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Oestrogenic Endocrine Disruption in fish - developing biological effect measurement tools and generating hazard data

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Steve Killeen

Head of Science

Executive summary

This report describes the results from the programme *'Oestrogenic Endocrine Disruption in Fish – Developing biological effect measurement tools and generating hazard data'*.

The programme's main aim was to develop a standardised test to assess the effects of endocrine active chemicals (EACs) on fish reproduction. EACs are compounds that can interfere with hormone systems and affect fish growth, development and/or reproduction. EACs that have a strong environmental relevance and that act in different ways were studied. They included the natural steroid, oestrone (E1), that acts as an oestrogen, a pesticide, linuron, that acts as an anti-androgen and a fungicide, fenarimol, that disrupts sex steroid biosynthesis by inhibiting the aromatase enzymes that convert testosterone into oestrogen. Hazard data were also generated for each of these chemicals to use in risk assessment. Another key aim was to find out whether specific biomarkers (for example, vitellogenin [VTG] and secondary sex characters [SSCs]) could reveal the mechanisms by which the selected EACs relate to reproductive health. This work also enabled conclusions to be drawn about how useful the selected biomarkers are for monitoring environmental discharges.

The fathead minnow (*Pimephales promelas*) was chosen for this work. As a member of the carp family, the fathead minnow represents a major part of the UK freshwater fisheries. It is a robust organism for laboratory studies on reproduction and is used in OECD and USEPA standardised regulatory tests. The biomarkers studied included the female egg yolk precursor protein, VTG, and the male SSCs. VTG is normally produced only in maturing females in response to increased circulating concentrations of endogenous oestrogens. Males exposed to exogenous oestrogen, though, will produce high concentrations of VTG, so this protein is often used as a biomarker to assess exposure to oestrogenic substances. Maturing male fish develop SSCs in response to increased circulating concentrations of endogenous androgens. Exposing males to anti-androgenic or oestrogenic chemicals inhibits the formation of these features. Exposing females to androgens induces the formation of the male SSCs. The SSCs may therefore have value as a biomarker for assessing exposure to chemicals that act via the androgen receptor. Studies on the dynamics of the VTG and SSC responses in male fathead minnows using the reference oestrogen (oestradiol-17β; E2) demonstrated that these biomarkers were highly responsive. Exposure to E2 induced a rapid and concentration-related induction of VTG, after only two and four days of exposure to 100 and 32 ng E2/L, respectively ($p < 0.05$). Exposure to 100 ng/L of E2 induced a maximal VTG response on day 14. A significant decrease in plasma VTG concentrations occurred only after 41 days of depuration in clean water ($P < 0.01$). A concentration-related increase in the plasma VTG concentrations was still evident after 41 and 70 days of depuration ($P < 0.01$). A concentration-dependent reduction in the prominence of the SSCs was also observed following exposure of the males to E2 for 14 days ($P < 0.05$). This effect was still evident after 41 days of depuration ($P < 0.05$). The availability of assays for VTG in many fish species and the ease of measuring SSC responses confer a practical utility for studies on EACs and their mixtures that can be applied to studies in environmental monitoring (either through

in situ caged fish work or in wild fish populations).
The first study clearly demonstrated that biomarkers are robust in their response to oestrogens and that the induced effects are persistent. A clear link between changes in biomarker responses and adverse health effects has, though, yet to be demonstrated. For this reason, tests that directly assess chemical effects on reproduction are necessary. In the fish reproduction test method used here, pairs of breeding fathead minnow were held in tanks supplied with flow-through water. Reproductive output (including egg production and embryo survival) was assessed for 21 days. After that, a chemical was supplied via the water, and reproductive output was measured for a further 21 days. The test is highly integrative, and any chemical that affects the females' ability to produce and release eggs, or the sexual drive of either sex could affect reproductive output. The test system developed was robust, with excellent consistency across the experiments. Pairs of fathead minnows under dilution water control conditions produced between 80 and 93 eggs/female/day, with the individual pairs of fish spawning between 263 and 358 eggs/spawn every three to four days. The coefficient of variation for fecundity estimates within each experiment conducted was less than 30 per cent, based on egg numbers for the individual pairs of fish (n = between 36 and 48 pairs within each experiment). The coefficient of variation between the four experiments, based on mean estimates of egg numbers for all pairs of fish was 14 per cent. This shows that measurements of fecundity in pairs of fathead minnow are highly consistent both within and between different stocks of fish and provides a robust short-term test for assessing reproductive performance in pair-breeding fish.

The fish reproduction test successfully detected the effects of each of the three test chemicals, with their different modes of action. All of the chemicals reduced egg production in a concentration-dependent manner ($P < 0.01$). A reduction in total egg number, relative to the controls, was observed in fish exposed to oestrone at concentrations of 307 and 781 ng/L ($P < 0.05$), linuron at concentrations of 842 and 2074 μg/L ($P < 0.05$), and fenarimol at a concentration of 497 μg/L ($P < 0.05$). The effects of oestrone on reproduction were observed only at concentrations that were toxic to males. The effects of linuron and fenarimol on reproduction were observed at concentrations below their reported 96-hour LC50 values for the rainbow trout (3.15 and 4.1 mg/L, respectively; data were not available for the fathead minnow). The effect on reproduction for all three chemicals appeared to be a result of an increase in the time between successive spawnings ($P < 0.01$) rather than a decrease in the number of eggs/spawn ($P > 0.05$). Spawning stopped completely at the highest test concentration of oestrone (781 ng/L) and linuron (2074 μg/L). There was no evidence that exposing adult fish to the test chemicals caused any effect on survival to hatch of the F1 generation. Neither was there any sign of a trans-generational effect of adult exposure for the test chemicals on somatic growth and sexual development in the F1 generation.

The biomarker responses confirmed that each test chemical affects the reproductive axis in a different way. Exposure to oestrogen (oestrone) resulted in a concentration-dependent induction of the oestrogen-dependent biomarker, VTG, in both the males and females ($P < 0.001$). A concentration-dependent suppression of the prominence of the androgen-dependent dorsal fatpad was also observed in males ($P < 0.01$), though no effect was observed on the prominence or number of nuptial tubercles ($P > 0.05$). Exposure to the anti-androgen (linuron) resulted in a concentration-related

reduction in the prominence of the SSCs (dorsal fatpad and nuptial tubercles; $P < 0.01$) in males and in a reduction in plasma VTG concentration in both the males and females ($P < 0.01$). The reason for this reduction in plasma VTG concentration is not known, but changes in the sex steroid dynamics (balance between endogenous steroid oestrogens and androgens) are probably responsible. Exposure to the aromatase inhibitor resulted in a concentration-dependent decrease in plasma VTG concentrations in the females ($P < 0.01$), but did not affect SSCs ($P > 0.05$). The reduction in plasma VTG concentrations in the females exposed to fenarimol probably resulted from a reduced level of circulating concentrations of endogenous oestrogen, as a consequence of inhibition of aromatase that converts testosterone to oestradiol-17β. Analysis of the relationship between the biomarker responses and the effects on reproduction demonstrated that the induction of VTG in males and females exposed to oestrogen correlated with a reduced reproductive success in those individuals ($P < 0.01$). In males exposed to the anti-androgen, the suppression in the prominence of the SSCs and the reduction of plasma VTG concentrations correlated with a reduced reproductive success in those individuals ($P < 0.01$). So these biomarkers can help identify the mechanisms of action of EACs where impacts are seen on reproduction in the pair-breeding assay and may help define potential adverse effects on fish populations.

Project objectives

1. To determine the variability in, and temporal dynamics of, biomarker and somatic responses in the fathead minnow (*Pimephales promelas*) exposed to reference oestrogenic EACs.

2. To validate reproductive performance and biomarker responses in pair-breeding fathead minnows exposed to a range of reference EACs.

3. To determine the trans-generational effects of exposure to reference EACs.

4. To assess how useful biomarkers for EACs are for monitoring and managing environmental discharges.

5. To develop standardized methods appropriate for future evaluation of endocrine active environmental samples.

6. To generate data for hazard and risk assessment purposes of selected substances prioritised in the Environment Agency's March 2000 strategy announcement.

Contents

Introduction

Surveys of wild fish populations in the UK have identified a high incidence of intersex in various species in both rivers (Jobling et al., 1998; van Aerle et al., 2001) and estuaries (Kirby et al., 2004) that receive effluent discharges from sewage treatment works (STWs). Intersex is when testicular and ovarian tissues exist at the same time in the gonads of fish that normally have either a testis or an ovary (ie, in fish that are normally gonochoristic). In UK rivers, the severity of the intersex condition is related to the concentration of STW effluent in the river (Jobling et al., 1998). There is also a link between the severity of intersex and a reduced reproductive capability (reduced gamete viability in males), with a complete inhibition of reproduction in more severe cases (Jobling et al., 2002a, 2002b). This raisesconcerns about the long-term consequences of exposure to STW effluents on the reproductive health of wild fish populations and demonstrates that aquatic organisms need to be protected against the chronic effects of STW effluents.

There is a reciprocal relationship between the number of intersex roach caught at any one site and the number of males (Jobling et al., 1998). This indicates that the intersex condition is a consequence of the feminisation of males, rather than the masculinisation of females. Observations of elevated concentrations of VTG in the intersex roach and in caged male rainbow trout and roach placed downstream of STW effluent discharges support this. VTG is an egg yolk precursor produced at high concentrations in mature females in response to increased concentrations of endogenous oestrogens (Van Bohemen et al., 1982; Ng & Idler, 1983; Tyler, 1991). Male fish possess very little, if any, detectable VTG in their plasmabut they do possess the VTG gene(s) (LeGuellec et al., 1988) and will produce VTG when exposed to exogenous oestrogen (van Bohemen et al., 1982; Lazier & MacKay, 1993; Sumpter & Jobling, 1995). Attempts to identify the chemical cause of intersex have, therefore, focused on chemicals that mediate their effects via the oestrogenic pathway. The major oestrogenic chemicals in STW effluents are the natural (oestradiol and oestrone) and synthetic (ethinyloestradiol) steroidal oestrogens and some alkylphenolic chemicals (Desbrow et al., 1998; Rodgers-Gray et al., 2001; Gibson et al., 2005). These chemicals are prevalent in the wider aquatic environment and frequently occur as mixtures (Johnson et al., 2004). In the laboratory, the individual oestrogenic chemicals induce VTG synthesis at environmentally relevant concentrations (Routledge et al., 1998; Panter et al., 1998; Thorpe et al., 2000, 2001). They also feminise the male reproductive duct (van Aerle et al., 2001), reduce the prominence of thesecondary sexual characteristics (Harries et al., 2000; Panter et al., 2005) and inhibit reproduction (Harries et al., 2000; Shioda & Wakabayashi, 2000; Länge et al., 2001; Zillioux et al., 2001), albeit at higher concentrations than those found in the environment. In addition it has been shown that when present as mixtures, oestrogenic chemicals interact in a concentration additive manner, *in vivo*, to produce a higher oestrogenic response than that produced by the individual chemicals (Thorpe et al., 2001, 2003; Tinwell & Ashby, 2004). Much of the focus has been on chemicals that mediate their effects via the oestrogenic pathwaybut laboratory investigations have also shown that chemicals that alter steroid biosynthesis or act as anti-androgens can feminise males and inhibit reproduction (Bayley et al., 2002; Ankley et al., 2002; Panter et al., 2004; Jensen et al., 2004).

Recent surveys conducted by the Environment Agency, in collaboration with WRc-NSF Ltd and the University of Exeter (Johnson et al., 2004) have shown that, as well as oestrogenic activity, STW effluents throughout England and Wales also have anti-androgenic effects. The chemical(s) responsible for this anti-androgenic activity within STW effluents have yet to be identified. It is not yet known whether anti-androgenic chemicals (and other EACs) contribute to intersex in the environment but their ability to feminise males and inhibit reproduction highlights the need to consider these chemicalswhen assessing the potential health effects of STW effluents. Tests developed should, therefore, be capable of capturing the different modes of action that might impact on the health of the fish populations of UK rivers.
Intersex is a valuable tool for assessing the degree of sexual disruption in wild fish populations and for demonstrating a link between exposure to STWs and the reduced reproductive capacity of individual fish. Inducing intersex, though, requires the exposure of fish embryos and early life stages for prolonged periods of time. In roach, intersex may not become evident for up to two years (Jobling et al., 2005). Long-term exposures are expensive and time-consuming and can be difficult to maintain. Inducing intersex is, therefore, unlikely to be practical for screening biological impacts in controlled experiments. Many short-term tests have been developed that are more readily applied to the assessment of the effects of EACs and STW effluents on the reproductive axis. These range from measuring changes in biomarker responses (e.g. VTG induction in males) to directly assessing reproductive capacity. To protect the fish populations of UK rivers, though, we need a clear understanding of the biological consequence of any change in the target endpoint for the population. Such a relationship has yet to be clearly demonstrated for the more widely employed biomarkers of reproductive health, (e.g. VTG induction, male secondary sexual characters). For this reason, tests that directly assess effects on reproduction are necessary as they demonstrate a clear link between exposure to chemical(s) and an individual's reduced reproductive capacity. Where reproduction is completely inhibited, it's easy to predict the consequences for the population. In this regard, the adult fish reproduction test (Harries et al., 2000; Ankley et al., 2001) offers considerable promise. This highly integrative test assesses effects on reproductive performance (frequency of spawning and number of eggs spawned). In the reproduction test, sexually mature fish are established as breeding pairs in the test system for a pre-exposure period of three weeks. This provides pair-specific baseline fecundity data. Once breeding is established for all fish, the substance of interest is introduced to the test system and the breeding pairs exposed for three weeks to see what impact exposure has on their reproduction. This test allows the effects of all classes of chemicals, and their mixtures, on the reproductive axis to be determined, irrespective of exactly how those chemicals act. That information can be obtained by using biomarkers specific to particular classes of chemicals.

The overall aim of this programme of work was to validate an adult fish reproduction test for assessing the effects of EACs on reproduction with a view to developing a standardised protocol. The adult fish reproduction test has been successfully applied to OECD freshwater test species including the fathead minnow (Harries et al., 2000; Ankley et al., 2001) and Japanese medaka (Shioda & Wakabayashi, 2000). The fathead minnow was selected as the test organism here. As a representative the ecologically important and ubiquitous Cyprinidae family (which includes roach, gudgeon and many other British freshwater fish), the fathead minnow, is one of the most widely used fish species in ecotoxicology, in support of regulatory programs, in

both Europe (OECD, 1992) and North America (EPA 1982, 1986, 1987, 1994). It is relatively easy to culture in the laboratory (EPA, 1987) and its reproductive cycle can be controlled through alterations in temperature and photoperiod, ensuring that adequate numbers of mature fish are available all year round. In addition, the fathead minnow is a repetitive spawner. Under favorable conditions, females can produce batches of eggs every three to five days for many weeks (Gale and Buynek, 1982; Jensen et al., 2001). This means that the fathead minnow's reproduction can be studied over a prolonged period of time. An advantage of the species is that it will readily spawn when the individuals are held as male:female pairs. Compared to group spawning systems, this generates a more statistically robust test design: any fish that do not spawn can easily be identified and eliminated from the test during the pre-exposure period. In addition, any changes in the biomarker responses can easily be compared to reproductive performance in the relevant individual(s), so the relationship between biomarkers and reproduction can be studied. Another advantage of the fathead minnow is that it has easily quantifiable secondary sexual characteristics that can be used as biomarkers for (anti-)androgenic effects. Measurements of androgen-dependent secondary sexual characteristics can be included, along with the oestrogen-dependent induction of VTG. So the fathead minnow pair-breeding reproduction test can be used to reveal how the chemicals work, as well as the effect they have on reproduction. The first major objective (chapter 1) was to assess the dynamics and persistence of the selected biomarker responses in male fathead minnows. To achieve this, groups of adult male fathead minnows were exposed to graded concentrations of a reference oestrogen, E2 for 14 days. Sub-samples of fish were collected at intervals within this period to establish the time-course of response for both the induction of VTG and changes in the prominence of the SSCs (nuptial tubercles and dorsal fatpad). Recovery was then determined in fish held for a further 70 days under control conditions. During this period of depuration, sub-samples of the fish were removed at intervals to determine the time required for VTG to clear from the plasma of the exposed fish, and for any effects on the SSCs to recover.

The second and main objective was to validate the adult fish reproduction test for assessing the effects of EACs on reproduction, with a view to developing a standardised test protocol that could readily be applied to assess effects on reproduction. To achieve this, fundamental information on the reproductive capacity of this species under the test conditions had to first be established, and a standardised approach for quantifying egg production developed (Chapter 2). Once this was done, a series of exposures to reference EACs was conducted to determine how sensitive the reproduction test was for assessing the effects of chemicals with different modes of action on the endocrine system (Chapter 3). There is some evidence that chemicals can accumulate in embryos as a result of maternal transfer from the chemically exposed adult female (Miller, 1993; Fisk & Johnston, 1998; Metcalf et al., 2000; Thibaut et al., 2002; Schwaiger et al., 2002; Hammerschmidt & Sandheinrich, 2005) resulting in early life stage toxicity of the offspring (Nakayama et al., 2005).

Given the sensitivity of early life stages to the effects of EACs (van Aerle et al., 2002; Brion et al., 2004), the potential for the test EACs to affect the offspring after exposure of the breeding adults was evaluated (third objective). Embryo hatching success was compared, under control conditions, for embryos collected from the

adult fish during both the pre-exposure and chemical exposure phases of each of the adult reproduction tests. In addition, hatchlings from the exposures to the feminising chemicals (oestrogen and anti-androgen) were held until 80 days post-hatch to evaluate the potential for possible trans-generational effects on somatic growth and sexual development.

The fourth major objective was to evaluate the potential for changes in the oestrogenic and (anti-) androgenic biomarker responses to indicate adverse effects on reproduction. To achieve this, plasma VTG concentrations and the prominence of the male SSCs were quantified and compared with reproductive output, at the individual level, for each of the reproduction tests.
Information from these tests will be used to evaluate the repeatability of the results and therefore the applicability of the test for standardisation (fifth objective).

Test chemical selection

The reference chemicals were selected according to their relevant environmental risk, in fulfilment of the sixth and final objective. The first major objective (chapter 1) was to assess the dynamics and persistence of the selected biomarker responses in male fathead minnows. So a reference oestrogen that would produce a potent oestrogenic response was required. Candidate test substances included the natural steroidal oestrogen (E2) and the synthetic steroidal oestrogen, ethinyloestradiol-17α (EE2). Both of these chemicals have been widely used as reference oestrogenic positive controls in tests for EACs and induce a rapid and potent vitellogenic response in male fish (Thomas-Jones et al., 2003; Shultz et al., 2001). In addition, both chemicals reduce male SSCs (Bjerselius et al., 2001; Länge et al., 2001; Bringolf et al., 2004; Parrot & Blunt, 2005). Investigations into the pharmacokinetics of EE2, though, show that plasma concentration-time profiles of this chemical are influenced by enterohepatic recirculation, with the major fraction of the EE2 conjugates being deconjugated and reabsorbed into the bloodstream (Shultz et al., 2001). As a consequence of recirculation in the fish, the individual is effectively re-dosed with EE2, resulting in a continued induction of the vitellogenic response even after exposure ceases. To avoid this influence of a ‘re-dosing’ effect on our investigations into the persistence of VTG in the plasma, the natural endogenous oestrogen, E2, was therefore favoured for use in this first study.

Selection of the reference EACs to validate the reproduction test was influenced by the overriding need to develop a test that could assess the feminising effects of substances on reproduction. Chemicals that bind the oestrogen receptor as an agonist, or the androgen receptor as an antagonist can feminise fish and inhibit reproduction. Given that both oestrogenic and anti-androgenic activity has been demonstrated in STW effluents discharging into UK rivers (Johnson et al., 2004), the focus of this project was, therefore, on validating the reproduction test for these mechanisms of action. These effluents, though, contain a complex cocktail of chemicals. Many may be able to target the endocrine system via additional target sites. It was felt, therefore, that the test should also be assessed for its ability to detect the effects of chemicals that act at a target site other than a sex steroid receptor. One of the best-documented examples of endocrine disruption in the aquatic environment is the induction of imposex in molluscs as a consequence of exposure to tributyltin. Tributyltin exerts its effect through the competitive inhibition of

the aromatization of androgens to oestrogens (Bettin et al., 1996). In fish, exposure to tributyltin during early life masculinises females (Shimasaki et al., 2003; McAllister & Kime, 2004) and causes irreversible sperm damage (McAllister & Kime, 2004). Though tributyltin is not likely to occur in STW effluents, it was felt that this mechanism of action and its effect on reproduction should be addressed within this project.

