, I added to the container one of five nuptially coloured conspecific males, which matched the focal fish's size to within 5 mm. I recorded the behaviour of the focal fish on a data recorder for 22 min after the first orientation towards the male conspecific. I recorded the following behaviours: time spent within one body length of the nest, time spent within one body length of the conspecific animal, zigzags (a courtship display), bites at the conspecific, and pokes at the nesting substrate.

Next, I presented the focal male with a gravid female conspecific, 'pretreatment female' observation. Between 4 and 6 h after the pretreatment male observation, I selected a gravid female to induce a courtship response in the focal male. I confined the female to the plastic container, and recorded the same behaviours following the procedure described above.

After the pretreatment female observation, I removed the male from his tank and randomly assigned him to either the control group (exposed to 0 ng/litre ethinyl oestradiol) or the experimental group (exposed to 15 ng/litre ethinyl oestradiol). I transferred the male to a tank that contained the same type of materials as before except that I added 57.9 ml of distilled water to the 37.9 litres of water in the control males' tanks and 57.9 ml of a 0.00001 g/litre ethinyl oestradiol solution to the 37.9 litres of water in the experimental males' tanks. I prepared the ethinyl oestradiol solution following the methods of Purdom et al. (1994). I confirmed the presence of low concentrations of ethinyl oestradiol in a subset of experimental tanks using a commercially available ELISA kit (Ridascreen Ethinylloestradiol, R-Biopharm, Darmstadt, Germany).

After a male completed a nest in the new tank, I subjected him to the third behavioural observation. Using the same procedure described for the pretreatment male observation, I observed the focal male's response to another male conspecific, 'post-treatment male' observation. Between 4 and 6 h after the post-treatment male observation, I subjected the focal male to the fourth and final observation, during which I recorded his response to a different female conspecific ('post-treatment female').

Thus I observed males in this experiment on four occasions: pretreatment male, pretreatment female, post-treatment male, post-treatment female. I obtained complete data sets (pretreatment male, pretreatment female, post-treatment male, post-treatment female) for 20 males (10 control, 10 experimental fish).

After behavioural testing, I used a lethal dose of MS-222 to kill 10 of the males in the experiment (5 experimentals, 5 controls) and collected blood for hormone analysis via caudal severance. I centrifuged blood from each male individually to separate the red blood cells from the plasma, then froze the plasma samples and sent them to Dr Ian Mayer, University of Stockholm, for measurement of 11-ketotestosterone (11KT) and oestradiol (E2). Plasma levels of 11KT and E2 were measured by radioimmunoassay, as described in Mayer et al. (1990) but without prior steroid separation by thin layer chromatography. The samples were run in a single assay to avoid possible intra-assay variation. The detection limits for the assays were 0.2–0.3 ng/ml.

Ethical Note

I restricted the movement of the stimulus animals in plastic containers and although the focal male often attempted to attack these animals, the container prevented contact and none of the stimulus animals suffered injuries. To prevent unnecessary distress, I used each stimulus animal less than once every 4 h.

Data Analysis

Ln-transformation successfully homogenized variances in the behavioural data. I treated data from the 'male' and 'female' observations separately.

To reduce the number of variables tested, I performed principal components analysis (PCA) on the post-treatment observations. I employed varimax rotation when necessary to obtain higher resolution. The varimax rotation is an orthogonal variance-maximizing procedure. I obtained the rotated loading matrix by multiplying the unrotated loading matrix by a transformation matrix (Tabachnick & Fidell 1996). I then examined the relationship between plasma hormone levels and behaviour by regressing component scores on levels of E2 and 11KT. I used the post-treatment data because plasma samples were taken shortly following the post-treatment observations.

I subtracted individuals' pretreatment behaviours from their post-treatment behaviours. I performed PCA on the difference between the pre- and post-treatment behaviours. I tested differences between the experimental and control groups' component scores using one-way analysis of variance (ANOVA). All statistical tests were two tailed.

RESULTS

The ELISA for ethinyl oestradiol was not sensitive enough to give a precise concentration but indicated that concentrations in the experimental tanks were between 10 and

Table 1. Principal component matrices and percentage variance explained for PCA on the post-treatment scores for male three-spined stickleback*

Behaviour	Conspecific	Nest
Post-treatment male†		
Bite	0.972	−0.0000187
Poke	−0.00371	0.959
Time with intruder	0.957	0.145
Time at nest	0.00341	0.948
Zigzag	0.464	0.757
% Variance explained	41.6	48.2
Post-treatment female‡		
Bite	0.960	0.160
Poke	0.142	0.966
Time with intruder	0.948	0.103
Time at nest	−0.00153	0.948
Zigzag	0.414	0.788
% Variance explained	40.3	49.8

Values represent factor loadings for each behaviour on each factor. High values indicate heavy loadings on the factor and the opposite for low values.