Selection of the specific test chemicals was influenced by the need for the Environment Agency to generate data for hazard and risk assessment purposes of selected substances prioritised in the Environment Agency's March 2000 strategy announcement (objective 6). One candidate compound that was considered, due to its prevalence in STW effluents, was nonylphenol (NP). NP disrupts follicle stimulating hormone synthesis/secretion (a key hormone in the control of gonad development; Harris et al., 2001), as well as binding both the oestrogen receptor as an agonist (Routledge & Sumpter, 1996) and the androgen receptor as an antagonist (Sohoni & Sumpter, 2000). Exposure to NP, though, has already been shown to feminise male fathead minnows and inhibit reproduction at a concentration of 57 μg/L (Harries et al., 2000). NP has also been shown to decrease reproduction (total number of eggs spawned) in the Japanese medaka at a concentration of 101 μg/L (Kang et al., 2003), to feminise the gonad of both the mosquitofish (50 μg/L; Dreze et al., 2000) and the Japanese medaka (24.8 μg/L; Kang et al., 2003) and to inhibit semen production in adult rainbow trout exposed during the spawning period (750 ng/L; Lahnsteiner et al., 2005). Intermittent exposure of adult rainbow trout to NP at a concentration of 10 μg/L reduces hatching success as a consequence of trans-generational effects mediated by the endocrine system (Schwaiger et al., 2002). The most extensive data set for NP is that provided by Yokota et al (2001) who demonstrated the effects of NP on the reproductive status of medaka (*Oryzias latipes*) over two generations of continuous exposure. NP was shown to reduce the reproductive potential of medaka at a concentration of 17.7ug/L as a consequence of reduced fertility in the F0 generation. In the F1 generation, induction of testis-ova was observed in the gonads of fish exposed to 8.2 μg/L of NP, at 60 dph (Yokota et al., 2001). Given the extensive datasets available for NP, it was designated a low priority test substance for study in this project.

Oestrone

The first chemical proposed for use was the natural oestrogen, oestrone (E1). The steroidal oestrogens, E1, E2 and EE2 are prevalent in STW effluents at concentrations ranging from a few ng/L upto 80, 50 and 7 ng/L, respectively (Desbrow et al., 1998; Routledge et al., 1998; Rodgers-Gray et al., 2000; Johnson et al., 2004). The effects of E2 and EE2 have been widely studied in fish. A number of datasets demonstrate their effects on reproduction (Kramer et al., 1998; Van den Belt et al., 2001; Länge et al., 2001; Bringolf et al., 2004; Nash et al., 2004; Seki et al., 2005; Parrot & Blunt, 2005). In the fathead minnow, E2 and EE2 inhibit reproductive success at concentrations of 299 ng/L (Kramer et al., 1998; Bringolf et al., 2004) and 3.5 ng/L (Parrot & Blunt, 2005), respectively. In contrast, though E1 induces VTG in male fish with a similar potency to E2 (Panter et al., 1998; Routledge et al., 1998; Thorpe et al., 2003), there isn't much data on its affects on the reproductive health of fish.

Given that E1 is typically present in the environment at higher concentrations than either E2 or EE2, information about its effects on fish reproduction is badly needed to understand fully the potential hazard it poses in the environment.

Linuron

The second chemical proposed for use was the herbicide linuron. A recent survey sponsored by the Environment Agency demonstrated that STW effluents possessing oestrogenic activity also inhibited the binding of the reference androgen, dihydrotestosterone, to the androgen receptor in an *in vitro* recombinant yeast assay (Johnson et al., 2004). In fish, exposure to the reference anti-androgen, flutamide, demasculinizes males (Bayley et al., 2002; Panter et al., 2004) and inhibits reproduction (Jensen et al., 2004). There is very little information about the effects of less potent anti-androgenic chemicals on the reproductive success of fish. Linuron is a phenylurea derivative used in the pre- and post-emergence control of annual grass and broad-leaved weeds, and some seedling perennial weeds. It is found in UK rivers, but only at concentrations below 1 μg/L (Environment Agency, 1999). Linuron was selected for study here because it is structurally similar to flutamide and competitively inhibits testosterone binding to the androgen receptor (Bauer et al., 1998). In fish, exposure to the reference anti-androgen, flutamide, inhibits reproduction at a concentration of 651 μg/L (Jensen et al., 2004). The reproductive health effects of linuron are less well studied in fish, but in male rats, exposure to linuron reduces the weight of the accessory sex organs (Cook et al., 1993). This suggests that it is likely to impact on the reproductive axis of fish.

Fenarimol

In selection of the third test chemical, chemicals that could impact the endocrine system via a pathway other than directly binding to sex steroid receptors were considered. Fenarimol is a pyrimidin-5-yl benzhydrol fungicide used on golf courses and commercial ornamental crops to control powdery mildews. Fenarimol was chosen because it possesses structural similarities to the reference aromatase inhibitor fadrozole. In fish, exposure to fadrozole reduces plasma VTG concentrations (Ankley et al., 2002; Panter et al., 2004) and inhibits reproduction at a concentration of 2 μg/L (Ankley et al., 2002) through inhibiting aromatase activity. Very little information regarding the effects of less potent aromatase inhibiting chemicals on the reproductive success of fish is available. In rodents, fenarimol reduces fertility and the size of the liveborn litter and increases the gestation period (reviewed in van Hoeven-Arentzen, 1995). These effects are thought to arise from fenarimol inhibiting the aromatase-catalysed conversion of testosterone to oestradiol-17β within the hypothalamus. This is supported by the results of *in vitro* studies showing that fenarimol does not bind to the oestrogen or androgen receptors, but acts as a moderately weak inhibitor of aromatase activity (reviewed in van Hoeven-Arentzen, 1995).

1. The dynamics of VTG induction and effects on secondary sexual characteristics in male fathead minnows exposed to a reference oestrogen (Oestradiol-17β)

Abstract

The induction of VTG (the female yolk protein) in male fish is a widely used biomarker for the effects of oestrogenic chemicals. The androgen-dependent male secondary sexual characteristics also respond to the effects of oestrogens and other EACs. Oestrogens and anti-androgens have been shown to reduce the prominence of the secondary sex characters (SSCs) in males, while androgens induce the formation of SSCs in females. Male SSCs may, therefore, have considerable value as biomarkers for measuring alterations in the functioning of the steroidogenic pathway. Their reliability as a biomarker of exposure is, though, limited by a lack of standardised methods for quantifying changes in their appearance. The aim of this experiment was to develop a method of quantifying changes in the SSCs of the male fathead minnow and to establish the dynamics and persistence of the effects of oestrogen exposure on both the SSCs and plasma VTG concentrations in male fathead minnows. In the experiment, sexually mature male fathead minnows were exposed to E2 in a flow-through system for 14 days. They were then transferred to clean aquaria receiving dilution water only and maintained for a further 70 days. Subgroups of fish were sacrificed at regular intervals, during the exposure and depuration period, to determine effects on somatic growth, gonadosomatic index (GSI; weight of the gonad relative to body weight), prominence of the SSCs (tubercle number and fatpad size), and plasma VTG concentrations. Differences in fatpad sizes between individuals were quantified by determining the wet weight of the fatpad. In control males, though, fatpad weight related to the total wet body weight of the males ($P < 0.05$). So all statistical analyses of chemical effects on fatpad weights were conducted using analysis of covariance (ANCOVA) with body weight as a covariate. Exposure to E2 induced a rapid and dose-related induction of VTG after only two and four days of exposure to 60 and 29 ng E2/L, respectively ($p < 0.05$). Exposure to 60 ng E2/L induced a maximal VTG response on day 14. A negative correlation between E2 concentration and fatpad weight was observed ($p< 0.05$) on day 14 of exposure, but this effect was not significant compared to the controls, indicating that the SSCs are a less sensitive biomarker for exposure to oestrogens than the induction of VTG. The effects of E2 exposure on both plasma VTG and fatpad weight persisted, and a significant decrease in plasma VTG concentrations occurred only after 41 days depuration (day 55). At this point, the negative correlation between E2 concentration and fatpad weight was still evident ($P < 0.05$). After 70 days depuration, the fatpad appeared to have recovered, though plasma VTG concentrations were still elevated ($P < 0.05$).

Introduction

Biomarkers are used widely in ecotoxicological investigations to quantify alterations in physiological function and to provide mechanistic information about how chemical effects occur. VTG has been widely used as such a biomarker in the study of endocrine active chemicals. VTG is an egg yolk precursor produced at high concentrations in mature females in response to increased concentrations of endogenous oestrogens (Van Bohemen et al., 1982; Ng & Idler, 1983; Tyler, 1991). Male fish have very little, if any, detectable VTG in their plasma, but they do possess the VTG gene (LeGuellec et al., 1988) and exposure to exogenous oestrogen induces production of VTG in males (van Bohemen et al., 1982; Lazier & MacKay, 1993; Sumpter & Jobling, 1995). Researchers have recently become aware that other chemicals in the environment may interact with other pathways within the endocrine system. This has led to the development of other biomarkers of exposure for endocrine active chemicals. In some species of fish, sex-specific secondary sex characters (SSCs) are evident at maturity (Smith & Murphy, 1974; Bayley et al., 2002; Ogino et al., 2004). The formation of these characters in males is under androgen-control. This offers the potential for their use as biomarkers of disruption of the androgen-mediated pathway (Smith & Murphy 1974; Borg, 1994; Ogino et al., 2004). Administering exogenous androgens to females and immature males results in the rapid formation of the male SSCs (Smith, 1973; Ankley et al., 2003), while exposing sexually mature males to anti-androgenic chemicals can reduce the prominence of the SSCs (Bayley et al., 2002; Panter et al., 2004). Exposing males to oestrogenic chemicals also reduces the prominence of the SSCs (Harries et al., 2001). This demonstrates that these features are also sensitive to the effects of chemicals that disrupt the endogenous androgen/oestrogen profile via mechanisms other than direct interaction with the androgen receptor. Male fathead minnows have two prominent SSCs: namely nuptial tubercles and a dorsal fatpad (Smith and Murphy, 1974). Changes in the prominence of these features have been measured through scoring the prominence of the features according to an assessment of their appearance (Smith 1978; Parrot & Blunt, 2005), through directly counting the number of tubercles on the head of the fish (Harries et al., 2000; Ankley et al., 2003; Panter et al., 2004) and by measuring the height of the fatpad above the dorsal musculature of the fish (Miles-Richardson et al., 1999; Harries et al., 2000). Scoring the SSCs according to their appearance, can, though, be subjective and difficult to quantify accurately on a consistent basis. In addition, the fatpad is not uniform in its formation, and measurements of the height of the fatpad vary depending on how it is measured. In this investigation, therefore, an alternative method of quantifying effects on the fatpad size was developed.

There is a prevalence of oestrogenic and anti-androgenic activity in UK rivers (Johnson et al., 2004). So robust and cost-effective screening assays that can quantify the effects of chemicals and the complex mixtures found in effluents on the reproductive axis of fish need to be developed. To understand more fully the possible usefulness of VTG and SSCs in tests for environmental oestrogens/androgens (and their antagonists), though, a greater understanding of how they respond to EAC exposure is required. This section provides this information by assessing the dynamics of these biomarkers responses in male fathead minnows exposed to a

reference oestrogenic chemical, E2, for up to 14 days. To futher our understanding of the biological consequence of alternations in these biomarker responses, their persistance after exposure to E2 was also determined during a period of depuration in clean water of up to 70 days.

Materials and methods

Test organisms

The male fathead minnows used in this study were bred at the Brixham Environmental Laboratory. Their average weight was5.592 ± 0.304 g (mean ± SEM; n = 16). Fish were kept under flow-through conditions in de-chlorinated water at 25.0 ± 1°C, with a 16-hour light:8-hour dark photoperiod, with 20 minute dawn and dusk transition periods. Throughout the exposures, fish were fed two per cent of body weight per day of Ecostart 17 1.0 mm fish food pellets (BIOMAR).

Water supply and test apparatus

The supply of de-chlorinated water to the laboratory dosing system was monitored every day for conductivity, hardness and free chlorine, and twice a week for alkalinity and total ammonia. The conductivity of the test water ranged from 204 to 234 μS/cm, the hardness from 41.0 to 52.0 mg/L (as $CaCO_3$) and free chlorine was < 2.0 μg/L. Alkalinity ranged from 17.4 to 25.4 mg/L and ammonia (as $N\text{-}NH_3$) ranged from < 0.01 to 0.05 mg/L. Dissolved oxygen concentrations and pH levels were determined in the individual tanks on days 0 and 1 and then twice weekly throughout the exposure period. In all experiments, the dissolved oxygen concentration remained >80 per cent of the air saturation value throughout the exposures, and pH values ranged between 6.7 and 7.5. Water temperatures were monitored constantly throughout the exposure period; they ranged between 24.1 and 24.9°C. Dilution water and test chemical flow rates were checked at least twice a week. The flow-rate provided a 99 per cent replacement time of approximately four hours. The test vessels had a working volume of 45 L and were made of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

Experimental design

Male fathead minnows were exposed to a dilution water control, ethanol solvent control and nominal concentrations of E2 at 10, 32 and 100 ng/L (n = 72 fish/treatment) for 14 days. The test chemical, E2 (98 per cent purity; Lot 70K1206), was purchased from Sigma, Poole, Dorset, UK. Stock solutions of E2 were prepared in HPLC grade ethanol (Fisher Scientific) and dosed to glass mixing vessels using a syringe pump, at a rate of 2.0 μL/min, to mix with the dilution water flowing to the mixing vessels at a rate of 750 mL/min. The stock solutions were renewed every seven days. The solvent control vessel received the same rate of addition of ethanol, such that the water in all test vessels, except the DWC, contained 2.6 μL ethanol per litre. After the 14-day exposure period, all remaining fish were transferred to clean 45 L glass aquaria receiving dilution water only.

Measurement of E2 concentrations

Water samples (50 mL) were collected from each tank into solvent-cleaned flasks on days 0, 7 and 14 of the exposure and extracted under vacuum (5 mL min^{-1}) on to preconditioned solid phase extraction columns. To assess the efficiency of the extraction procedure, 50 mL dechlorinated water was spiked with 32 ng/L E2 and extracted under the same conditions. Two procedural blanks (a methanol blank and 50 mL HPLC grade water) were also extracted.. Estradiol-17β was eluted from the columns using 5 mL methanol and stored at –20°C for subsequent analysis. At the time of analysis, the methanol was removed under a stream of nitrogen and the extracts resuspended in 1 mL ethanol. The extracts were analysed using a commercially available enzyme immunoassay for E2 (Cayman Chemical Company, USA).

Fish sampling

Fish were sampled at the outset (day 0, n = 16) of the experiment and then randomly sampled subgroups (n = 8) after 2, 4, 7, 14, 28, 42, 55 and 85 days. Fish were sacrificed via a Schedule I method using a lethal dose (200 mg L^{-1}) of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) (Sigma), buffered with 1M NaOH to pH 7.3. Total wet body weight and gutted body weight of the fish were recorded to the nearest 0.01 g and standard and total lengths measured to 0.1 mm. Condition factor was derived by expressing the cube of the total fish length as a percentage of the gutted body weight. Blood was collected by cardiac puncture, using a heparinised syringe (5000 units heparin mL^{-1}), centrifuged (7,000 g; 5 min, 15°C) and the plasma removed and stored at -20°C until it was needed for VTG analysis. Plasma samples were tested for VTG using a carp ELISA (Tyler *et al*., 1999). The gonads were removed, wet-weighed to the nearest 0.01 mg, and the GSI derived by expressing the gonad weight as a percentage of the total body weight minus the gonad weight. The number of tubercles on each males snout were recorded and the dorsal fat pad removed and wet weighed to the nearest 0.01 mg.

Statistical analyses

In the first instance, possible relationships between the endpoints measured were investigated in the control fish. To do this, all solvent and dilution water control data for any one parameter were pooled. The analyses were conduced using the CORREL function in Microsoft Excel 2000. Significance levels for relationships between the parameters measured were determined using critical values of the correlation coefficient, r (Zar, 1999). To investigate effects of the test chemical, E2, at each sampling time point, data were compared to the solvent control using SigmaStat version 2.01 (SPSS (UK) Ltd., Surrey, UK). Data meeting the assumptions of normality and homogeneity of variance were analysed using one-way analysis of variance (ANOVA), followed by a pair-wise multiple comparison procedure (Tukey Test). Data that failed to meet the assumptions of both normality and homogeneity of variance were analysed using a Kruskal-Wallis one-way analysis of variance on ranks.

This was followed by a pair-wise multiple comparison procedure (Dunnett's Method for equal sample sizes and Dunn's Method for unequal sample sizes). The fatpad weight data were log 10 transformed to normalise the data and then analysed using the general linear model (ANCOVA) in Minitab version 14.1, with body weight as a covariate.

Results

Chemistry

Measured exposure concentrations of E2 are shown in Table 1. Mean measured concentrations of E2 in the 10, 32 and 100 ng/L test tanks were between 60 and 127 per cent of nominal: 12.7, 29.3 and 60.0 ng/L, respectively. The measured concentration of E2 in the spiked sample was 36.3 ng/L, which was 113 per cent of nominal. E2 was also detected in both the DWC and solvent control tanks and the two procedural blanks at a concentration of between 8 and 9 ng/L.

Relationships between size of the fish and sexual endpoints studied in the control fish

There were no increases in the total wet body weight, condition factor, SSCs (tubercle number and fatpad weight), GSI or plasma VTG concentrations in the DWC or solvent control fish throughout the 14-day exposure period or during the 70-day period of depuration ($P > 0.05$). Pooled data from the initial control fish and DWC fish from each sampling point revealed a positive correlation between the total wet body weight of the fish and both the weight of the gonad ($n = 80$, $p < 0.001$) and the weight of the fatpad ($n = 80$, $p < 0.001$). The total wet body weight of the fish also correlated positively with the number of tubercles ($n = 80$, $r = 0.499$, $p < 0.001$). Positive correlations were also observed between the two SSCs (fatpad weight and tubercle number, $n = 80$, $p < 0.001$) and between the wet weight of the gonads and both the number of tubercles ($n = 80$ $p < 0.001$) and the fatpad wet weight ($n = 80$, $p < 0.001$). There was no relationship between plasma VTG concentrations and total wet body weight, condition factor (CF), gonad weight, fatpad weight or tubercle number in the control fish.

Growth

At the beginning of the experiment (day 0), the male fathead minnows weighed (as mean $\pm$ SEM) 5.592 ± 0.304 g ($n = 16$) and had a condition factor of 1.092 ± 0.030 ($n = 16$). There were no concentration-related effects of E2 on total wet body weight or CF of the fish at any of the sampling points throughout the 14-day exposure period. During the depuration period, a negative correlation between concentration of E2 and the total fish wet weight and CF on day 55 ($p < 0.01$) was observed, but this effect was not significant when compared to the solvent control. No other concentration-related effects of E2 on total wet weight or CF were observed during the depuration period.

Secondary sexual characteristics

At the onset of the experiment (day 0), the mean fatpad weight and tubercle number in the subgroup of males sampled was 156.9 ± 23.42 mg and 10 ± 2 (n = 16), respectively. Exposure to E2 resulted in a concentration-related decrease in both the fatpad weight ($P < 0.05$) and tubercle number ($P < 0.05$) after 14 days of exposure, but these reductions in the SSCs were not significant when compared to the control fish ($P > 0.05$). During the depuration period, the negative correlation between the concentration of E2 and both the tubercle number and fatpad weight persisted and was evident on day 42 ($p < 0.05$) and day 55 ($p < 0.05$). On day 55 only, the decrease in tubercle number in males that had previously been exposed to the highest concentration of E2 (60 ng/L) was significant compared to the control fish ($p < 0.05$). At the same time, (day 55), a negative correlation was observed between the number of tubercles and the concentration of plasma VTG in the individual fish ($p < 0.01$). By contrast, the apparent reduction in the fatpad weight was not significant compared with the control fish, and no correlation between fatpad weight and plasma VTG concentration ($p > 0.05$) was observed. No concentration-related effect of E2 on either tubercle number or fatpad weight was observed on day 84.

Gonad growth

The GSI in the male fish at the onset of the experiment was 1.145 ± 0.092 (n = 16). There were no concentration-related effects of the test chemical on the GSI at any of the sampling points during the exposure period, though there was a negative correlation between concentration and the wet weight of the gonad on day 14 of exposure ($p < 0.05$). During the depuration period, a negative correlation between concentration of E2 and gonad weight and GSI of the fish was observed on day 55 ($p < 0.01$), but this effect was not significant when compared to the solvent control. No other concentration-related effects of E2 on gonad wet weight and GSI were observed during the depuration period.

VTG induction

The concentration of VTG in the plasma of the male fathead minnows at the start of the experiment was 50 ± 30 ng/mL. At each of the sampling points during the exposure there was a concentration-dependent increase in plasma VTG concentration ($p < 0.01$). The lowest concentration of E2 tested (13 ng/L) caused a significant increase in plasma VTG concentrations on day 7 of exposure (VTG concentration of 80 ± 20 ng/mL, $p < 0.05$). There were no significant differences between the concentrations of VTG in the plasma of the solvent control fish and the fish exposed to an E2 concentration of 13 ng/L at any other time during either the exposure period or the depuration period ($p > 0.05$). Significant increases in plasma VTG concentration, compared with the solvent control fish were observed in fish exposed to an E2 concentration of 29 ng/L on days 4, 7 and 14 or exposure. At the highest concentration of E2 tested (60 ng/L), significant increases in plasma VTG concentrations, compared with the solvent control were observed after only two days of exposure (VTG concentration of 4370 ± 940 ng/mL). This VTG concentration progressively increased, reaching a maximum on day 14 (VTG concentration of 595445 ± 129127 ng/mL). After transfer to clean water, there were no significant

changes in the concentration of plasma VTG on days 28 and 42, compared to the concentrations observed on the last day of exposure (day 14) in fish exposed to E2 concentrations of 29 and 60 ng/L. On day 55, a significant decrease in plasma VTG concentration was observed compared to day 14, in fish exposed to both 29 ng/L (VTG concentration of 390 $\pm$ 160 ng/mL, $p < 0.05$) and 60 ng/L (VTG concentration of 8850 $\pm$ 1570 ng/mL, $p < 0.05$). These concentrations of plasma VTG did not decrease further between days 55 and 84.