*The results from this PCA were used in the regressions on plasma hormone levels.

†Cumulative variance explained: 89.8%.

‡Cumulative variance explained (varimax rotated): 90.1%.

Table 2. Principal component matrices and percentage variance explained for PCA on the difference between post- and pretreatment behaviours of male three-spined stickleback, or change in behaviour over time*

Behaviour	Conspecific	Nest
Post-treatment male–pretreatment male†		
Bite	0.903	0.283
Poke	−0.561	0.766
Time with intruder	0.944	0.199
Time at nest	−0.577	0.749
Zigzag	0.639	0.655
% Variance explained	55.3	33.9
Post-treatment female–pretreatment female‡		
Bite	0.896	0.245
Poke	0.414	0.866
Time with intruder	0.916	0.00857
Time at nest	0.09385	0.948
Zigzag	0.68	0.391
% Variance explained	45.7	37.4

*The results from this PCA were used to test for differences between the control and experimental groups (Table 4).

†Cumulative variance explained: 89.2%.

‡Cumulative variance explained (varimax rotated): 83.1%.

50 ng/litre. Ethinyl oestradiol was undetectable in the control tanks.

PCA successfully compressed the behavioural data into two components for each data set (Tables 1, 2). One component links together 'conspecific-related behaviours', with high loadings on time spent near the conspecific and rates of biting. The other component links together 'nest-related behaviours', with high loadings on rates of poking at the nest and time spent near the nest. Zigzags did not load consistently on either the

Table 3. Relationship between endogenous hormone levels of male three-spined stickleback and their behavioural responses to other male and female conspecifics

Component	11-Ketotestosterone			Oestradiol		
	β	R^2	P	β	R^2	P
Male conspecific	0.273	0.074	0.514	-0.440	0.194	0.275
Female conspecific	0.557	0.310	0.194	-0.815	0.665	0.025
Male nest	-0.788	0.621	0.020	0.580	0.337	0.132
Female nest	-0.444	0.197	0.319	0.669	0.448	0.100

The data are from both experimental and control groups. β represents the slope of the linear regression line. 'Male conspecific' refers to the component loaded with time spent near the male and bites in the presence of a male conspecific. 'Male nest' refers to the component loaded with time spent at the nest and pokes at the nest in the presence of a male conspecific. 'Female conspecific' refers to the component loaded with time spent near the female and bites in the presence of a female conspecific. 'Female nest' refers to the component loaded with time spent at the nest and pokes at the nest in the presence of a female conspecific.

'conspecific' or 'nest' component for either the male or female conspecific observations.

Plasma hormones levels at the end of the study were related to male behaviour in the post-treatment observations. Males with higher plasma levels of 11KT at the end of the experiment performed fewer nest-related behaviours in the presence of a male conspecific ($F_{1,6}=9.848$, $R^2=0.621$, $P=0.02$; Table 3) than males with lower levels of 11KT. Males with higher levels of E2 performed fewer female conspecific-related behaviours ($F_{1,5}=9.905$, $R^2=0.655$, $P=0.025$; Table 3) than males with lower levels of E2. In general, 11KT levels were positively related to conspecific-related behaviours and negatively related to nest-related behaviours, while the reverse was true of E2.

There was no difference between plasma levels of either E2 or 11KT between control and experimental males (two-tailed t test: 11KT: $t_9=0.555$, $P=0.61$; E2: $t_9=0.120$, $P=0.91$; Fig. 1). However, this result should be treated with caution because the power of this test was very low (E2: power=0.052; 11KT: power=0.073).

Exogenous oestrogen affected behaviour directed towards male conspecifics over the course of the study. As suggested by previous sensitization studies (Peeke & Veno 1973), control males increased rates of behaviour directed towards males (bites, time spent near the conspecific male), as indicated by the positive values of the component 1 scores for these individuals (Table 4). In contrast, males exposed to exogenous oestrogen reduced rates of male conspecific-related behaviours during this same period ($F_{1,19}=7.072$, $P=0.016$; Table 4). Adjusting the P value for two simultaneous tests on nonindependent data (nest and conspecific components), the Bonferroni-corrected P value for this test was 0.032.