Discussion

Concentrations of E2 were measured throughout the exposure period to determine the actual concentrations to which the fish were exposed. In general, nominal and actual measured concentrations were very similar. Any differences observed were probably due to either a small inaccuracy in the preparation of the stock solutions and/or inaccuracies in the dosing to the test vessels. E2 was also measured, albeit at low concentrations, in the control tanks (DWC and SC) and the analytical procedural blanks. The concentrations in the controls and blanks were very similar. This suggests that the source of contamination is likely to arise from the analytical procedure rather than the exposure system.

In this investigation it was found that the fatpad could easily be removed from the dorsal musculature of a male to determine its wet weight as a way of quantifying differences in its prominence between individuals. As far as we know, this is the first time this method of quantifying the fatpad size has been used. To date, investigations determining effect of fish age/or chemical exposure on the size of the fatpad have measured the thickness (or height) of the fatpad at its widest margin, and in some cases estimated fatpad surface area (Miles-Richardson *et al*., 1999; Harries *et al*., 2000). The advantage of using such measurements is that effects on the fatpad can be determined without harming the fish. They do not, though, take into account the full mass of dorsal fatpad. Measuring the wet weight of the fatpad provides a more accurate and sensitive method of quantifying changes in its size. Using pooled data from the control fish, though, it was demonstrated that the total weight of the fatpad was influenced by the total wet body weight of the male ($P < 0.05$). To measure effects of chemical exposure on the fatpad, it was therefore necessary to use the general linear model (one way analysis of covariance; ANCOVA), with body weight as a covariate. Positive correlations were also observed between the two SSCs (tubercle number and fatpad weight) and gonad weight. This indicates that the prominence of these features can be used as an external indicator of the males' sexual status. This relationship between the prominence of the SSCs and testis weight is consistent with expectation since the formation of the male SSCs is androgen dependent (Smith, 1974) and that, in the male carp,, plasma testosterone and 11keto-testosterone concentrations are related to testis weight (Degani et al., 1998).

No concentration-related effects of E2 on the body weight or the CF of the fish were observed during the 14-day exposure period. This is not surprising given the very limited growth of the fish observed during this period, the low concentrations of the test chemical employed and the short duration of the chemical exposure. During the depuration period, a negative correlation between concentration of E2 and fish

growth (weight and CF) was observed on day 55 (though this apparent effect was not significant compared to the SC). This negative correlation between E2 concentration and fish growth may be due to a delayed effect of the test chemical caused by the high circulating concentrations of VTG in the plasma of the exposed fish (the energy drain imposed by the induction and or metabolism of VTG).

On day 14 of exposure and on days 42 and 55 during the depuration period, there were negative correlations between the concentration of E2 and the FPI and tubercle number. When compared to the solvent control, though, this effect was significant only for reduction of tubercle number and only in fish exposed to an E2 concentration of 60 ng/L on day 55. The lack of a significant effect for the FPI, when compared to the solvent control, was probably due to the large variation in the fatpad weight of the control fish. Miles-Richardson et al. (1999) reported a reduction in the prominence of the breeding tubercles and the size of the fatpad in adult male fathead minnows exposed for 19 days to E2 at 1 nM and 100 nM, respectively. Similarly, Harries *et al*. (2000) showed that exposing adult male fathead minnows for three weeks to the alkylphenol, NP (at a dose of 58 μg/L) reduces the number of tubercles. In that study, they also found a dose-dependent effect of NP on fatpad thickness for males exposed to nonylphenol, but these effects were not significant compared to the control (Harries et al., 2000). Interestingly, Miles-Richardson and colleagues (1999) also demonstrated that the reduction of SSCs caused by 10 nM E2 in male fathead minnows exposed for 10 days persisted for about three months after transfer to clean water. This persistence of effect on the SSCs following exposure to an oestrogen is consistent with the results observed in our study. SSCs are also sensitive to the effects of androgens. Exposing female fathead minnows to methyltestosterone results in the formation of a dorsal fat pad and nuptial tubercles (Smith, 1974; Ankley et al., 2000; Pawlowski et al., 2004). Smith (1974) demonstrated that these effects took only three to six days to become apparent, but they then persisted for 18 days or more after exposure ceased. The results of these investigations, together with the data presented in this report demonstrate that the SSCs, are a valuable, sensitive, and easily measured endpoint for detecting both oestrogen and (anti-) androgen exposure. They should therefore be included in pair-breeding assays.

In our study, concentration-related effects of E2 on GSI were observed during the 14-day exposure period. A previous study on fathead minnow demonstrated that exposing male fathead minnows for 21 days to a nominal E2 concentration of 100 ng/L inhibited testicular growth (Panter et al., 1998). Though this E2 concentration is similar to the one used in our investigation, the exposure period used here was shorter, which may explain the lack of a response on gonad size. A more likely explanation, though, is that Panter et al. (1998) used maturing fish, where the gonads were undergoing a period of rapid growth, whereas the males in our study were already mature, with gonads at (or close) to their maximum size. During the period of depuration, there was a negative correlation between the concentration of E2 and GSI on day 55 (though, again, this was not significant compared to the SC). The reason for a negative correlation between E2 concentration and gonad growth is not known. It is probably due to an impact on the energy budget for recovery from high levels of circulating VTG, but there may be a more direct effect on the testis or pituitary gland.

Concentration-response curves for plasma VTG occurred on exposure to E2 after only two days' exposure, with a lowest observed effect concentration (LOEC) of 60 ng/L on day 2 and a LOEC of 29 ng/L on day 4 of exposure. Though the potency of the test chemical increased with duration of exposure, reaching a maximal response on day 14 of exposure, there was no decrease in the LOEC of 29 ng/L by day 14. This pattern of VTG induction is consistent with other studies in which fish were exposed to steroidal oestrogens via the water (Panter et al., 1998; Folmar *et al*., 2000; Thorpe *et al*., 2000). It reinforces the fact that induction of VTG is a rapid and sensitive response for indicating oestrogen exposure. The LOEC of 29 ng/L obtained for E2 in this investigation is slightly below that previously reported for other studies using early life stages of fathead minnows exposed for 30 days (50 ng/L; Tyler *et al*., 1999) and adult male fathead minnows exposed for 21 days (100 ng/L; Panter *et al*., 1998). The LOEC of 29 ng/L, though, is very consistent with LOEC values of between 5 and 14 ng E2/L obtained in earlier investigations with rainbow trout using the same experimental design (Thorpe *et al*., 2001, 2003). This suggests that the fathead minnow is just as sensitive as the rainbow trout to oestrogen exposure.
After transfer to clean water, there were no significant decreases in plasma VTG concentrations until day 55 (after 41 days in clean water), demonstrating the persistence of VTG in male fish. Schultz *et al*. (2001) reported this persistence of plasma VTG concentrations in male rainbow trout exposed via intra-arterial injection to a single dose of EE2. After induction by EE2, peak plasma concentrations of VTG were reached after 7-9 days. This was followed by a mono-exponential decline to near basal values by day 48, with the exception of the fish exposed to the highest dose of EE2 (10 mg/kg) in which VTG levels were still around 5-10 per cent of maximum values. This persistence of VTG was attributed to the persistence of EE2 in the fish. Most of the EE2 was conjugated and stored in the gall bladder, only to be released into the gut and de-conjugated when the gall bladder emptied. The de-conjugated EE2 was then reabsorbed into the blood, in effect, 're-dosing' the fish. E2 is rapidly eliminated from fish as a result of conjugation. It may be possible, though, that some E2 is also de-conjugated and reabsorbed into the blood, which would contribute to the persistence of VTG observed in our study. It may also be the case, though, that VTG itself has a long half-life in males where there is no clearance of VTG to the gonad. It should also be noted that immunoassays using polyclonal antibodies for VTG will detect some of the degradation products of VTG, as well as the parent glycolipophosphoprotein.

Of the various endpoints included in this investigation, VTG was the most sensitive to oestrogen, highlighting its usefulness as a biomarker for exposure to oestrogen. Very high concentrations of VTG can cause kidney failure resulting in death, particularly in males (Herman & Kincaid, 1988; Zillioux et al., 2001; Seki et al., 2002). At present, little else is known about the relevance of VTG induction in terms of the health of the organism. The effects observed during depuration in this investigation further indicate that VTG induction may be detrimental to the fish. Once induced and secreted into the blood, VTG was persistent, remaining at significant concentrations even after 70 days of depuration in clean water. The negative impacts on SSCs of E2 exposure were correlated with plasma VTG concentration on day 55. This suggests that a critical threshold for health effects arising from increased VTG synthesis may occur sometime after the initial oestrogen exposure. The effects seen on growth and fatpad weight on day 55 may be a consequence of a change in the partitioning of energy in favour of VTG induction and metabolism. A more comprehensive study would be

needed to quantify this assessment. Recoveries from the concentration-related effects of E2 on growth, GSI and SSCs seen on day 84 were accompanied by significant reductions in the quantity of plasma VTG.

Table 1. Mean measured concentrations of estradiol-17β (E2) in the test tanks over the 14-day exposure and in the procedural blanks and spiked sample.

Nominal (ng/L)	Measured tank concn. of E2 (ng/L)			Mean measured concn. E2 (ng/L) Mean ± SEM
	Day 0	Day 7	Day 14	
Water blank	5	11	11	9.0 ± 2.0
Methanol blank	7	10	9	8.7 ± 0.9
Spiked sample	36	41	32	36.3 ± 2.6
DWC	9	6	10	8.3 ± 1.2
SC	6	4	14	8.0 ± 3.1
10	12	8	18	12.7 ± 2.9
32	32	24	32	29.3 ± 2.7
100	56	50	74	60.0 ± 7.2

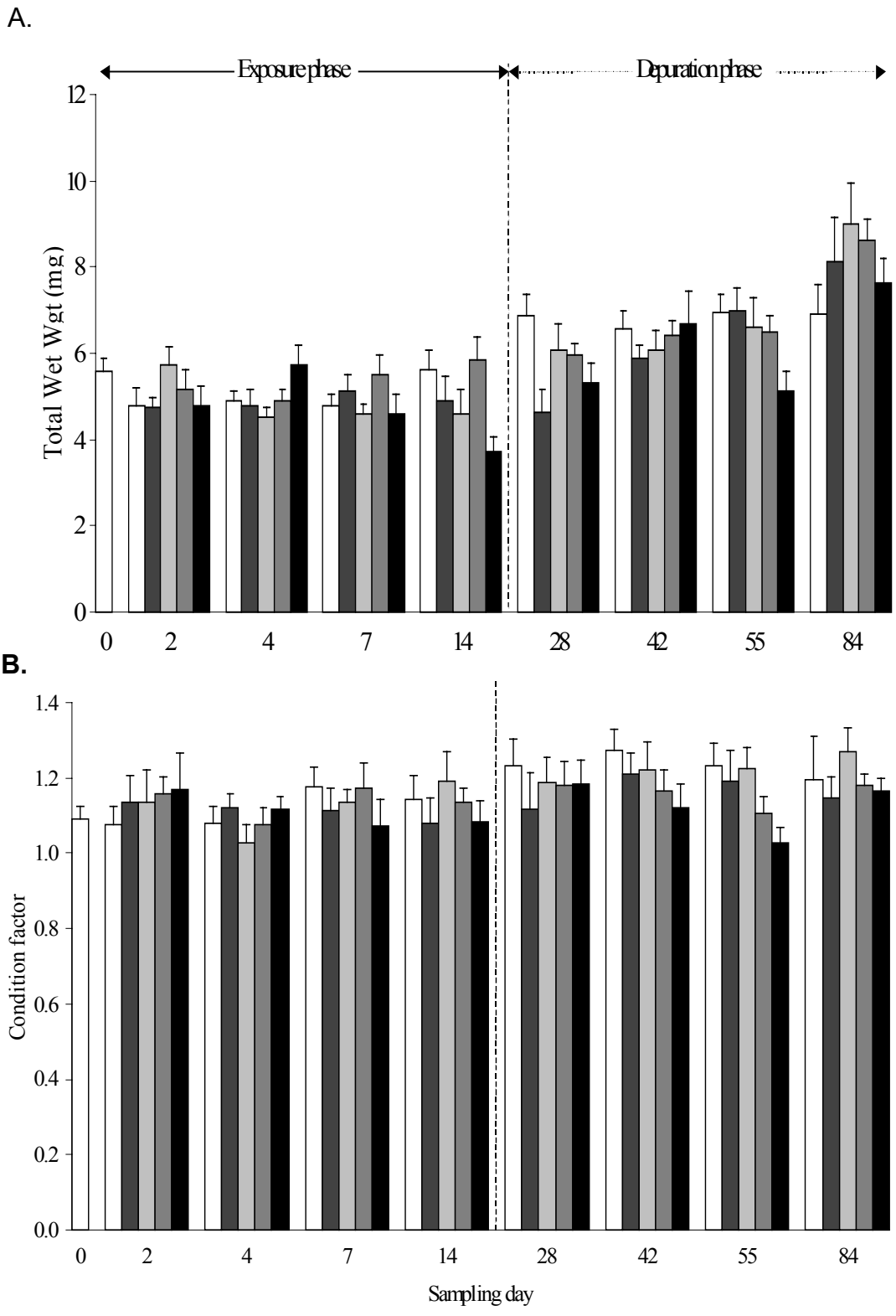


FIGURE 1. Total wet body weight (A) and condition factor (B) in male fathead minnow exposed to (□) dilution water control, (■) solvent control, (▒) 10 ng estradiol-17β (E2) L^{-1}, (▒) 32 ng E2 L^{-1} and (■) 100 ng E2 L^{-1}. Each column represents the mean ± standard error of the mean (SEM).

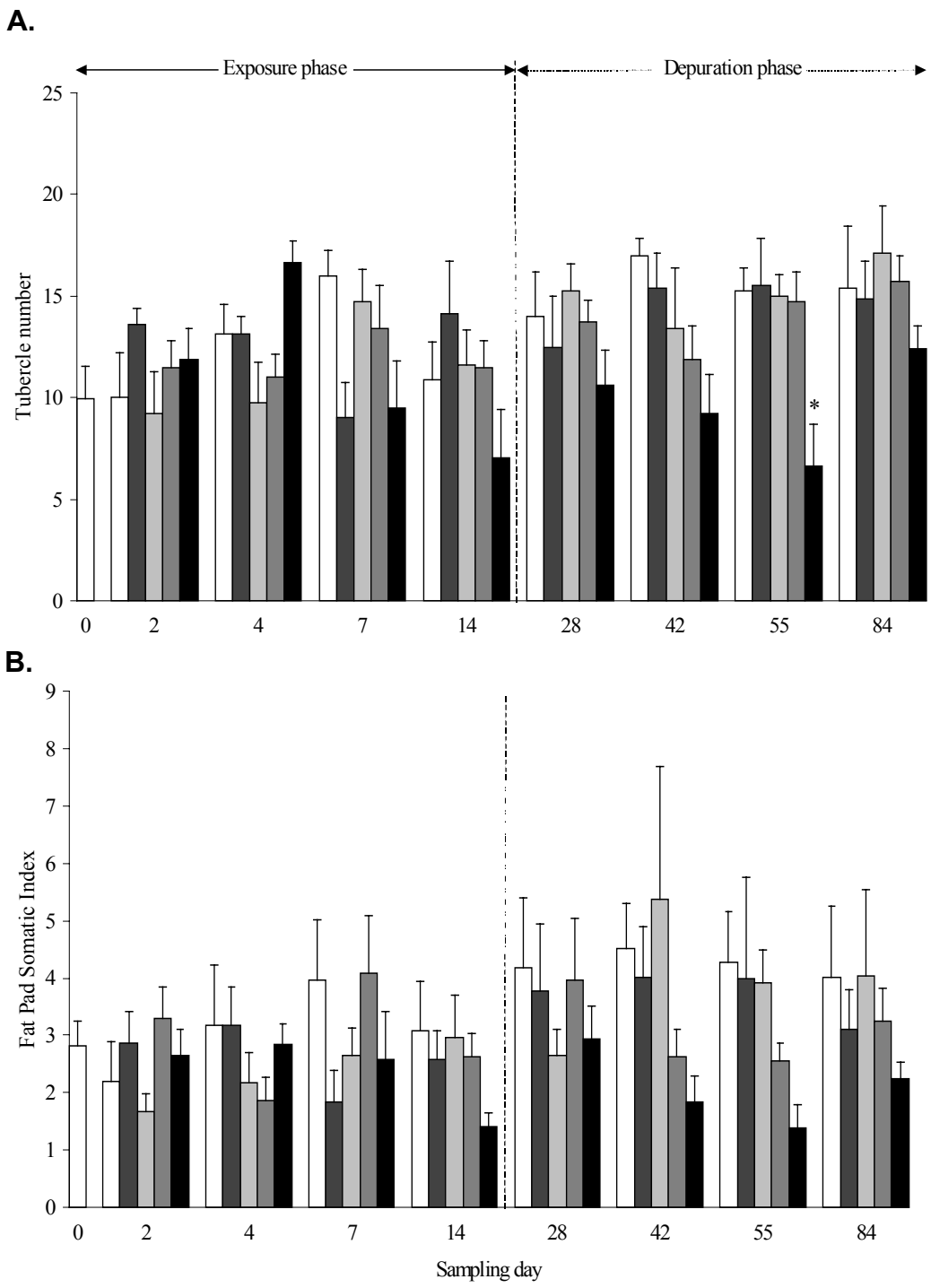


FIGURE 2. Tubercle number (A) and fatpad somatic index (B) in male fathead minnow exposed to (□) dilution water control, (■) solvent control, (▒) 10 ng estradiol-17β (E2) L^{-1}, (▒) 32 ng E2 L^{-1} and (■) 100 ng E2 L^{-1}. Each column represents the mean ± standard error of the mean (SEM). Significant differences from solvent control values at each sampling point are denoted as * $p < 0.05$ (Tukey Test).

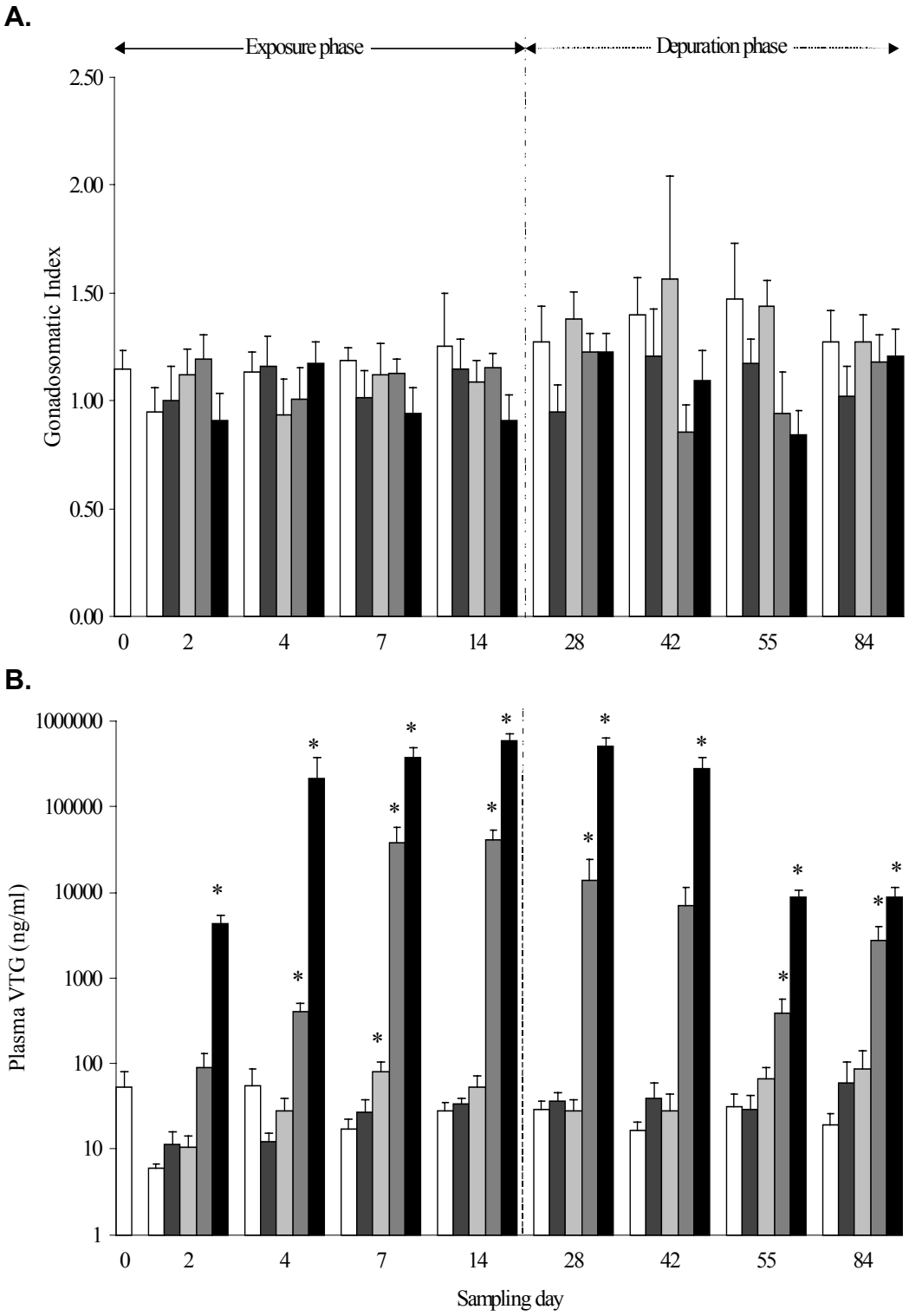


FIGURE 3. Gonadosomatic index (A) and plasma VTG (VTG) concentrations in male fathead minnow exposed to (□) dilution water control, (■) solvent control, (■) 10 ng estradiol-17β (E2) L^{-1}, (■) 32 ng E2 L^{-1} and (■) 100 ng E2 L^{-1}. Each column represents the mean ± standard error of the mean (SEM). Significant differences from solvent control values at each sampling point are denoted as * $p < 0.05$ (Dunnett's Test).

2 Fecundity dynamics in pair-breeding fathead minnow

Abstract

The fathead minnow, *Pimephales promelas*, is one of the most widely used fish species in chronic life-cycle and early life-stage tests in toxicology, but very little is known about its normal reproductive capacity, and standardized tests to measure reproductive performance are not well established. This is surprising, given that reproduction is a major endpoint in chronic fish tests. Under favourable conditions, fathead minnow spawn every three to five days over periods of many weeks. Reported fecundity estimates, though, vary widely in the literature both between and within studies, and this has raised questions about the robustness of this species as a testing organism for assessing reproductive effects. The reported variation in fecundity estimates for this species may relate partly to its reproductive strategy. Fathead minnows typically spawn on the under-surface of objects. Laboratory investigations to study fecundity in this species often use an inverted U-shaped tile to collect the spawned eggs, but not all of the eggs stick successfully to the tile surface. In this study, extensive and comprehensive assessments were made on fecundity estimates in pair-breeding fathead minnows using various egg-collection strategies. Reproduction was assessed in a total of 172 pairs of fish, in four independent experiments, each lasting between three and four weeks. These results demonstrate that using a screened collection tray placed beneath the spawning tile, not only increased fecundity estimates by between 25 and 67 per cent, but importantly also reduced intra-assay variation from > 50 per cent to <30 per cent. In addition, the inter-assay variation fell from 39 per cent to 14 per cent. This suggests that the reported variability of fathead minnow fecundity estimates is likely to be a consequence of the egg collection systems traditionally used, which don't take into account eggs that haven't stuck to the spawning tile. This revised test system demonstrates that egg production in fathead minnows is a highly robust endpoint against which chemical effects can now be challenged more effectively.

Introduction

The fathead minnow, *Pimephales promelas*, represents the ecologically important and ubiquitous Cyprinidae family of fish. It is one of the most widely used fish species in chronic life-cycle and early life-stage tests in toxicology, in support of regulatory programs, in both Europe (OECD, 1992) and North America (EPA 1982, 1989, 1991, 1994). More recently, this species has also been shown to a suitable test organism for assessing the effects of endocrine active chemicals on reproductive function (Harries et al., 2000; Ankley et al., 2001; Länge et al., 2001; Bringolf et al., 2004; Jensen et al., 2004; Parrot & Blunt, 2005). In the fathead minnow test, reproductive performance (frequency of spawning and number of eggs spawned) is evaluated over a period of two to three weeks. This is done in replicate groups (Ankley et al., 2001 Bringolf et al., 2004; Jensen et al., 2004) or in pairs (Harries et al., 2000) of sexually mature fish held under control conditions. After the pre-exposure period, the fish are exposed to the test substance. Reproductive performance is monitored for a further two to three weeks to evaluate the effects of the test chemical(s) on reproductive function. Assessments on embryo viability, hatching success and growth and development of the F1 generation, provide valuable further additions in this test and allow the direct or indirect (i.e. trans-generational) effects of chemicals on early life stages to be assessed. Under optimal conditions, the fathead minnow has a short lifecycle: it reaches sexual maturity within three to four months. In addition, the species is easy to culture in the laboratory, and its reproductive cycle can be controlled through alterations in temperature and photoperiod (EPA, 1987). This makes the fathead minnow an ideal test species for studies on its reproductive biology and for assessing the effects that chemicals have on reproduction.

A significant uncertainty in using the fathead minnow to study reproduction and sexual development is a general lack of knowledge concerning the basics of its reproduction in the context of laboratory holding and testing. It is know that the fathead minnow is a repetitive spawner, with females producing batches of eggs every three to five days over periods of many weeks (Gale and Buynek, 1982; Jensen et al., 2001). Fecundity estimates for the fathead minnow, though, vary widely in the literature. They don't provide consistent reference data on which to base fecundity as an endpoint for chemical impact assessments. Mean fecundity rates in the fathead minnow are reported to be between 2.5 and 26 eggs/female/day in a number of laboratories studying reproduction in this species (Ankley et al., 2001; Länge et al., 2001; Jensen et al., 2001, 2004; Bringolf et al., 2004; Pawlowski et al., 2004; Parrot & Blunt, 2005). In two laboratories, though, considerably higher fecundity rates of between 43 and 112 egg/female/day have been observed (Gale and Buynek, 1982; Harries et al., 2000). The reasons for these marked differences aren't clear because of differences in the studies' experimental conditions, but they could be down to the way in which researchers collected eggs for fecundity estimates. Fathead minnow eggs are spawned on the under-surfaces of submerged objects. Laboratory investigations to study fecundity in this species, therefore, use an inverted U-shaped tile to collect the spawned eggs. Some eggs do not adhere to this substrate, however, and have been observed to fall to the bottom of the tank where they are often predated (McMillan, 1972). Gale and Buynek (1982) and Harries et al. (2000), therefore, placed a screened tray underneath the spawning tile to catch any eggs that didn't stick to the spawning surface. In the studies that reported lower

fecundity estimates, however, a screened tray wasn't used, which could explain why the fecundity estimates differ between the studies reported.

In this study, comprehensive assessments were made of fecundity and embryo viability in pair-breeding fathead minnows held under optimal laboratory conditions. Initial studies set out to establish the proportion of eggs spawned that actually stuck to the spawning tile and how this varied between pairs of breeding fish. Further fecundity estimates within and between pair-breeding fathead minnows were made according to the egg collection method used. In addition, the hatching success of embryos adhered to the spawning tiles was compared to the hatching success of those that did not. The study also looked at variability in hatching success across spawnings for individual pairs of fish and between different pairs of fish. In one experiment, growth and development of the F1 fish were also studied to determine the normal level of variation in these parameters in offspring from different adult pairs.

Materials and methods

General experimental conditions

Test organisms

Details regarding the supply of the fathead minnows for each investigation are provided in the relevant sections. All fish were held for at least three months in the husbandry unit at AstraZeneca's Brixham Environmental Laboratory, Devon, UK, before any tests started, to ensure that they were free from disease and at the stage of reproductive development required for the pair-breeding studies.
A minimum of two weeks before the start of each study, sexually mature males and females (determined by the development of secondary sexual characteristics) were separated to prevent any spawning activity and acclimated to the test conditions. During all acclimations and experiments, the fish were maintained in de-chlorinated water at 25.0 $\pm$ 1°C, with a 16-hour light:8-hour dark photoperiod, with 20-minute dawn and dusk transition periods. Fish were fed frozen brine shrimp (Tropical Marine Centre, Hertfordshire, UK), twice daily (approximately 0.9 g/feed/tank), and Ecostart 17 pelleted fish food (approximately 0.1 g/feed/tank) BIOMAR, Houghton Springs Fish Farm, Dorset, UK), once daily. Uneaten food was siphoned from the tanks once a day.

Water quality

The supply of de-chlorinated water to the laboratory dosing system was monitored daily for conductivity, weekly for alkalinity, hardness and free chlorine, and monthly for total ammonia. Throughout all studies, the test water's conductivity ranged from 206 to 282 $\mu S\ cm^{-1}$, its alkalinity ranged from 20.0 to 30.6 mg L^{-1}, and its hardness from 38.3 to 50.0 mg L^{-1} (as $CaCO_3$). Free chlorine remained below 2.0 $\mu g\ L^{-1}$ and ammonia (as $N\text{-}NH_3$) was below 10 $\mu g\ L^{-1}$. Dissolved oxygen concentrations and pH levels were determined in the individual tanks on days 0 and 1 and then twice weekly throughout the exposure period. In all experiments, the dissolved oxygen concentration remained >70 per cent of the air saturation value, and pH values ranged from 6.9 to 8.0. Water temperatures were monitored constantly throughout the exposure period and ranged between 24.3 and 25.2°C.
Fish in all experiments were held under flow-through conditions. The dilution water was dosed into each tank via a gravity-fed system at a nominal rate of 80 mL/min. This flow rate provided a 95 per cent replacement time of six hours. Dilution water flow rates were checked at least once a week; they remained within 20 per cent of nominal.

Test apparatus

The test vessels used for the adult pair-breeding studies had a working volume of 12 litres (305 x 150 x 355 mm; length x width x depth). They were made of glass with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions. Screens were placed around the sides of all test vessels to prevent fish in adjacent tanks from interacting with one another and to minimise disturbance caused by researchers. The spawning substrates consisted of a tile (80 mm length of

110 mm diameter PVC half guttering; EPA, 1987) placed above a screened collection (Sc) tray. The Sc tray was a rectangular glass tray, measuring 130 x 110 x 30 mm (length x width x depth) covered with 0.5 cm^2 stainless steel mesh (the mesh allowed eggs to pass through to the collection vessel). In experiment I, a non-screened collection (N-Sc) tray was also used. The N-Sc tray consisted of a rectangular glass tray measuring 130 x 260 mm (length x width). The spawning substrates were checked for eggs once a day at 10.30am and the tiles and trays replaced with clean ones, regardless of the presence or absence of eggs.

The test vessels used for the hatching trails and for maintenance of the F1 generation fish had a working volume of nine litres (305 x 205 x 210 mm; length x width x depth). They were made of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

Experimental design

To begin each test, males and females were selected at random and placed as pairs into replicate test vessels containing a spawning substrate. The fish were acclimated to the spawning test conditions for a minimum of seven days, before any detailed assessments of reproductive performance were made. After this initial acclimation period, the reproductive behaviour and spawning activity of each pair were measured every day.

Fecundity assessments

Experiment I

The fathead minnows used in this experiment were supplied as juveniles (about six months old) by Aquatic Research Organisms (Hampton, USA) and held for 10 months at Brixham Environmental Laboratory, before starting the experiment. The body weights (as means $\pm$ SEM) of the male and female fish at the start of the experiment were 6.67 $\pm$ 0.23 g (n = 30) and 2.82 $\pm$ 0.10 g (n = 30), respectively. In total, 40 pairs of fish were used in experiment I. These were divided randomly into four groups, according to the type of spawning substrate used: a total of 10 replicate pairs of fish were assigned to each group. The first group was provided with a spawning tile and N-Sc tray. The second were given a spawning tile and Sc tray. The third group were initially provided with a spawning tile and N-Sc tray, but after 14 days, a Sc tray replaced the N-Sc tray. The fourth group initially received a spawning tile and Sc tray, but after 14 days, a N-Sc tray replaced the Sc tray. It has previously been observed, both in our laboratory (personal observations) and those of others(McMillan, 1972;Gale and Buynek, 1982; Harries et al., 2000), that eggs fall to the bottom of the tank during spawning. So it was not felt appropriate to include a group without any form of tray system. After an acclimation period of 10 days, the spawning activity of each pair of fish was measured daily for 28 days.

Experiments II, III and IV

The fathead minnows used in experiment II were supplied as juveniles (age unknown) by Osage Catfisheries Inc. (Missouri, USA) and held for a period of three

months at Brixham Environmental Laboratory, before the start of the experiment. The fathead minnows used in experiments III and IV were bred at Brixham Environmental Laboratory. At the beginning of each experiment, they were approximately five and eight months old respectively. A total of 36, 48 and 48 pairs of fish were used in experiments II, III and IV, respectively. The body weights (as means ± SEM) of the male and female fish at the start of each experiment were 4.21 ± 0.13 g and 2.21 ± 0.06 g respectively in experiment II, 4.47 ± 0.11 g and 2.08 ± 0.08 g respectively in experiment III, and 5.52 ± 0.12 g and 2.69 ± 0.11 g respectively in experiment IV. In each experiment, the reproductive behaviour and spawning activity of each pair of fish was monitored daily, for 24 days.

Embryo viability

Experiment I

To compare the viability of eggs in the collection tray with those adhered to the tile, eggs were collected randomly from the spawning tile and tray, where there were more than 50 eggs on each substrate. Embryos from the spawning tile were rinsed and put into a Petri-dish with dechlorinated water. A total of 50 eggs were taken at random from the Petri-dish and transferred to replicate incubation cups (25 eggs/cup). This procedure was repeated for eggs taken from the corresponding N-Sc or Sc tray. The incubation cups were suspended from an oscillation unit into tanks receiving a continuous supply of dilution water, as described for the adult exposures. Embryo viability was assessed daily, and any dead embryos discarded to minimise the risk of fungal contamination. When there were fewer than 50 eggs on either the spawning tile or tray, all the eggs were counted and then removed from the spawning surfaces and discarded with no assessment of viability.

Experiments II, III and IV

To assess normal variation in hatching success between separate spawning events for an individual pair of fish, and between fish pairs, hatching trials from three separate spawning occasions were undertaken for each pair of fish used in experiments II, III and IV. For each pair, eggs were collected randomly, on three separate spawning occasions over the 24-day period. To minimise possible effects arising as a consequence of fungal contamination (more likely to occur in the Sc tray where there was an accumulation of faeces and uneaten food) eggs from the spawning tile were collected in preference to those in the tray, but if there weren't enough eggs on the spawning tile, eggs were also collected from the Sc tray (provided that they hadn't been there more than 12 hours, i.e. had not developed beyond the blastula stage).
Fifty eggs/embryos were collected at each spawning, rinsed in dechlorinated water and put in replicate incubation cups (25 eggs per cup). The incubation cups were suspended from an oscillation unit into tanks receiving a continuous supply of dilution water, as described for the adult exposures. Embryo viability was assessed daily and any dead embryos discarded to minimise the risk of fungal contamination. After seven days, any unhatched eggs were considered nonviable and discarded. After hatching, fry not needed for further investigation were killed using a lethal dose of anaesthetic.

Growth and sexual differentiation of F1 fish

Experiment III

For experiment III, survival, growth and sexual differentiation (ratio of males:females) were assessed in offspring from several pairs of adult fathead minnows. This was to determine normal variation in offspring with different parents. Twenty fry were selected at random from the final hatching trial for each of 20 randomly selected adult pairs and released into glass tanks (working volume of nine litres) receiving a continuous supply of dilution water only. A separate tank was assigned for the fry from each adult pair. The fry were maintained until 80 dph and survival rates assessed daily. At 80 dph, all surviving fish were killed in a lethal dose (500 mg/L) of MS222 buffered with 1M NaOH to pH 7.3. The total wet body weight of the fish was recorded to the nearest 0.01 g and standard and total lengths measured to 0.01 mm. The sex of the fish was determined by gross examination of the gonads. Where the sex could not easily be identified macroscopically, the fish were fixed in Bouin's solution (24-hour fixation of whole body) and later sectioned and examined histologically. Additional fish, selected at random, were also fixed for histological examination to confirm the accuracy of the gross examination of the gonads.
From the day they hatched, all fry were fed three times a day with live rotifers (Mannin Sea Farms, Isle of Mann), for the first week, then three times a day with live brine shrimp nauplii (Argent Marine Labs, San Francisco, USA) for the second week. After this, fry were fed twice a day with live brine shrimp nauplii and once a day with Ecostart 17 pelleted fish food until they reached 30 dph. From 30 to 80 dph, the fry were fed twice a day with frozen adult brine shrimp and once a day with Ecostart 17 pelleted fish food.

Statistical analyses

All results are expressed as mean ± standard error of the mean (SEM). Data meeting the assumptions of normality and homogeneity of variance were analysed using one way analysis of variance (ANOVA) followed by a pair-wise multiple comparison procedure (Tukey Test). In experiment I, an all pair-wise multiple comparison procedure (Bonferroni t-test) was used. Data that failed to meet the assumptions of both normality and homogeneity of variance were analysed using a Kruskal-Wallis one way analysis of variance on ranks. This was followed by a pairwise multiple comparison procedure (Dunnett's Method for equal sample sizes and Dunn's Method for unequal sample sizes). In all experiments, strengths of association between pairs of variables were measured using the Pearson product movement correlation coefficient.

Results

In all experiments, water quality parameters remained within acceptable OECD guideline levels (see Table 1). No mortalities were observed in any of the experiments, but some pairs of fish had to be excluded from the data analyses because the females in the breeding pairs became unable to release their eggs. In some cases, additional pairs of fish were also excluded from the analyses because they spawned on the sides of the tank, in addition to, or occasionally instead of, on the spawning substrate, making it difficult to count the eggs accurately.

Fecundity assessments

Experiment I

All pairs of fish spawned regularly, with spawning intervals of between 2.7 and 14 days (mean 4.75, mode 4.3) for the individual pairs. Table 2 shows the mean total egg numbers and egg batch sizes over each 14-day collection period for each group of fish. This is based on the number of eggs collected from the spawning tile only and the number collected from the spawning tile and Sc or N-Sc tray. Mean (± SEM) egg batch sizes, based on eggs collected from the spawning tiles only, ranged from 69 ± 36 up to 484 ± 109 eggs/spawn, with an overall mean spawning batch of 258 ± 13 egg/spawn (for all pairs). Total egg production over a period of 14 days varied from 130 for one pair of fish up to 2,547 for another, with an overall mean of 893 ± 64. No significant differences were observed in egg batch size or total egg number between each of the four groups either within, or between, the two 14-day monitoring periods ($P > 0.05$), based on the tile-only egg collection data.

Including eggs collected from the N-Sc tray increased the mean spawn size by 11 per cent to 286 ± 20 eggs/spawn (range between 93 ± 30 and 525 ± 127 eggs/spawn for the individual pairs) and the total number of eggs (in 14 days) by 10 per cent to 910 ± 102 eggs (range between 165 and 2553 eggs for the individual pairs). The apparent higher fecundity estimates obtained using the N-Sc were not, though, significantly different from those obtained using the tile only ($P > 0.05$). Including eggs collected from the Sc tray, however, did significantly increase both egg batch size ($P < 0.01$) and total egg number ($P < 0.05$), compared to that for the tile only. Estimates of fecundity using the Sc tray increased the egg batch size by 39 per cent to an overall mean of 358 ± 14 eggs/spawn (range between 254 ± 25 and 520 ± 90 eggs/spawn for the individual pairs) and the total number of eggs in 14 days by 44 per cent, to 1,286 ± 84 eggs (range between 307 and 1944 eggs). As well as giving a higher fecundity estimate, the inclusion of eggs in the Sc trays also reduced the variation in egg batch size ($P < 0.05$), with a decrease in the coefficient of variation (CoV) from 36 per cent (tile only data) to 19 per cent (tile and Sc tray). A reduction in the CoV for total fecundity over a period of 14 days, from 57 per cent (tile only) to 33 per cent (tile and Sc tray), was also observed, but this was not significant ($P > 0.05$). By contrast, using the N-Sc tray did not affect the variability of the fecundity estimates compared with the estimates derived using the tile-only data ($P > 0.05$).

Experiments II, III and IV

In all experiments, fish acclimated to the test conditions relatively quickly and established regular spawning patterns. Spawning intervals varied between pairs of fish, from 2.4 to 4 days (mean 3.3, mode 3.0) in experiment II, from 2.4 to 8 days (mean 3.5, mode 3.4) in experiment III and from 2.4 to 6 days (mean 3.8, mode 3.4) in experiment IV. Mean egg batch sizes, based on numbers of eggs spawned on the tiles only, were 88 ± 12 (ranging from 5 ± 3 to 234 ± 57), 187 ± 15 (ranging from 30 ± 24 to 437 ± 27) and 230 ± 20 (ranging from 43 ± 12 to 588 ± 46) in experiments II, III and IV, respectively. Mean total egg numbers (over a 24 day period) were 639 ± 87 (range from 23 to 1871), 1394 ± 130 (range from 208 to 3057) and 1532 ± 134 (range from 171 to 3526) in experiments II, III and IV, respectively. Consistent with the results of experiment I, some pairs of fish failed to attach all of the eggs spawned to the under-surface of the spawning tile. The percentage of each batch of eggs deposited on the spawning tile varied between 2.1 ± 1.4 and 68.8 ± 15.2 per cent (mean 28.2 ± 4.0 per cent) in experiment II. It was between 10.3 ± 4.9 and 90.1 ± 1.7 per cent (mean 56.4 ± 3.3per cent) in experiment III and between 11.5 ± 3.4 and 96.9 ± 0.6per cent (mean 64.2 ± 3.7 per cent) in experiment IV. Mean egg batch sizes were higher when the Sc tray was used ($P < 0.05$). They increased by 198 per cent in experiment II (mean 263 ± 12; range 139 ± 13 to 394 ± 14), by 59 per cent in experiment III (mean 298 ± 12; range 156 ± 24 to 485 ± 31) and by 47 per cent in experiment IV (mean 339 ± 16; range 211 ± 20 to 672 ± 53). Similarly, using the Sc tray increased mean total egg numbers (over a 24 day period; $P < 0.05$). They rose by 202 per cent in experiment II (mean 1927 ± 99; range 934 to 3149), by 56 per cent in experiment III (mean 2170 ± 114; range 871 to 3448) and 46 per cent in experiment IV (mean 2232 ± 108; range 928 to 4033). As well as increasing the fecundity estimates for each pair of fish, though, using the Sc tray also reduced the variation in fecundity estimates between the individual pairs of fish. The CoV for egg batch sizes was reduced from 75 to 24 per cent in experiment II, from 50 to 25 per cent in experiment III and from 52 to 28 per cent in experiment IV. The CoV in total egg numbers (over the 24 day period) between individual pairs of fish also fell through use of the Sc tray from 74 to 28 per cent in experiment II, from 57 to 32 per cent in experiment III and from 52 to 29 per cent in experiment IV. Reductions in the CoV were also observed in egg batch size between separate spawning occasions for each individual pair of fish. The mean within-pair variation in egg batch size fell from 100 per cent (range 58 to 176 per cent) to 28 per cent (range 10 to 63 per cent) in experiment II. It fell from 65 per cent (range 17 to 211 per cent) to 28 per cent (range 7 to 54 per cent) in experiment III. And in experiment IV the mean within-pair variation in egg batch size fell from 50 per cent (range 10 to 157 per cent) to 26 per cent (range 9 to 58 per cent).