There were no significant effects of exogenous oestrogen on behaviour directed towards female conspecifics (female conspecific component) or on patterns of nest-related behaviour displayed in the presence of either males (male nest component) or females (female nest component) (Table 4). However, there was a nonsignificant difference in the direction of change in behaviour in control and experimental animals: whereas control animals decreased nesting behaviours over time, experimental animals increased nesting behaviours. Similarly,

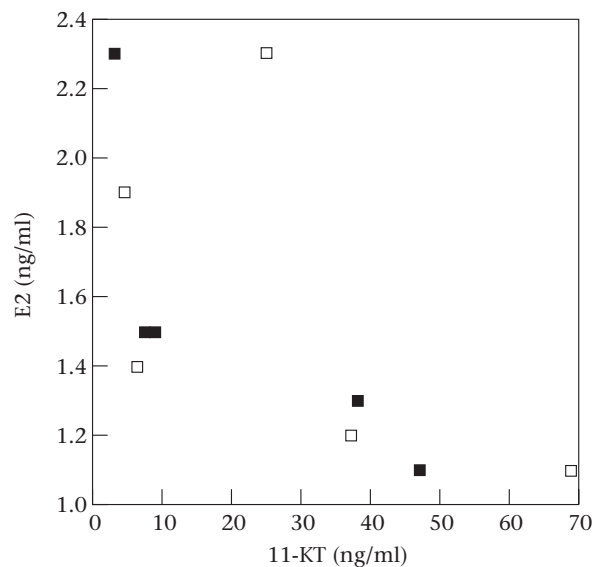


Figure 1. Relationship between levels of endogenous oestradiol (E2) and 11-ketotestosterone (11KT) in male three-spined stickleback. The linear regression of E2 on 11KT was marginally significant ($F_{1,8}=4.81$, $P=0.06$). ■: Experimental; □: control.

whereas control animals increased their response to a female conspecific over time, experimental males decreased their courtship response over time.

DISCUSSION

Males exposed to environmentally relevant levels of an oestrogenic endocrine disrupter decreased their aggressive response to a conspecific male over time, whereas control males increased their aggressive response. Levels of aggressiveness are related to territory ownership (Whoriskey & FitzGerald 1994), territory size (Assem 1967) and reproductive success. Thus, exposure-induced reductions in aggressiveness could have effects on the fitness of individual males and on population-level characteristics such as population growth rate.

There are several mechanistic explanations for the behavioural differences found in this experiment. One

Table 4. Effects of exposure to ethinyl oestradiol on courtship and aggressive behaviour of male three-spined stickleback

Component	$\bar{X} \pm SE (N)$		F	df	P
	Control	Experimental			
Male conspecific	0.518±0.279 (10)	-0.518±0.272 (10)	7.072	1, 19	0.016
Female conspecific	0.202±0.378 (9)	-0.182±0.279 (10)	0.688	1, 18	0.418
Male nest	-0.0082±0.350 (10)	0.0082±0.295 (10)	0.127	1, 19	0.726
Female nest	-0.325±0.355 (9)	0.293±0.281 (10)	1.899	1, 18	0.186

Values represent component scores from PCA on the difference between the post- and pretreatment observations. Component labels are as in Table 3.

hypothesis is that exogenous oestradiol caused males to down-regulate endogenous androgen production, thereby reducing the frequency of behaviours related to plasma androgen. This hypothesis is consistent with the nearly significant negative correlation between E2 and 11KT plasma concentrations as well as the nonsignificant but positive correlation between 11KT and aggression towards males. Another hypothesis is that the liver of treated males released vitellogenin into the blood (Hansen et al. 1998), which caused males to become less active and less responsive. This explanation is less likely because under this hypothesis, we would expect decreases in all types of male activities but I found no effect of treatment on the frequency of nest-related activities.

In addition, this study provides evidence for the role of endogenous gonadal steroids in male stickleback behaviour. Unlike other behavioural endocrinological studies that have shown that androgens affect the presence or absence of nest building and territorial behaviour (Hoar 1962; Wai & Hoar 1963; Baggerman 1966; Borg 1981, 1987; Borg et al. 1993; Borg & Mayer 1995; Bornestaf et al. 1997), this study quantified the relationship between levels of hormone and levels of behaviour. In general, the data suggest that E2 is negatively related to conspecific-related behaviours and that 11KT is negatively related to nest-related behaviours. More specifically, I found a significant negative correlation between plasma levels of E2 and the frequency of female conspecific-related behaviours, which suggests a possible negative causal relationship between E2 and courtship behaviour. The (nonsignificant) negative effect of exogenous ethinyl oestradiol on courtship behaviour is consistent with the relationship between endogenous oestradiol and courtship behaviour.

Male reproductive behaviour is just one of many types of behaviour likely to be affected by endocrine disrupters. Because oestrogens play important roles in female reproduction and physiology, oestrogenic endocrine disrupters might have more consequential effects on females than on males. In addition, chronic developmental exposure to low levels of hormones in the environment might affect ontogeny and sexual differentiation (Guillette et al. 1999).

This study contributes to the growing literature on endocrine disrupters that should attract the attention of animal behaviourists studying field-caught animals.

Anthropogenic activities are inadvertently creating experiments by introducing evolutionarily novel environmental conditions and selective pressures. Studies such as this show that very low levels of environmental contaminants may be affecting animal behaviour in subtle yet potentially important ways.

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