In every experiment, pairs of fish that spawned a large number of eggs per spawning were also better at depositing a higher percentage of their eggs on to the spawning tile ($P < 0.02$, experiment II; $P < 0.01$, experiments II and IV). It was noted, though, that in all experiments (II, III and IV) egg batch size increased as a function of time, with a batch size in the final spawning (over a 24-day period) almost twice that of the first spawning ($P < 0.01$). Mean egg batch sizes increased from 169 ± 18 to 340 ± 20 in experiment II, from 175 ± 30 to 325 ± 23 in experiment III and from 236 ± 25 to 340 ± 32 in experiment IV, over 24 days. It is possible that the observed relationship

between the percentage of eggs on the spawning tile and egg batch size was an indirect result of the pairs of fish becoming more proficient over time at depositing their eggs on the spawning tile. The results from experiments II and III appear to support this. There, the percentage of eggs deposited on the spawning tile increased from 7 to 45 per cent, in experiment II ($P < 0.01$) and from 40 to 92 per cent in experiment III ($P < 0.01$) in the successive batches over the 24-day monitoring period. In experiment IV, though, there was no evidence that the individual pairs of fish got better at depositing their eggs on the spawning tile with time ($P > 0.05$). In experiment III, egg batch size was also positively related to female body weight. A two-fold increase in body weight was associated with an increase in egg batch size by 108 eggs/spawning ($P < 0.05$) and an increase in total egg number by 890 eggs over a period of 24 days ($P < 0.05$). Similarly, in experiments II and IV, there was an apparent larger egg batch size and total egg number in bigger females, but this was not significant in either of these experiments ($P > 0.05$). In each experiment (II to IV), there was no evidence that the percentage of eggs deposited on the spawning tile was influenced by either the body weight of either the male ($P > 0.05$) or female ($P > 0.05$) fathead minnow in the breeding pair, or by the male:female body weight ratio ($P > 0.05$).

Embryo viability

Experiment I

The hatching successes of embryos collected from both the spawning tile and tray within six to eight hours of spawning (confirmed through identification of the embryonic stage, i.e. early cleavage through to morula) were compared. Embryos that were collected from the spawning tiles hatched within four to five days, with a mean survival to hatch of 85 ± 2.6 per cent. Embryos collected from the spawning tray also hatched within four to five days, but their survival to hatch was lower than those collected from the tile (mean 63 ± 5.7 per cent ; $P < 0.05$). Throughout the exposure, embryos that had been left in the tray for approximately 24 hours post spawning showed a greater than 80 per cent mortality and often did not develop beyond the tail-free stage. By contrast, the embryos left on the spawning tile underwent normal embryonic development with no increase in the percent mortality (15 per cent) from the time they were removed from the tile. Uneaten food and faeces tended to adhere to the outer surface (egg capsule) of the eggs found in the tray, but not to those on the tile. When the eggs from the tray were thoroughly cleaned by rinsing in dechlorinated water and any debris physically removed from their surface, embryo survival to hatch increased to more than 85 per cent; it did not differ from the hatching success of embryos removed from the spawning tile ($P > 0.05$).

Experiments II, III and IV

In all experiments, embryos hatched within four to five days. Mean survivals to hatch for embryos selected from the individual pairs of fish ranged from 68 ± 7.0 to 100 ± 0.0 per cent (mean 90 ± 1.9 per cent) in experiment II. In experiment III, mean survival to hatch ranged from 80 ± 1.7 to 100 ± 0.0 per cent (mean 97 ± 0.67 per cent) and from 72 ± 3.7 to 100 ± 0.0 per cent (mean 94 ± 1.2 per cent) in experiment

IV. Within each experiment, 61, 95 and 84 per cent of the pairs of fish consistently spawned embryos that showed a greater than 90 per cent hatching success. The CoV between the three sets of hatching trials for each pair of fish remained below 10 per cent. In all experiments, the CoV increased with reduced hatching success ($P < 0.001$), reaching levels of 34, 11 and 16 per cent in experiments II, III and IV, respectively.

Growth and sexual differentiation of F1 fish

Experiment III

Survival of the F1 fish to 80 dph varied between offspring from the different adult pairs and ranged from 60 to 100 per cent (overall mean survival of 93 $\pm$ 3 per cent, n = 480). For all pairs of fish, the critical period for offspring survival was between 0 and 6 dph: 92 per cent of the total mortalities happened during this period. Variations in sex ratiosbetween offspring from different adult pairs were also observed, with the percentage of males in each aquaria ranging from 31 to 80 per cent (overall mean sex ratio of 54 per cent males, n = 480). Growth parameters for the offspring from the individual adult pairs are shown in figure 3. Mean wet body weights of the male and female offspring, from 23 of the 24 adult pairs, varied between 1.21 $\pm$ 0.18 and 1.99 $\pm$ 0.25 g, and between 0.94 $\pm$ 0.070 and 1.40 $\pm$ 0.18 g, respectively. Offspring from the remaining adult pair (adult pair number 11) appeared to grow at a faster rate, and the mean wet body weights of the males and females at 80 dph were 2.70 $\pm$ 0.12 g ($P < 0.001$) and 1.54 $\pm$ 0.10 g ($P < 0.05$), respectively. Mean total body lengths also varied between 48 $\pm$ 1.7 and 59 $\pm$ 1.6 mm for the male offspring from 23 of the 24 adult pairs. Males from adult pair 22 were shorter (46 $\pm$ 1.6 mm, $P < 0.001$). Female body lengths did not vary significantly between the offspring from different adult pairs (range 45 $\pm$ 1.02 to 50 $\pm$ 0.89 mm, $P > 0.05$). Condition factors varied between 0.99 $\pm$ 0.013 and 1.15 $\pm$ 0.02 for the male offspring from 22 of the adult pairs. Higher condition factors were observed in male offspring from the adult pair that produced the heavier fish (adult pair number 11; CF of 1.30 $\pm$ 0.08, $P < 0.001$) and from the adult pair that produced the shorter males (adult pair number 22; CF of 1.26 $\pm$ 0.12, $P < 0.001$). Condition factors varied between 0.96 $\pm$ 0.034 and 1.10 $\pm$ 0.095 for the female offspring from 20 of the 24 adult pairs. They were significantly higher (1.14 $\pm$ 0.041, 1.15 $\pm$ 0.025, 1.24 $\pm$ 0.072, 1.25 $\pm$ 0.041, $P < 0.001$) in female offspring from the remaining four adult pairs.

To determine whether the observed variations in survival, sex ratios and growth parameters between offspring from different adult pairs were parent-specific, offspring from a second spawning event for four of the adult pairs were kept until 80 dph. Comparison of the F1 data (Table 3) from the two separate spawning events for each adult pair demonstrated that both F1 survival and sex ratio varied between the two batches of offspring from the same adult pairs. No significant differencesin male or female body weights, lengths, or condition factors between the batches of offspring from the same adult pairs were observed (Table 3, $P > 0.05$).

Discussion

In these investigations, comprehensive fecundity estimates for adult pairs of fathead minnow held under controlled experimental conditions were determined. The fathead minnow is a robust test organism for assessing reproduction, and no adult fish died during any of the experiments. In all experiments, though, it was sometimes necessary to exclude pairs of fish from the data analyses because the females became unable to release their eggs. This typically occurred during the first week, after reproduction had begun. It was accompanied by a swelling of the abdomen with, in more severe cases, some haemorrhaging around the oviduct. It is not known why some females stop releasing their eggs, but this phenomena has been observed previously in our laboratory (unpublished data) and in other laboratories working with the fathead minnow (Harries et al., 2000; Jensen, personal communication). As detailed in the results section, some pairs of fish spawned on the walls of the tanks. These fish were excluded from the analysis because it was impossible to accurately determine how many eggs they had produced. In the wild, fathead minnows can spawn on vertical surfaces, such as plant stems (McMillan, 1972). It is possible that the use of the screens on the sides of the tanks encouraged this type of spawning behaviour by creating shaded areas that the fish favoured for egg desposition. Careful observations of the spawning activity of the individual pairs of fish and daily examination of the walls and base of the aquaria for eggs are therefore required to ensure appropriate assessments of fecundity.

In all experiments, fish quickly acclimated to the test conditions and established regular spawning activity, producing batches of eggs on average every 3.3 to 4.8 days. These spawning frequencies are consistent with those reported by others working with the fathead minnow (Gale and Buynek, 1982; Harries et al., 2000; Jensen et al., 2001; Ankley et al., 2001). Mean fecundity rates, based on egg numbers on the spawning tile only, were 64 $\pm$ 5.0, 27 $\pm$ 3.6, 58 $\pm$ 5.4 and 64 $\pm$ 5.6 eggs/female/day, in experiments I, II, III and IV, respectively. With the exception of experiment II, these fecundity estimates are more than two-fold higher than the 2.5 to 26 eggs/female/day, reported by laboratories that have used the standard spawning tile design to collect eggs (Ankley et al., 2001; Jensen et al., 2001; Länge et al., 2001; Jensen et al., 2004; Bringolf et al., 2004; Pawlowski et al., 2004; Parrot & Blunt, 2005). It is not clear, though, why the fecundity estimates were higher in experiments I, III and IV, than in experiment II. Fish were held under identical conditions (e.g. photoperiod, temperature, flow rates, water quality, diet) in all four experiments. The experimental conditions used here were similar to those used by Jensen et al. (2001), who also studied fecundity in pairs of fathead minnows. In this study, fish differed in both age and body weight between the experiments, but there was no evidence of a consistent age- or size-related effect on fecundity. As an illustration of this, females used in experiment III were both smaller and younger in age (five months; 2.08 g) than those used in experiment I (16 months; 2.82 g), but their fecundity was comparable. Jensen et al. (2001) used fish of similar ages to those in experiments III and IV, but fecundity was three times lower in their study. The fish in this investigation came from a variety of suppliers, and so the differences in fecundity between this work and that reported in the literature by others are unlikely to be a result of using different populations of fathead minnow. It is possible that the difference in estimates is a result of differences in the ability of the individual

fish to deposit their eggs on the spawning tile. This is supported by the observation that fecundity estimates in this study depended on how the eggs were collected. When the spawning tile was used along with a screened egg collection tray to catch eggs that did not adhere to the spawning tile, mean fecundity rates were higher at 92 $\pm$ 6.0, 80 $\pm$ 4.1, 90 $\pm$ 4.7 and 93 $\pm$ 4.5 eggs/female/day in experiments I, II, III and IV, respectively. This resulted in a reduction in the inter-assay coefficient of variation, from 53 per cent (based on mean fecundity rates determined using egg numbers on the tile only) to seven per cent when eggs collected from the screened tray were included in the fecundity assessments. To date, only two other laboratories have used a screened egg collection tray along with the standard spawning tile to assess fecundity (Gale and Buynek, 1982; Harries et al., 2000). The fecundity estimates obtained in those investigations were between 73 and 109 eggs/female/day (Gale and Buynek, 1982) and between 43 and 112 eggs/female/day (Harries et al., 2000) and are highly consistent with those observed here.. These results imply that, in addition to almost doubling the fecundity estimates, using the screened egg collection tray reduces the apparent variation in fecundity estimates both within and between laboratories. It is, therefore, likely that fecundity estimates are highly comparable between different fish stocks. The reported variations in fecundity estimates are likely to be the result of sub-optimal ways of collecting eggs. In contrast to the screened egg collection tray, using a non-screened tray was not a good way to collect the eggs that did not stick to the tile. Though using the non-screened tray did result in higher estimates of fecundity, the variation in egg batch sizes and total egg numbers between individual pairs of fish was not reduced ($P > 0.05$). The screen probably stops the adult fish from eating these eggs/embryos (McMillan and Smith, 1974; Harries et al., 2000).

It is not known why some pairs of fish are better at attaching their eggs to the spawning tile than others. During spawning, a male fathead minnow lifts and presses the ventral surface of the female against the underside of the spawning object so that eggs and sperm can be released at the same time (McMillan, 1972). The ability of the pair to successfully deposit the eggs on the tile could conceivably be influenced by either the male or female body size, or, more importantly, by the ratio of male:female body size. This was not found to be the case here ($P > 0.05$). In experiments II and IV, though, an unexpected relationship between egg batch size and the ability of the fish to deposit their eggs on the spawning tile was observed. Pairs of fish that spawned a larger number of eggs per spawning were more successful at putting a higher proportion of their eggs on the spawning tile ($P < 0.02$, experiment II; $P < 0.01$, experiments II and IV). As a consequence, using the screened tray reduced variation in both egg batch sizes and total egg production both within and between individual pairs of fish in each experiment. The average within- and between- pair variation (CoV) in egg batch size fell from >50 per cent to <29 per cent ($P<0.05$), and the CoV for total fecundity from >52 per cent to < 33 per cent ($P<0.05$), based on the four studies. It is not known why the size of the spawn would influence the ability of the fish to deposit their eggs on the spawning tile. A more likely explanation is that, over time, the individual pairs of fish became more proficient at depositing their eggs on the spawning tile. The results from experiments II and III support this. The percentage of each batch of eggs successfully deposited on the spawning tile increased from 7 to 45 per cent in experiment II ($P < 0.01$) and from 40 to 92 per cent in experiment III ($P < 0.01$) over the 24-day monitoring period. In experiment IV, there was no evidence of such a relationship ($P > 0.05$). These fish were, though, highly

successful at sticking their eggs on the spawning tiles from the start of spawning. It is possible, therefore, that the apparent relationship between egg batch size and the percentage of the spawn deposited on the spawning tile was an indirect result of the increase in egg batch size with time in all experiments ($P < 0.01$). The final batch size spawned (over a 24-day period) was almost twice that of the first spawn in each experiment ($P < 0.01$). Much of the increase in egg batch size occurred during the first seven days of spawning ($P < 0.01$). After the first week of spawning activity, egg batch size did not change throughout the remaining period of the study ($P > 0.05$). These results are consistent with the observations of Gale and Buynek (1982), who found that the first spawning tended to be smaller. It is therefore recommended that reproduction tests with the fathead minnow exclude these initial spawning events and do not make any assessments of fecundity until at least seven days after spawning starts.

Fathead minnow reproduction tests sometimes also look at the effects of chemical treatment on the F1 generation under control (trans-generational effects) or exposure conditions (Ankley et al., 2001). Embryos selected from group spawning fathead minnows have a greater than 90 per cent survival to hatch. Under optimal conditions such as those adopted in these investigations, fish reach sexual maturity within 90 to 120 dph and the normal sex ratio in a population is approximately 50:50 males:females. In the pair-breeding reproduction test, it is possible to study these parameters in offspring from individual pairs of fish rather than groups, but the normal variation in F1 survival, growth and development has yet to be defined. These investigations showed high rates of survival to hatch, but this did appear to be pair-specific. In each investigation, a high proportion of the adult pairs (65, 95 and 84 per cent of the adult pairs in experiments II, III and IV, respectively) consistently spawned embryos that showed a greater than 90 per cent hatching success in three independent hatching trials. Embryos from the remaining subset of the adult pairs, though, consistently showed a reduced hatching success in all three hatching trials. So embryo viability and survival to hatch may depend on the adult pairing. This demonstrates the need to establish hatching success over multiple spawning events for all adult pairs before starting any exposure study involving F1 effects. This is easy to do in the pair-breeding reproduction assay. In the group-breeding assay, it would be more difficult to identify the parents of each batch of eggs (without perhaps the use of costly molecular approaches). In experiment I, there was no evidence that the successful attachment of the eggs to the spawning tile was related to their viability. Visual observation of the eggs in the collection tray indicated that these eggs had been fertilised and were developing at the same rate as those stuck to the tile. If left in the tray for more than 24 hours, though, all of these embryos died, but if they were removed from the tray within 24 hours of spawning, the embryos continued to develop successfully, with a mean survival to hatch of 63 ± 5.7 per cent. The hatching success of embryos collected from the spawning tiles was significantly higher at 85 ± 2.6 per cent ($P < 0.05$). The difference between the two hatching success values was probably due to faecal matter and waste food that accumulates in the egg collection trays. Indeed, when eggs from the spawning tray were cleaned thoroughly by rinsing and physically removing any contaminates from their surface, survival to hatch increased to greater than 85 per cent.

In experiment III, a subset of fry from the final hatching trial conducted for each adult pair were maintained under control conditions until 80 dph. The critical period for

survival of the fry occurred during the first week post hatch, but survival to 80 dph was generally very high (> 60 per cent) for offspring from all adult pairs. The mean sex ratio of 54per cent males (for offspring from all pairs) was consistent with expectation, ut variations in the sex ratios between offspring from the individual adult pairs (31 to 80 per cent males) were observed. This highlights the need for caution when assessing the effects of chemicals on the sex ratio of offspring from a small number of pairs of adult fish for each treatment group. Differences in the growth parameters for the offspring from the different adult pairs (Figure 3) were also observed, but differences in survival rates complicated the analyses. A fall in the survival correlated to an increase in male condition factor and female weight, length and condition factor ($P < 0.05$). This suggests a density dependence effect. The feeding rate was reduced in these tanks because there were fewer fish, but their growth rate probably increased because of the reduced competition for space. Comparisons of the mean condition factors for the two sets of offspring from each adult pair (Table 3) indicate that somatic growth is consistent between the two sets of offspring from each adult pair, but different between offspring from different adult pairs. This suggests that the adult pairing may influence somatic growth of the offspring. , This, though, is based on a small dataset; further investigation would be required to confirm this result. No conclusive findings can be derived from this part of the experiment, but when considered with the results of the hatching studies, though, they highlight the need to understand the normal variability of hatching success, growth and development in offspring from different adult pairs before determining chemical effects on the F1 generation.

These results demonstrate that the fathead minnow is a robust test organism highly suited for studies that investigate the effects of chemicals on reproductive function. Fecundity estimates were shown to depend on the method of egg collection, as a consequence of differences in the ability of the individual pairs to attach their eggs to the undersurface of the spawning tile. Placing a screened egg collection tray beneath the spawning tile significantly increased fecundity estimates to between 80 and 93 eggs/female/day and reduced the coefficient of variation both within and between experiments. Embryo hatching success in this species was easy to assess and generally high (> 90 per cent). Survival to hatch, though, is parent-specific. This highlights the need to establish the variability in this parameter for each adult pair before starting any chemical exposure.

Table I. Water quality measurements in experiments I to IV

	Expt I	Expt II	Expt III	Expt IV
Conductivity (μS/cm)	210 – 259	212 – 269	214 - 282	206 – 258
Hardness (mg/L)	38.3 – 47.6	39.0 – 50.0	41.7 – 50.0	39.3 – 49.7
Free chlorine (μg/L)	< 2.0	< 2.0	< 2.0	< 2.0
Alkalinity (mg/L)	21.6 – 29.2	22.6 – 30.6	20.0 – 29.2	22.0 – 24.6
Ammonia (mg/L)	< 0.01	< 0.01	< 0.01	< 0.01
PH	7.12 – 7.70	6.90 – 7.87	7.07 – 8.01	7.01 – 7.64
Dissolved oxygen (mg/L)	> 6.4	> 6.0	> 6.3	> 6.6
Temperature (˚C)	24.3 – 24.8	24.5 – 25.0	24.8 – 25.2	24.7 – 25.0

Table II. Mean (± SEM) fecundity estimates (total egg production over 14 days and egg batch size) in fathead minnows held under control conditions and provided with either a spawning tile placed above a non-screened (N-Sc) or screened (Sc) egg collection tray. Egg numbers counted on just the spawning tile, and on the spawning tile with its associated tray are shown.

Group	Type of tray	Total Egg Production		Egg Batch size	
		Tile only	Tile + Tray	Tile only	Tile + Tray
1	N-Sc	724 ± 125	841 ± 113	241 ± 28	286 ± 31
	N-Sc	714 ± 141	837 ± 162	229 ± 43	266 ± 46
2	Sc	1304 ± 141	1542 ± 110	301 ± 21	362 ± 24
	Sc	1183 ± 174	1470 ± 137	297 ± 40	376 ± 40
3	N-Sc	1079 ± 85	1168 ± 351	232 ± 46	264 ± 38
	Sc	757 ± 173	1133 ± 176	203 ± 27	321 ± 19
4	Sc	775 ± 159	1039 ± 168	273 ± 38	371 ± 24
	N-Sc	763 ± 188	851 ± 223	296 ± 34	327 ± 37

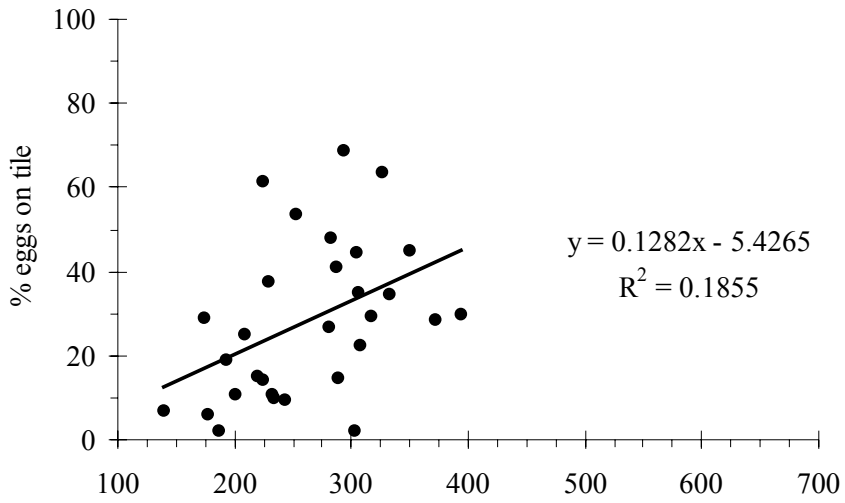
Table III. Mean survival, sex ratio (as % males) and growth parameters at 80 days post hatch in offspring collected from two different spawning events for each of four pairs of adult fathead minnow.

Fish pair no.	1		2		3		4	
Spawning event	A	B	A	B	A	B	A	B
% Survival	100	80	100	100	60	80	95	95
% males	35	56	50	40	75	56	47	47
Males:								
Weight (g)	1.61	1.46	1.81	1.90	1.70	1.27	1.77	1.99
Length (mm)	52	51	54	54	52	46	54	55
Condition Factor	1.02	1.07	1.11	1.14	1.15	1.26	1.08	1.12
Females:								
Weight (g)	1.07	1.13	1.06	1.23	1.40	1.12	1.06	1.02
Length (mm)	45	46	46	47	48	46	47	46
Condition Factor	1.07	1.04	1.06	1.15	1.24	1.14	1.00	0.99

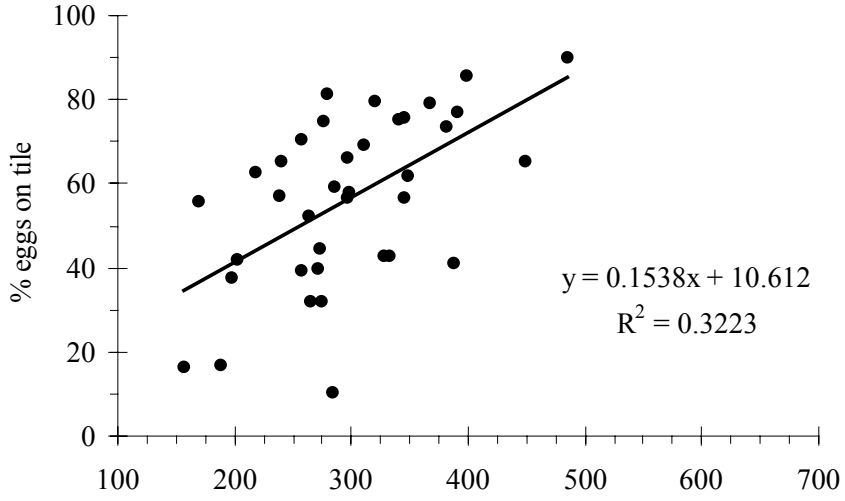
Figure 1. Picture of the modified egg collection system, showing the commonly used inverted U-shaped tile that has been placed above a screened egg collection tray.
Figure 2. Assessment of the relationship between the percentage of eggs in each

spawn attached to the spawning tile and the size of the egg batch spawned in experiments II (A), III (B) and IV (C).

A.



B.



C.

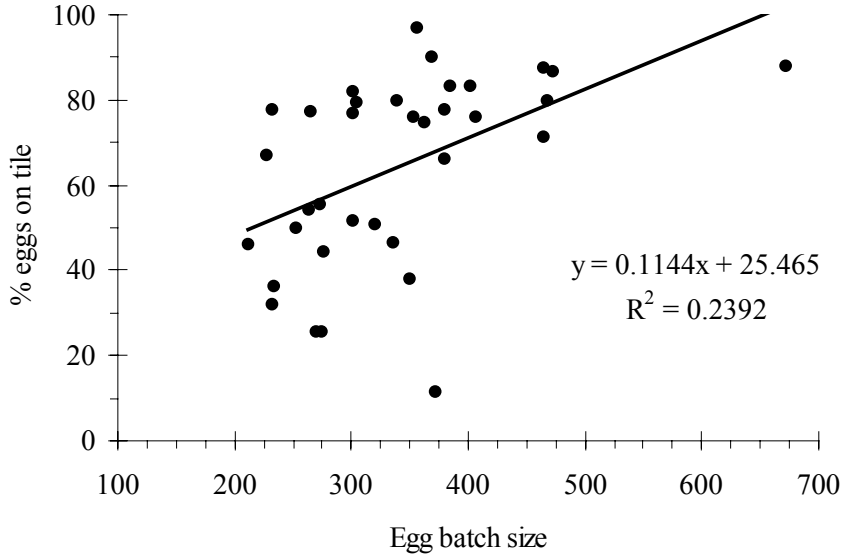


Figure 3. Assessment of the relationship between fecundity (egg batch size and total number) and female body weight in experiments II (A), III (B) and IV (C).

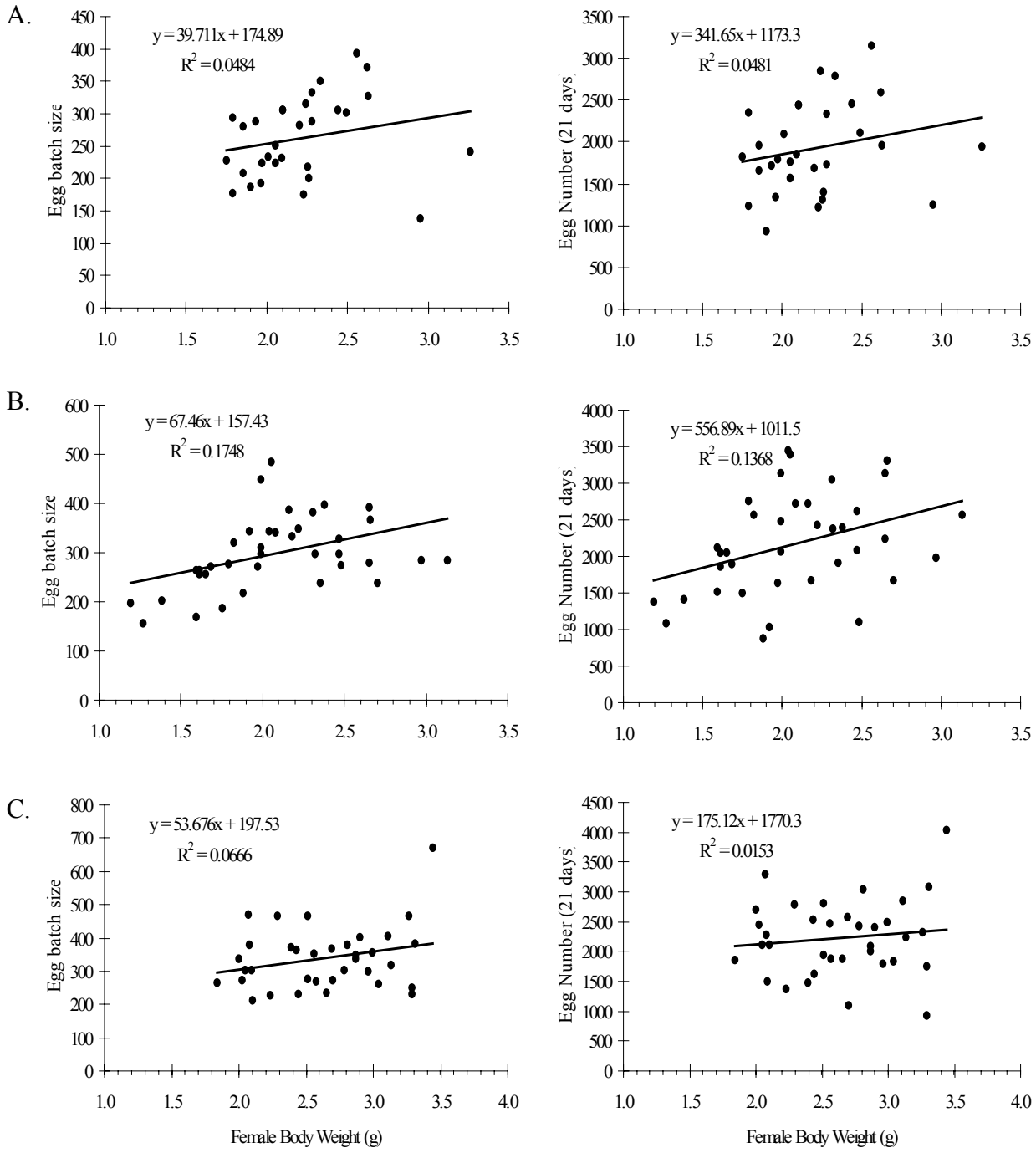
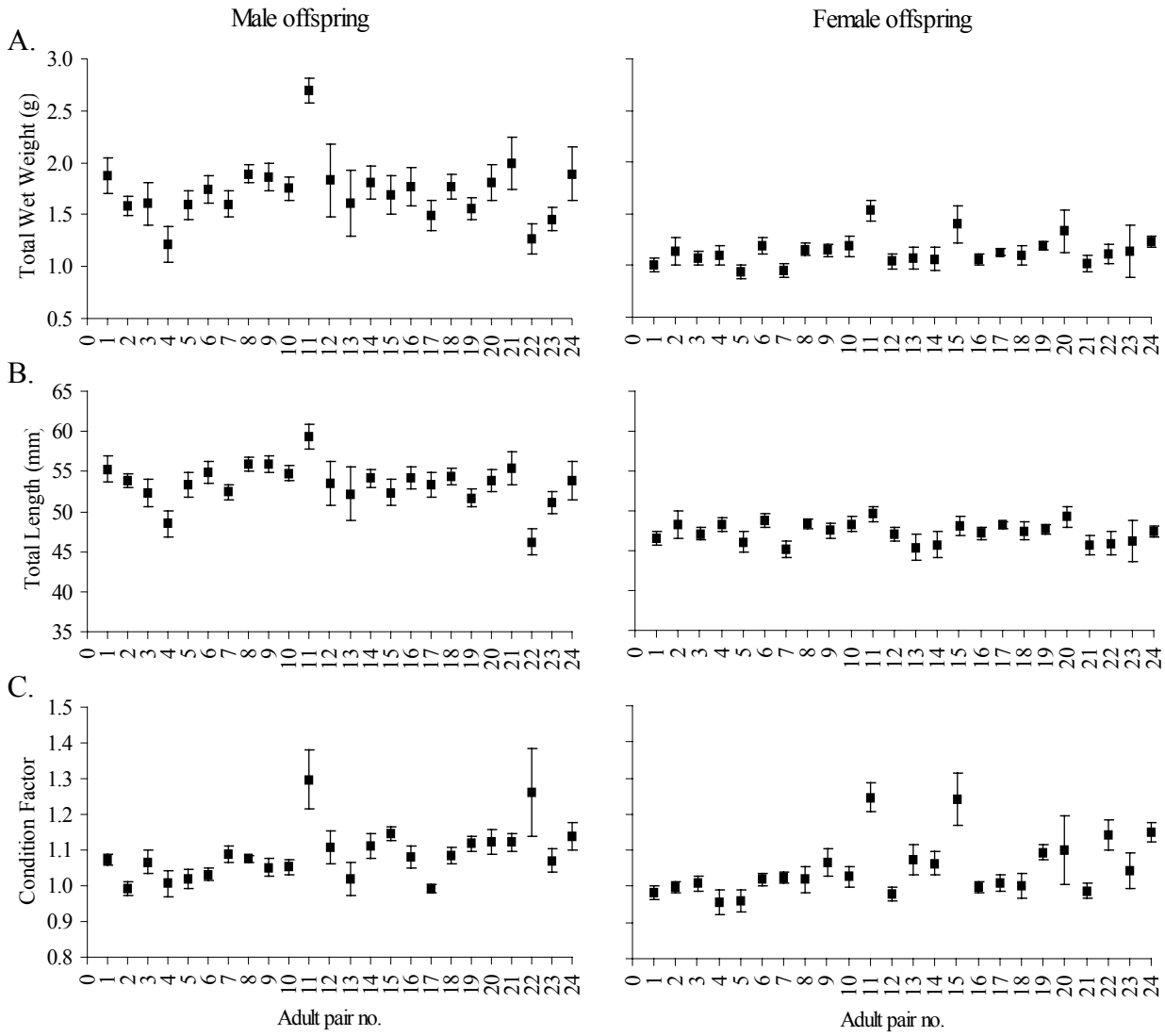


Figure 4. Variability in mean (± SEM) total wet body weight (A), length (B) and condition factor (C) in male and female fathead minnows offspring, at 80 days post hatch, from different adult pairs.



3. Reproductive effects and associated biomarker responses in pair-breeding fathead minnows exposed to an oestrogen, anti-androgen and aromatase inhibitor

Abstract

The fathead minnow adult pair-breeding assay is a highly integrative test that allows assessment of chemical effects on reproduction by comparing egg numbers in individual pairs of fish spawning over a 21-day pre-exposure period, followed by a 21-day chemical exposure period. In these investigations, the validity of the pair-breeding test was assessed for different classes of EACs. They included an oestrogen (oestrone at concentrations of 0, 34, 98, 307 and 781 ng/L), an anti-androgen (linuron at concentrations of 0, 18, 41, 90, 189, 207, 435, 842 and 2074 μg/L) and an aromatase inhibitor (fenarimol at concentrations of 0, 51, 94, 178 and 497 μg/L). Exposure to each of the test chemicals resulted in a concentration-related reduction in total fecundity ($P < 0.01$), over a 21-day period. Reductions were significant compared to the controls at concentrations of 307 and 781 ng oestrone/L, 842 and 2074 μg linuron/L and 497 μg fenarimol/L ($P < 0.05$). These effects were the result of a reduction in the number of spawning events during the exposure period ($P < 0.05$), with no evidence of an effect on egg batch size ($P > 0.05$). In males, the highest concentrations of oestrone that reduced egg production had wider adverse health effects. At the end of the 21-day exposure period, concentration-related elevations in plasma VTG concentrations were observed in fish exposed to oestrone ($P < 0.01$). By contrast, concentration-related reductions in plasma VTG concentrations were observed in males exposed to linuron ($P < 0.01$) and females exposed to fenarimol ($P < 0.01$). Concentration-related reductions in the prominence of the male secondary sexual characteristics were observed in males exposed to oestrone ($P < 0.01$) and linuron ($P < 0.01$). Including the biomarker responses, therefore, provided valuable information about how each test chemical affected the reproduction axis.

In each experiment, during both the acclimation period and exposure periods, the fertilized embryos collected from each pair of fish, were incubated in clean water to assess effects of the adult exposures on hatching success. Hatched fry from adults exposed to either oestrone or linuron were also kept in clean water up to 80 days post-hatch. This was to assess possible trans-generational effects on survival, growth and sexual differentiation. There was no evidence that exposing adult fish to oestrone, linuron or fenarimol had any impact on embryo survival to hatch or (for oestrone and linuron) on survival, growth or sex ratio of the F1 generation.

Introduction

Disruption in sexual development in wild fish exposed to STWs effluents in the UK is characterised by the feminisation of males (Purdom et al., 1994; Harries et al., 1996; Jobling et al., 1998; van Aerle et al., 2001; Rodgers-Gray et al., 2001). The hypothesis is that these feminising effects are the result of exposure to oestrogen receptor agonists. There is, indeed, good evidence to support this (Desbrow et al., 1998; Routledge et al., 1998; Rodgers-Gray et al., 2001). Feminising effects have, though, also been induced in laboratory studies in males exposed to chemicals that mediate their effects by blocking the androgen pathway (Bayley et al., 2002, 2003; Jensen et al., 2004; Panter et al., 2004). Oestrogenic chemicals are widespread in UK Rivers (Williams et al., 2003; Johnson, 2004), but there has so far been little attempt to establish the prevalence of other classes of feminising chemical in the environment. In a recent survey sponsored by the Environment Agency, Johnson et al. (2004) showed that wastewater treatment works that were oestrogenically active also possessed anti-androgenic activity (assessed using *in vitro* recombinant yeast assays containing either the human oestrogen or androgen receptor. The effects of wastewater treatment work effluents on other endocrine receptors have not been well investigated. *In vivo* assays to assess the endocrine disrupting effects of chemicals and environmental mixtures need, therefore, to capture these different mechanisms of action, not just the oestrogenic pathways. Many of the existing *in vivo* tests focus on the effects of chemicals on biomarkers of exposure, such as the oestrogen-dependent induction of VTG and the androgen-dependent induction of male SSCs. The biological relevance of these biomarker responses has yet to be established, though. Essentially, nothing is known about their relevance at the individual or population level. Standardised and robust i*n vivo* tests that assess the fundamental effects of EACs on growth, development and reproduction are therefore needed. In this respect, a short-term (six week) reproductive performance test, which assesses chemical effects on fecundity (total egg number, egg batch size and number of spawnings), offers great promise (Harries et al., 2000; Ankley et al., 2001). Successful breeding in fish involves a complex interplay between behavioural and physiological responses that are controlled by the hypothalamic-pituitary-gonadal-axis. Any substance that interferes with these control processes could affect reproduction. The reproductive performance test is, therefore, highly integrative. It means that the biological effects of individual chemicals or chemical mixtures on the reproductive axis can be assessed, irrespective of their mechanism of action. The primary endpoint in the reproduction test is an assessment of egg production, but test is also designed to allow other endpoints to be incorporated. Relevant biomarkers in the exposed adult fish can be measured to find out how a particular chemical affects reproduction. By maintaining the embryos until they hatch, the effect of a chemical on gamete quality can also be determined. Possible long-term effects in the F1 generation can also be measured. This involves assessing survival, growth and sexual differentiation in offspring that have been reared until they have undergone sexual differentiation (this takes between 60 and 90 dph under optimal conditions in the fathead minnow; van Aerle et al., 2004).

The fathead minnow pair-breeding assay was originally described by Harries et al. (2000). In the following investigations, this was further developed and validated for different classes of EACs. The three selected EACs included the natural oestrogen, oestrone, an anti-androgen, linuron, and an aromatase inhibitor, fenarimol. The

rationale for the selection of these test chemicals are provided in the introduction. Biomarker endpoints were appraised by comparing the effects of each chemical on reproduction with the oestrogen-mediated induction of VTG and the androgen-mediated formation of the male SSCs. Relationships between the biomarker responses and reproductive performance were examined to see whether changes in biomarkers indicate adverse-health affects in fish. To determine whether chemical effects on adults would impact on the F1 generation, embryos were removed from the control and chemically exposed adult pairs and maintained until hatch. In experiments where fish were exposed to a model oestrogen and an anti-androgen, a subset of the hatched embryos was also maintained for a further 80 days to assess for possible trans-generational effect of EACs on survival, growth and sexual differentiation of the offspring.

Materials and methods

General experimental details

Test organisms

Details about the original supply of the fathead minnows for each investigation are provided in the relevant sections. All fish were held for a minimum of three months in the husbandry unit at AstraZeneca's Brixham Environmental Laboratory, Devon, UK, before any tests began. This was to ensure that they were free from disease and at the right stage of reproductive development to start the pair-breeding studies.
At least two weeks before the start of each study, sexually mature males and females (identified by the development of secondary sexual characteristics) were separated to prevent any spawning activity, and acclimated to the test conditions. During the acclimations and each experiment, all fish were kept in de-chlorinated water at 25.0 ± 1°C, with a 16-hour light:8-hour dark photoperiod, with 20 minutes dawn and dusk transition periods. Fish were fed frozen brine shrimp (Tropical Marine Centre, Hertfordshire, UK), twice a day, and Ecostart 17 pelleted fish food (BIOMAR, Houghton Springs Fish Farm, Dorset, UK), once a day.

Water quality

The supply of de-chlorinated water to the laboratory dosing system was monitored every day for conductivity, every week for alkalinity, hardness and free chlorine, and every month for total ammonia. Throughout all studies, the test water's conductivity ranged from 206 to 282 $\mu S\ cm^{-1}$, alkalinity ranged from 20.0 to 35.8 mg L^{-1}, the hardness from 38.3 to 53.3 mg L^{-1} (as $CaCO_3$). Free chlorine remained below 2.0 $\mu g\ L^{-1}$, and ammonia (as $N\text{-}NH_3$) was below 10 $\mu g\ L^{-1}$. Dissolved oxygen concentrations and pH levels were determined in the individual tanks on days 0 and 1, and then twice weekly throughout the exposure period. In all experiments, the dissolved oxygen concentration remained >70 per cent of the air saturation value, and pH values ranged from 6.9 to 8.0. Water temperatures were monitored constantly throughout the exposure period; they ranged between 24.3 and 25.2°C.
In all experiments, fish were held under flow-through conditions. The chemical was dosed, via a peristaltic pump, to glass mixing vessels receiving the dilution water

through a gravity-fed system. From the mixing vessels, the diluted test solutions flowed into flow-splitting chambers and then into the individual test vessels at a nominal rate of 80 mL/min. This flow rate provided a 95 per cent replacement time of about six hours. Chemical dosing rates were checked at least twice per week and the dilution water flow rates at least once per week. All flows were maintained within 20 per cent of nominal.

Test apparatus

The test vessels had a working volume of 12 L and were made of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions. Screens around the sides of all test vessels prevented fish in neighbouring tanks from interacting with one another and minimised disturbance by researchers' movements. The spawning substrates consisted of a tile (80 mm length of 110 mm diameter PVC half guttering; EPA, 1987) placed above a screened collection (Sc) tray. The Sc tray was a rectangular glass tray, measuring 130 x 110 x 30 mm (length x width x depth) covered with 0.5 cm^2 stainless steel mesh (the mesh allowed eggs to pass through to the tray). Spawning substrates were checked for eggs once a day at 10.30 am, and the tiles and trays replaced with clean ones, regardless of whether there were any eggs there or not.

Experimental design

To start each test, males and females were selected at random, individually wet weighed and placed as pairs into replicate test vessels containing a spawning tile placed above a mesh-screened tray. A representative number of fish from the same batch were sampled for further analysis, as described below. Further pairs of fish from the same batch introduced to the test system were held temporarily under the same conditions as used in the test, until it was confirmed that the breeding pairs were compatible. In a few cases, some breeding pairs were incompatible and were replaced by randomly selected animals. Full records of all such exchanges were kept. The fish were acclimated to the test conditions for a minimum of 10 days, before the start of the pre-exposure period. During this time, their reproductive performance was assessed to confirm that each pair was spawning successfully. After this initial acclimation period, the pre-exposure phase began. Reproductive behaviour and spawning activity for each pair were assessed daily, for at least three weeks. The data from this pre-exposure period established the reproductive capacity of the individual pairs of fish. It provided pair-specific baseline data for statistical comparison with the subsequent exposure phase. On the final day of the pre-exposure phase, after the daily assessments of reproductive performance (i.e. after 11 am), dosing of the test chemical to the individual tanks was initiated. Survival, reproductive behaviour and spawning activity for each pair of fish were assessed every day, for an exposure period of three weeks. This determined the effects of the test chemical on the health and reproductive capacity of the individual pairs of fish.

Fish sampling

Subgroups of fish were sampled at the start of experiments I (n = 12), II (n = 16), III (n = 16) and IV (n = 16). Any spare pairs of fish from the acclimation periods that were not used to replace incompatible pairs of fish (ie, that were surplus to

requirements for the test), were sampled at the start of the exposure period (day 0) to confirm the physiological status of the fish. All experimental adult fish were sampled at the end of the exposure period. Fish were sacrificed by a schedule I method using a lethal dose (500 mg/L) of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) (Sigma), buffered with 1M NaOH to pH 7.3. Total wet body weight of the fish was recorded to the nearest 0.01 g and standard and total lengths measured to 0.01 mm. Condition factor was derived by expressing the cube of the total fish length as a percentage of the body weight. Blood was collected by cardiac puncture, using a heparinised syringe (1000 Units heparin/mL), centrifuged (7,000 g; 5 min, 4°C), the plasma removed and stored at -80°C until required for VTG analysis. Plasma samples were assayed for VTG using a carp ELISA [Tyler *et al*., 1999]. To quantify secondary sex characters, the tubercles on the snout of each fish were counted. The dorsal fatpad was removed and wet weighed to the nearest 0.00001g. The prominence of the tubercles was also recorded using Smith's (1978) grading system: 0, no visible sign of tubercles; 1, tubercles visible as white disks, not protruding above body surface; 2, project above body surface; 3, prominent but not sharp; 4, prominent and sharp; 5, tubercles prominent and sharp but starting to run together so that not all individual tubercles can be distinguished. Figure 1 gives a visual representation of the grading system used and shows the appearance of both the tubercles and dorsal fatpad at each stage of development. Gonads were removed and wet weighed to the nearest 0.00001g to determine the gonadosomatic index (GSI; wet weight of the gonad relative to the body weight of the fish).

Statistical analyses

All results are expressed as mean ± standard error of the mean (SEM). Data that met the assumptions of normality and homogeneity of variance were analysed using one way analysis of variance (ANOVA) followed by a pair-wise multiple comparison procedure (Tukey Test). Data that failed to meet the assumptions of both normality and homogeneity of variance were analysed using a Kruskal-Wallis one way analysis of variance on ranks, followed by a pairwise multiple comparison procedure (Dunnett's Method for equal sample sizes and Dunn's Method for unequal sample sizes). Analyses of effects on the fatpad weight were conducted using analysis of covariance (ANCOVA) using body weight as a covariate. In all experiments, strengths of association between pairs of variables were measured using the Pearson product movement correlation coefficient. A Kolmogorov-Smirnov test was used to determine effects of exposure to the test chemicals on fecundity (mean cumulative egg production) relative to the 21-day dilution water pre-exposure data for each treatment group.

Experiment I

The fathead minnows used in this study were supplied as juveniles by Osage Catfisheries Inc. (Missouri, USA) and held for about three months at Brixham Environmental Laboratory, before the start of the experiment. The body weights (as means ± SEM) of the male and female fish at the start of the experiments pre-exposure phase were 4.19 ± 0.10 g (n = 44) and 2.20 ± 0.05 g (n = 44), respectively. The test chemical, E1 (99 per cent purity; Lot 30K1168), was purchased from Sigma, Poole, Dorset, UK. A stock concentrate of E1 was prepared in HPLC grade methanol

(Fisher Scientific) and stored in the fridge. This was used this to prepare daily stock solutions of the test chemical, by spiking 1 L dechlorinated water with the desired concentration of the stock concentrate. The stock solutions of E1 were dosed to glass mixing vessels by means of a peristaltic pump, at a rate of 0.375 mL min^{-1}, to mix with the dilution water flowing to the mixing vessels, via a flow-splitting chamber, at a rate of 750 mL min^{-1}. The SC vessel received the same rate of addition of methanol, such that the water in all test vessels contained 0.9 μL methanol per litre. Nominal concentrations of E1 used in the exposure phase of the test were 0, 32, 100, 320 and 1000 ng/L.

The actual concentrations of E1 were monitored throughout the exposure period. To measure E1 on days 1, 4, 7, 14 and 21 of the exposure, 500 mL (control and 32 ng/L), 250 mL (100 ng/L) and 100 mL (320 and 1000 ng/L) water samples were collected from one tank per treatment in to solvent-cleaned flasks. The water samples were spiked with d_2-estradiol (Aldrich; 98 per cent D atom); 0.05 ng/L (control and 32 ng/L), 0.2 ng/L (100 ng/L), 2.5 ng/L (320 ng/L) and 5 ng/L (1000 ng/L) and extracted under vacuum (50 mL min^{-1}) onto preconditioned 47 mm C_{18} ENVI-18™ solid phase extraction disks (Supelco). To assess the efficiency of the procedure, a 250 mL HPLC grade water (Rathburn) sample, spiked with 25 ng/L E2 and 0.2 ng/L d_2-estradiol was also extracted. The disks were dried, under full vacuum, for 15 minutes, then eluted with 15 mL methanol (HPLC grade; Rathburn) under gravity. The methanol was removed under a stream of nitrogen and the sample extracts derivatised: 200 μL of pyridine (Aldrich; 99.8 per cent pure) and 300 μL of bis(trimethysilyl)trifluoroacetamide with 10 per cent trimethylchlorosilane (BSTFA + 10 per cent TMCS; Aldrich) were added to each extract and the sample heated to 60˚C for 30 minutes. The reagents were removed under nitrogen and the sample residues resuspended in 250 μL (control, spike, 32 and 100 ng/L), 500 μL (320 ng/L) and 1000 μL (1000 ng/L) dichloromethane (DCM; HPLC grade; Rathburn). The final extracts were then transferred to a low volume auto-sampler vial for analysis by tandem GCMS. The analysis conditions were as follows; sample volume, 1.0 μL; GC column, HP5 MS 30 m × 0.25 mm (id) fused silica with 0.25 μm film thickness; injector temperature, 280˚C; column program, (1) 75˚C for 5 min, (2) increase to 300˚C at 10˚C min^{-1}. The MS was operated in the electron impact ionisation mode (70eV) with selected reaction monitoring (SRM). Parent ions for the E1 derivative (*m/z* 324), and for deuterated E2 (*m/z* 418) were stored and fragmented to give daughter ions (*m/z* 257 for E1 derivative and m/z 287 for deuterated E2). The limit of detection was 5 ng/L.

Experiments II and III

The fathead minnows used in experiments II and III were bred at Brixham Environmental Laboratory, and were five and eight months old, respectively, at the onset of each experiment. The body weights (as means ± SEM) of the male and female fish at the start of experiment II were 4.5 ± 0.11 g and 2.1 ± 0.08 g, respectively, and they were 5.5 ± 0.13 g and 2.6 ± 0.08 g, respectively, at the start of experiment III.

The test chemical, linuron (Technical grade 99 per cent purity; Lot C02271060), was a gift from Aventis CropScience. Solvent-free stock solutions of linuron were

generated using liquid-solid saturator columns (Kahl et al., 1999). A U-shaped glass column (1.2 m long, 25 mm i.d.) was packed with glass wool and then coated with a saturated solution of linuron (prepared by dissolving 35 g of linuron in 100 mL of acetone). The solvent was evaporated from the column under a gentle vacuum. Filtered water (0.45 μm) was continuously pumped through the column at a rate of ~ 20 mL/min to produce the stock concentrate. This method proved to be effective in both experiments, producing consistent stock concentrations that were near to the expected water solubility values (63.8 mg/L at 20˚C; Tomlin, 1997).

Measured concentrations of the linuron stock solutions were approximately 67 and 59 mg/L in experiments II and III, respectively. In experiment II, the stock solution of linuron was dosed at a rate of 0.26, 0.55, 1.19 and 2.63 mL/min, to glass mixing vessels receiving the dilution water at a rate of 800 mL/min, to give nominal exposure concentrations of 0, 22, 46, 100 and 220 μg/L. In experiment III, the stock solution of linuron was dosed at a rate of 2.5, 5.2, 11.4 and 25.0 mL/min, to glass mixing vessels receiving the dilution water at a rate of 670 mL/min, to give nominal exposure concentrations of 0, 220, 460, 1000 and 2200 μg/L.

Concentrations of linuron were measured throughout the exposure periods of both experiments. Water samples were collected from the outlet of the saturation column at least three times per week and from the centre of each tank on days 1, 2, 7, 14 and 21 of the exposure period. In the samples collected from the saturation column, concentrations of linuron were expected to be at or near solubility. To prevent precipitation before starting the analysis, these samples were diluted 50:50 with methanol. The actual concentrations of linuron on both the saturation column and in the individual test vessels were verified using liquid chromatography. A Jasco PU980 LC pump was used to isocratically pump 60:40 acetonitrile:water stationary phase at 1 mL/min. Separation was achieved on a HPLC Zorbax SB-Phenyl 250 mm □ 4.6 mm (id) column. The compound was detected using a Jasco UV-975 ultraviolet spectrometer at 214 nm. The limit of quantification was 2.5 μg/L for a 200 μL injection.

Experiment IV

The fathead minnows used in experiment IV were bred at Brixham Environmental Laboratory, and were about seven months old at the start of the experiment. The body weights (as means ± SEM) of the male and female fish at the start of the experiment were 3.14 ± 0.159 g and 1.31 ± 0.073 g, respectively.

The test chemical, fenarimol (99.8 per cent purity), was purchased from Sigma, Poole, Dorset, UK. Solvent-free stock solutions of fenarimol were generated using liquid-solid saturator columns (Kahl et al., 1999). A U-shaped glass column (1.2 m long, 25 mm i.d.) was packed with glass wool and then coated with 10 g of fenarimol dissolved in 75 ml acetone. The solvent was evaporated from the column under a gentle vacuum. Filtered water (0.45 μm) was continuously pumped through the column at a rate of ~ 20 mL/min to prepare the stock concentrate. Concentrations of fenarimol eluting from the saturation column were 50 mg/L during the first day of use, but gradually dropped to 12 mg/L by the fourth day. Saturation columns were therefore prepared every three days to help keep the stock solutions at the nominal concentrations. Mean measured concentrations of the fenarimol stock solutions were

37 ± 3.4 mg/L over the 21 days of study. The stock solution of fenarimol was dosed at a rate of 0, 2.2, 4.8, 10 and 20 mL/min, to glass mixing vessels receiving the dilution water at a rate of 800 mL/min, to give nominal exposure concentrations of 0, 100, 220, 460 and 1000 μg/L. The actual concentrations of fenarimol were monitored throughout the exposure period. Water samples were collected from the outlet of the saturation column at least three times per week and from the centre of each tank on days 1, 2, 7, 14 and 21 of the exposure period. Additional samples were collected from the centre of a single tank for each treatment at regular intervals, to ensure that the sampling regime included tank measurements from each saturation column prepared. All samples were diluted 50:50 with acetonitrile before analysis. The samples from the saturation column were diluted further with 50:50 acetonitrile:deionised water to give an overall dilution of six-fold. The actual concentrations of fenarimol on both the saturation column and in the individual test vessels were verified using liquid chromatography. A Jasco PU1580 LC pump was used to isocratically pump 65:35 acetonitrile:water stationary phase at 1 mL/min. Separation was achieved on a Hypersil H5BDS-C18 250 mm □ 4.6 mm (id) column. The compound was detected using a Jasco UV-975 ultraviolet spectrometer at 220 nm. The limit of quantification was 2.0 μg/L for a 50 μL injection.

Results

Experiment I

The tank water concentrations of E1 were stable and relatively close to the target values of 32, 100, 320 and 1000 ng/L. The respective mean (SEM, n = 4) measured concentrations were 34 ± 5.7, 98 ± 16, 307 ± 26, 781 ± 90 ng/L over the course of the 21-day exposure period. A small quantity of E1, 7.7 ± 2.2 ng/L was also measured in the control tanks. In the spiked sample, E1 was measured at 94 per cent of nominal.

Before the start of exposure, three pairs of fish were randomly selected from the 14 spare pairs. These replaced pairs where either the females did not release their eggs or the fish were spawning on the sides of the tank. In the highest test concentration, one pair of fish was removed from the test system and sacrificed via a schedule 1 method because the female stopped releasing her eggs and some haemorrhaging was visible around her oviduct. No further mortalities were observed among either the control fish or those exposed to E1 at concentrations of 34 and 98 ng/L. In the higher treatments, the females remained healthy and active, but two males exposed to 307 ng/L of E1 and three males exposed to 781 ng/L became pale compared to the controls. Their abdomens swelled once spawning had stopped, and they subsequently died. The remaining males in the 307 ng/L treatment, were healthy throughout the exposure. Surviving males in the highest oestrone treatment appeared pale, with abdominal swelling and were therefore sacrificed via a schedule I methodon day 16 of exposure.

During the 21-day pre-exposure period, mean (± SEM) total egg production, number of spawnings and number of eggs per spawning were 1876 ± 210, 6.7 ± 0.21 and 281 ± 28 per female, respectively. There were no significant differences in fecundity during the three-week pre-exposure period between any of the groups, or between the pre-exposure and exposure period in the control group. Exposure to E1 resulted

in a concentration dependent decrease in fecundity ($P < 0.01$). There were significant reductions in the total number of eggs spawned per female, compared with that of the same breeding pairs during the pre-exposure period, of 51 per cent and 66 per cent in fish exposed to 307 ($P < 0.05$) and 781 ng E1/L ($P < 0.01$), respectively. The decrease in total egg production was mostly a result of a decrease in the number of spawning events ($P < 0.05$) rather than a decrease in the number of eggs per spawning ($P > 0.05$).

The mean ($\pm$ SEM) weights, lengths and condition factors of the fathead minnow, sampled at the start of the pre-exposure period, were 4.815 ± 0.209 g, 69.1 ± 1.67 mm and 1.460 ± 0.048 (n = 6) for the males, and 2.143 ± 0.053 g, 57.2 ± 0.52 mm and 1.149 ± 0.043 (n = 6) for the females. During the 42-day reproduction test, a small increase in body weight, length and condition of the control fish was observed. At the end of the exposure phase, the mean weights, lengths and condition factors had increased to 5.752 ± 0.177 g, 71.4 ± 1.20 mm and 1.592 ± 0.096 (n = 6), respectively, in the males, and 2.860 ± 0.245 g, 60.0 ± 0.83 mm and 1.316 ± 0.069 (n = 6), respectively, in the females. Exposure to E1 resulted in a concentration-dependent increase in the condition factor of the males ($P = 0.001$), which was significant relative to the control males at the highest test concentration (781 ng/L; $P < 0.05$). Male wet body weight was not affected by exposure to E1, but body length decreased in males exposed to E1 at concentrations of 307 and 781 ng/L fish ($P < 0.05$). In the females, exposure to E1 did not affect total wet body weight or condition factor, but a concentration-related decrease in total length of the E1 exposed females ($P < 0.001$) was observed, with a significant decrease relative to the control females at the highest test concentration (781 ng/L; $P < 0.05$).

All males had developed secondary sexual characteristics (dorsal fatpad and tubercles) at the onset of the pre-exposure period (day -21). The mean fatpad weight was 278.8 ± 18.89 mg, and the mean number of tubercles was 23 ± 2 (n = 6). During the 42-day reproduction test, there were no significant changes in the SSCs of the control fish. A concentration-dependent decrease in the fatpad weight was observed in the E1 exposed males ($P < 0.001$) at the end of the exposure period (day 21), but this was only significant relative to the control males at the highest concentration of oestrone tested (781 ng/L, $P < 0.05$). There was no evidence of a concentration-related effect on tubercle number or prominence ($P > 0.05$).

The concentration of VTG in the plasma of the male and female fathead minnows at the onset of the experiment was 0.254 ± 0.085 μg/mL and 1508 ± 194 μg/mL. No changes in plasma VTG concentrations were observed in the control fish during the pre-exposure or exposure periods. Exposure to E1 resulted in a concentration-dependent increase in plasma VTG concentration in the males ($P < 0.0001$), with a significant increase, relative to the controls, in males exposed to the three highest concentrations of E1 tested (98, 307 and 781 ng/L; $P < 0.05$). In females, there was not a clear concentration-dependent effect of E1 on plasma VTG concentrations, though significant increases, relative to the controls, were observed in females exposed to the two highest concentrations of E1 tested (307 and 781 ng/L; $P < 0.05$). Mean gonadosomatic indices in male and female fathead minnows at the onset of the experiment were 1.433 ± 0.197 and 11.28 ± 1.826, respectively. No changes in GSI were observed in the control fish during the pre-exposure, or exposure periods.

Exposure to E1 resulted in a concentration-dependent decrease in GSI in the females ($P < 0.05$), but this decrease was not significant relative to the controls at any concentration tested. There was no evidence of an effect of E1 exposure on GSI in males ($P > 0.05$).

The mean hatching success of embryos selected from the control adults was 91 ± 2.8 per cent and did not vary between the pre-exposure and exposure periods ($P > 0.05$). There was no evidence of an effect of exposure of the adults to E1 on the hatching success of the embryos ($P > 0.05$). Hatched fry selected from the control adults showed a mean survival to 80 dph of 87 ± 4.4 per cent. Mean sex ratio (percentage of males), total wet weight and condition factor of the surviving offspring were 57 ± 4.3 per cent, 1.44 ± 0.038 1.08 ± 0.009, respectively. There was no evidence that exposing the adult fish to oestrone affected the survival of the offspring to 80 dph or the sex ratio ($P > 0.05$). Total wet weight and condition factor were lower in the offspring collected from adults exposed to 34 and 781 ng/L of oestrone ($P < 0.05$), but this was not concentration dependent ($P > 0.05$).

Experiments II and III

The tank water concentrations of linuron were stable and relatively close to the target values of 22, 46, 100 and 220 μg/L, in experiment II, and 220, 460, 1000 and 2200 μg/L, in experiment III. The respective mean (n = 40) measured concentrations were 18 ± 0.27, 41 ± 0.26, 90 ± 0.44, 189 ± 1.4 μg/L over the course of the 21 day exposure period in experiment II, and 207 ± 3.8, 435 ± 8.6, 842 ± 14, 2074 ± 34 μg/L in experiment III. Linuron was not detected in the control tanks in either experiment. In each experiment, before exposure, five pairs of fish were randomly selected from the eight spare pairs and used to replace pairs where either the female had failed to release her eggs, or where the fish were spawning on the sides of the tank. In the 460 μg/L test concentration, one female also became egg bound after the onset of exposure. This pair of fish was removed from the test system, but not replaced. No other mortalities were observed in either the control fish or those exposed to linuron during either experiment.

During the pre-exposure period, mean total egg production, number of spawnings and egg number per spawning, in the control fish were 2087 ± 256, 6.38 ± 0.263 and 326 ± 35.1 per female, respectively, in experiment II, and 2156 ± 313, 5.67 ± 0.211 and 389 ± 69.4 per female, respectively, in experiment III. In both experiments, there was no evidence of a change in the spawning activity of the controls between the pre-exposure and exposure periods. Exposure to linuron did not appear to affect egg production in experiment II ($P > 0.05$), but in experiment III, where fish were exposed to higher concentrations of linuron, a concentration-dependent decrease in fecundity ($P < 0.01$) was observed. Total egg production fell by 25 per cent, 60 per cent and 98 per cent in fish exposed to linuron at concentrations of 435, 842 and 2074 μg/L, respectively. This decrease in total egg production relative to the control was significant at the two highest test concentrations (842, $P < 0.05$; 2074 μg/L, $P < 0.01$). A concentration-related decrease in the number of spawning ($P < 0.05$) was observed, but there was no evidence of an effect of linuron exposure on egg batch size ($P > 0.05$).

In experiment II, the mean weights, lengths and condition factors of the fathead minnows sampled at the onset of the pre-exposure phase (day -21) were 3.55 ±

0.185 g, 67.5 ± 0.816 mm and 1.15 ± 0.039 (n = 8) for the males, and 1.61 ± 0.049 g, 51.7 ± 0.333 mm and 1.22 ± 0.044 (n = 8) for the females. During the 42-day reproduction test, increases in body weight, length and condition of all the fish were observed. At the end of the exposure phase (day 21), the mean weights, lengths and condition factors of the control fathead minnows were 5.68 ± 0.242 g, 70.7 ± 0.862 mm and 1.61 ± 0.062 (n = 8) for the males, and 2.22 ± 0.192 g, 56.7 ± 1.394 mm and 1.20 ± 0.041 (n = 8) for the females. The mean weights, lengths and condition factors of the fathead minnows, sampled at the onset of the pre-exposure period, in experiment III, were 5.56 ± 0.394 g, 73.7 ± 1.427 mm and 1.37 ± 0.050 (n = 8) for the males, and 2.29 ± 0.196 g, 56.2 ± 0.990 mm and 1.27 ± 0.055 (n = 8) for the females. As in experiment II, increases in body weight, length and condition of all the males were observed during the course of the experiment. The body length of the females also increased during the course of the experiment, but there was no apparent increase in the body weight of the females and so their condition factor decreased. At the end of the exposure phase (day 21), the mean weights, lengths and condition factors of the control fathead minnows were 6.81 ± 0.351 g, 75.1 ± 1.920 mm and 1.61 ± 0.063 (n = 8) for the males, and 2.34 ± 0.265 g, 59.4 ± 1.620 mm and 1.10 ± 0.049 (n = 8) for the females. Exposure to linuron did not affect total wet body weight, length or condition factor in the male or female fish in the first experiment ($P > 0.05$). In experiment III, the condition factor significantly decreased in males exposed to linuron at a concentration of 842 μg/L ($P < 0.05$), but this did not appear to be concentration-related ($P > 0.05$). In the females, a concentration-related reduction in the condition factor was observed ($P < 0.05$), but there was no evidence of a significant effect when compared to the controls.

Secondary sex characters (dorsal fat pad and tubercles) were evident in all males at the onset of the pre-exposure period (day -21) in both experiments. In experiment II, the mean fatpad weight was 335 ± 42.4 mg and the mean number and prominence of tubercles was 13.0 ± 0.945 and 2.25 ± 0.250, respectively (n = 8). In experiment III, the mean fatpad weight was 354 ± 89.4, and the mean number and prominence of tubercles was 15.4 ± 1.085 and 3.25 ± 0.313, respectively (n = 8). In both experiments, the SSCs increased in prominence during the course of the 42-day reproduction test. At the end of experiment II, the mean fatpad weight in the controls was 387 ± 27.9 mg and the mean number and prominence of tubercles was 19.9 ± 0.915 and 4.00 ± 0.000, respectively (n = 8). At the end of experiment III, the mean fatpad weight in the controls was 580 ± 94.6 mg, and the mean number and prominence of tubercles was 15.0 ± 0.447 and 3.67 ± 0.333, respectively (n = 8). In experiment II, concentration-dependent decreases in both tubercle number and fatpadwere observed in the linuron exposed males ($P < 0.05$), but the effect on tubercle number was not significant when compared to the dilution water control ($P > 0.05$). The effect on the fatpad was significant only at the highest test concentration (189 μg/L; $P < 0.05$). There was no evidence of an effect of linuron treatment on the prominence of the tubercles in experiment II ($P > 0.05$). In experiment III, concentration-related decreases in tubercle number and prominence and fatpad weight were observed in males exposed to linuron ($P < 0.01$). The observed decrease in tubercle number was not significant relative to the controls ($P > 0.05$), but a significant decrease in the prominence of the tubercles was observed at the highest test concentration (2074 μg/L; $P < 0.05$). All concentrations of linuron (207 to 2074 μg/L) resulted in a significant decrease in the fatpad weight ($P < 0.05$).

Plasma VTG concentrations in the male and female fathead minnows sampled at the start of the pre-exposure period were 0.243 ± 0.067 μg/mL and 1713 ± 145 μg/mL, respectively in experiment II, and 0.050 ± 0.015 μg/mL and 102 ± 7.37 μg/mL, respectively in experiment III. No changes in plasma VTG concentrations were observed in the control fish during the pre-exposure or exposure periods ($P > 0.05$). In experiment II, exposure to linuron increased plasma VTG concentrations in both the males and the females at the lowest exposure concentration (22 μg/L; $P < 0.05$), but there was no evidence of a concentration-related effect ($P > 0.05$). In experiment III, there was a concentration-related reduction in plasma VTG concentrations in the males ($P < 0.01$), with a significant decrease relative to the controls at the highest test concentration (2074 μg/L; $P < 0.05$). There was no evidence of an effect of linuron exposure on plasma VTG concentrations in the females in experiment III ($P > 0.05$).

Gonadosomatic indices in male and female fathead minnows at the onset of the experiment were 1.430 ± 0.148 and 14.14 ± 2.086, respectively in experiment II, and 1.510 ± 0.148 and 16.66 ± 1.976, respectively in experiment III. No changes in GSI were observed in the control or linuron exposed fish in either experiment ($P > 0.05$).

The mean hatching success of embryos selected from the control adults, in experiments II and III, was 91 ± 2.8 per cent and 94 ± 3.2 per cent, respectively. The hatching success of embryos from the control adults did not vary between the pre-exposure and exposure periods in experiments II or III ($P > 0.05$). There was no evidence of an effect of exposure of the adults to linuron on hatching success during either experiment ($P > 0.05$). Hatched fry selected from the control adults in experiment II showed a survival to 80 dph of 89 ± 9.7 per cent. Mean sex ratio (percentage of males), total wet weight and condition factor of the surviving offspring were 52 ± 8.4 per cent, 1.41 ± 0.067 1.08 ± 0.014, respectively. In the offspring selected from the control adults, there was no evidence that these parameters varied between the pre-exposure and exposure periods ($P > 0.05$). There was no evidence that exposing adult fish to linuron affected the survival, sex ratio or growth of the offspring to 80 dph ($P > 0.05$).

Experiment IV

Mean measured concentrations of fenarimol in the exposure tanks were 51 ± 1.6, 94 ± 5.2, 178 ± 9.9 and 497 ± 23.2 μg/L. Fenarimol was not detected in the control tanks.

During the acclimation and pre-exposure period of the experiment, three pairs of fish were randomly selected from the eight spare pairs and used to replace pairs where either the female had failed to release her eggs or where the fish were spawning on the sides of the tank. No mortalities occurred in either the control fish or those exposed to fenarimol during the experiment.

During the 21-day pre-exposure period, the mean total egg production, number of spawnings and egg number per spawning, in the control fish were 1868 ± 186, 6.43 ± 0.297 and 292 ± 26.3 per female, respectively. There was no evidence of a change in the spawning activity of the controls between the pre-exposure and exposure periods. Exposure to fenarimol resulted in a decrease in total fecundity at the highest concentration tested (497 μg/L; $P < 0.01$). At this concentration, mean total number of egg fell by 40 per cent (from 1662 ± 254 to 990 ± 341 eggs; $P < 0.05$) and the number of spawnings fell by 63 per cent (from 6.1 ± 0.34 to 2.3 ± 0.64; $P < 0.05$). In

contrast, ut egg batch size increased by 60 per cent (from 261 ± 30 to 431 ± 95 eggs/spawn; $P < 0.05$).

The mean weights, lengths and condition factors of the fathead minnows sampled at the start of the pre-exposure phase (day -21) were 3.14 ± 0.159 g, 64.9 ± 1.318 mm and 1.15 ± 0.032 (n = 8) for the males. They were 1.31 ± 0.073 g, 50.5 ± 1.104 mm and 1.01 ± 0.027 (n = 8) for the females. During the 42-day reproduction test, increases in body weight, length and condition of all the fish were observed. At the end of the exposure phase (day 21), the mean weights, lengths and condition factors of the control fathead minnows were 5.01 ± 0.303 g, 68.6 ± 1.470 mm and 1.54 ± 0.034 (n = 8) for the males, and 1.856 ± 0.069 g, 55.1 ± 1.128 mm and 1.114 ± 0.054 (n = 8) for the females. Exposure to fenarimol did not affect total wet body weight, length or condition factor in the male fish ($P > 0.05$). In the females, a concentration-related increase in total wet body weight was observed, but this was significant only at the highest concentration tested (497 μg/L; $P < 0.05$). There was no effect of fenarimol exposure on body length or condition factor in the females ($P < 0.05$).

All males had secondary sex characters (dorsal fatpad and tubercles) at the onset of the pre-exposure period (day -21). The fatpad weight was 281 ± 31.0 mg, and the mean number and prominence of tubercles was 13.6 ± 0.981 and 3.25 ± 0.164, respectively (n = 8). In both experiments, the prominence of the secondary sex characters increased during the course of the 42-day reproduction test. At the end of the experiment, the mean FPI of the controls was 367 ± 29.1 mg, and the mean number and prominence of tubercles was 22.0 ± 0.598 and 4.00 ± 0.189, respectively (n = 8). There was no evidence of a concentration-related effect of fenarimol on the FPI, tubercle number or prominence ($P > 0.05$).

Plasma VTG concentrations in the male and female fathead minnows sampled at the onset of the pre-exposure period were 0.048 ± 0.0084 μg/mL and 632 ± 73 μg/mL, respectively. No changes in plasma VTG concentrations were observed in the control fish during the pre-exposure or exposure periods ($P > 0.05$). There was no evidence of an effect of exposure to fenarimol on plasma VTG concentrations in the males ($P > 0.05$). In the females, a concentration-related decrease in plasma VTG concentrations was observed ($P < 0.01$), with an effect relative to the controls at the highest exposure concentration (497 μg/L; $P < 0.05$).

Gonadosomatic indices (GSI) in the male and female fathead minnows sampled at the start of the pre-exposure period were 1.36 ± 0.112 and 12.8 ± 1.21, respectively. No changes in GSI were observed in the control fish during the pre-exposure or exposure periods ($P > 0.05$). Exposure to fenarimol resulted in a concentration-related increase in male GSI ($P < 0.01$), which was significant, relative to the controls, at the highest exposure concentration (497 μg/L; $P < 0.05$). There was no evidence of an effect of exposure to fenarimol on female GSI ($P > 0.05$).

There was no evidence that exposing the adults to fenarimol had any effect on the hatching success of the embryos ($P > 0.05$).

Discussion

The results from this investigation demonstrate how useful the fathead minnow pair-breeding reproduction test is for assessing the effects of oestrogens, anti-androgens and aromatase inhibitors on the reproductive health of fish. The fathead minnow proved to be a robust test organism for conducting the breeding test, and no mortalities were observed in the control fish during the pre-exposure or exposure phase of the experiments. In all experiments, fish acclimated to the test conditions relatively quickly and established regular spawning patterns, spawning every 3.1, 3.3, 3.7 and 3.3 days, in experiments I, II, III and IV, respectively. These spawning frequencies are consistent with those reported by others working with the fathead minnow (Gale and Buynek,1982; Harries et al., 2000; Jensen et al., 2001). Mean total egg production was between 1,868 and 2,156 eggs/female during the pre-exposure phase of each experiment (inter-assay coefficient of variation of seven per cent). This demonstrates that reproduction is highly consistent between different stocks of fish, irrespective of differences in the age or body weight of the females at the start of the experiment. Total egg production did vary between the individual pairs of fish (coefficient of variation between 27 per cent and 35 per cent during the pre-exposure period of each experiment). But this variation was consistent for the individual pairs of fish assigned to the control group, when total egg production during the pre-exposure and exposure periods was compared (CoV between 11 per cent and 19 per cent in the four experiments). This illustrates the value of including the pre-exposure period in the experiment in order to establish the baseline fecundity for the individual pairs of fish within each treatment group.

The primary aim of these investigations was to find out whether the fathead minnow pair-breeding test could assess the effects of endocrine active chemicals with different modes of action on the reproductive axis. For this reason, the test concentrations for each chemical were selected to ensure biological activity rather than to reflect environmental relevance. Mean measured concentrations of oestrone ranged from 34 to 781 ng/L, concentrations of linuron ranged from 18 to 2074 μg/L, and concentrations of fenarimol ranged from 51 to 497 μg/L. These concentrations were close to nominal for both oestrone and linuron, > 78 per cent and 82 per cent respectively. Mean measured concentrations of fenarimol, though, were between only 38 per cent and 51 per cent of nominal in the exposure tanks. This was due in part to the fluctuating concentrations produced by the saturation column, but was also a consequence of the test chemical precipitating out of solution in the dosing lines. The test chemicals were not expected to be toxic to adult fathead minnow at the concentrations tested. Exposure to oestrone (at concentrations of 307 and 781 ng/L), though, did have an impact on male survival. Exposure to oestrone also inhibited somatic growth (length) of both the males and females. These effects were not expected given that Panter et al. (1988) had previously demonstrated that exposing non-spawning male fathead minnows to oestrone at a nominal concentration of 993 ng/L for 21 days did not affect survival. The apparent sensitivity of the males in this study was probably related to the physiological demands of spawning activity. Other studies on the effects of EACs on reproductively active fish reported an increased sensitivity of males to the toxic effects of oestrogenic chemicals (Kramer et al., 1998; Ankley et al., 2001; Länge et al., 2001; Seki et al., 2002; Kang et al., 2002, 2003; Nash et al., 2004). This increased sensitivity of

reproductively active males, though, appears to be specific to oestrogenic chemicals. Exposure to linuron and fenarimol did not affect survival or somatic growth relative to the controls ($P > 0.05$). The toxic effects of oestrogens are probably linked to their mechanism of action and, more specifically, to the induction of high levels of VTG in the males. Mortality was observed among males with levels of plasma VTG similar to those normally observed in reproductively active females (> 1 mg/ml of plasma). An excessive production of VTG has been implicated in male mortality in other investigations (Herman & Kincaid, 1988; Kramer et al., 1998; Schwaiger et al., 2000; Zillioux et al., 2001; Folmar et al., 2001; Seki et al., 2002; Kang et al., 2003). Exposure to each of the test chemicals resulted in concentration-related decreases in total egg production ($P < 0.01$), over the 21-day exposure period. There were significant reductions, compared to the pre-exposure period, at concentrations of 307 and 781 ng oestrone/L, 842 and 2074 μg linuron/L and 497 μg fenarimol/L ($P < 0.05$). To our knowledge, this investigation is the first to demonstrate an affect of oestrone and linuron on fecundity in fish. Previous studies have shown that other oestrogenic (Kramer et al., 1998; Harries et al., 2000; Shioda & Wakabayashi, 2000; Ankley et al., 2001; Zillioux et al., 2001; Brion et al., 2004; Nash et al., 2004; Bringolf et al., 2004), anti-androgenic (Ensenbach & Nagel, 1997; Bayley et al., 2003; Jensen et al., 2004) and aromatase inhibiting (Ankley et al., 2002) chemicals affect reproduction in fish.In this work, the reductions in total egg number in fish exposed to either oestrone or linuron resulted from reductions in the number of spawning events ($P < 0.05$), with no change in the size of the egg batch. Consistent with a reduction in the frequency of spawning, it was also observed that males exposed to concentrations of oestrone and linuron that reduced reproduction spent less time in spawning related activities (e.g. guarding and tending the spawning territories and courting the females). This was consistent with a reduction in the frequency of spawning. This effect on reproductive behavior, was not quantified. Exposure to the reference oestrogen, oestradiol-17β, suppresses sexual behavior in male fish (Bjerselius et al., 2001; Oshima et al., 2003). Also, reproduction can be reduced in fish by exposing the males, but not the females, to an oestrogen (Shioda & Wakabayashi, 2000; Länge et al., 2001) or anti-androgen- (Bayley et al., 2003). These observations suggest that the effects of oestrone and linuron on reproduction in this investigation could be the result of an effect on the male alone. Indeed, there was no evidence that exposure to linuron altered the reproductive physiology of the females, based on the endpoints measured in this investigation. In males, though, concentration-related decreases in plasma VTG concentrations, decreases in the prominence of the secondary sex characters (dorsal fatpad and nuptial tubercles) and a reduction in the number of nuptial tubercles were observed. The effects of linuron on reproduction were probably the result of an effect on the males alone, and were probably due to a reduced sexual drive in the males. By contrast, exposure to oestrone altered the reproductive physiology of both the males and females. This resulted in a concentration-related increase in plasma VTG concentrations in males and females, a decrease in the prominence of the dorsal fatpad in males, and a decrease in female gonadosomatic index. The reduced reproductive output in the pair-breeding fish exposed to oestrone was, therefore, probably the result of the altered reproductive physiology in both the males and females. Exposure to fenarimol (497 μg/L) reduced reproduction by decreasing the frequency of spawning, but unlike oestrone and linuron, exposure to fenarimol appeared to result in an increased egg batch size. This affect on egg batch size was not, though, concentration-related ($P > 0.05$). After the onset of exposure to

fenarimol (497 μg/L), all pairs of fish stopped spawning. On day 7 of the exposure, reproduction was 91 per cent lower than at the same time point during the pre-exposure period. The individual pairs of fish then appeared to recover, though exposure to the test chemical continued. By day 15 of exposure, all pairs of fish had resumed reproductive activity, but the batches of eggs spawned were two- to three-fold greater than during the pre-exposure period. As a consequence of this increase in egg batch size, reproduction in this group of fish was only 40 per cent lower than in the control period at the end of the 21-day exposure. Ankley et al. (2002) found that exposing reproductively active females to an aromatase inhibiting chemical, fadrozole, decreases brain aromatase activity and results in decreases in circulating endogenous plasma E2 and VTG concentrations. Though aromatase activity and E2 concentrations were not measured in this investigation, concentration-related decrease in plasma VTG concentrations in the fenarimol-exposed females would support the suggestion that this chemical acted as an aromatase inhibitor. The effect of fenarimol on reproduction was, therefore, probably the consequence of changes in the timing of oocyte maturation in the females, as a consequence of disruptions in the pathway of endogenous oestradiol production. In the fenarimol-exposed males, a concentration-related increase in GSI was observed that was inversely related to the number of spawnings. This was probably the result of an accumulation of sperm in the testis due to the extended spawning cycle of the females. Indeed Ankley et al. (2002) also observed a concentration-related increase in GSI following exposure of reproductively active males to an aromatase inhibitor (fadrozole). They reported a marked accumulation of sperm in the testis.

Further analysis of the data sets revealed strong inverse correlations between inductions of plasma VTG in oestrogen exposed males and females and reproductive success (total egg number and frequency of spawning). This relationship was exponential. Effects on reproduction were observed only when plasma VTG concentrations in both the males and females exceeded a threshold level. In this research, this threshold appeared to be an induction above 1 mg VTG/mL. Other studies (Kramer et al., 1998; Harries et al., 2000; Kang et al., 2002, 2003; Mills et al., 2003; Bringolf et al., 2004; Pawlowski et al., 2004) have shown a similar effect. They demonstrated that reproductive success falls only when induced plasma VTG concentrations in the oestrogen-exposed males and females exceeded levels normally measured in mature females. Collectively, these data demonstrate that induction of VTG to levels exceeding those measured in mature control females will result in adverse reproductive health effects. In addition, reductions in VTG in females exposed to the aromatase inhibitor, fenarimol, were strongly correlated with reductions in the frequency of spawning. This is the first investigation to demonstrate such a relationship. This implies that alterations in the circulating concentrations of VTG in mature females may impact on their reproductive capabilities. This further highlights the importance of this protein as a bioindicator of reproductive health. In the fish exposed to the anti-androgen linuron, there was a strong correlation between the prominence of the secondary sex characters (tubercle prominence and fatpad weight) and reproductive success (total egg number and spawning frequency). There was a weaker correlation between tubercle number and reproduction. This is the first investigation to demonstrate such a relationship. It illustrates the significance of these features to reproductive success. It's very easy to measure SSCs, and the process does not involve the use of complex assays for quantification. The weight of the fatpad was highly sensitive to linuron exposure with effects detected at a four-fold

lower concentration than those affecting reproduction. In addition, the effects on fatpad weight were highly reproducible between the two linuron-exposures. There were decreases of 34 per cent and 53 per cent in the weight of the fatpads from males exposed to 189 and 207 μg linuron/L, respectively, in the two experiments, relative to their respective controls. The use of this biomarker would provide studies of reproduction in the fathead minnow with valuable information about the status of the androgenic pathway and serve as a useful indicator of the reproductive health status of the exposed males.

In this investigation, there was no evidence that the selected test chemicals affected the survival of the embryos to hatch. There was a reduction in the growth of offspring from the adults exposed to the lowest and highest concentrations of oestrone tested, but there was no evidence that the intermediate concentrations of oestrone affected the growth of the offspring. It has previously been demonstrated that growth rates are variable between offspring from different adults (Chapter 1). The small, but significant, reduction in the growth of the offspring in these two groups was, therefore, most likely the consequence of natural variation. These data imply that trans-generational effects of EACs are unlikely in short-term exposures. Exposing adult fathead minnows to the anti-androgen flutamide (Jensen et al., 2004), and adult medaka to the aromatase inhibitor, TBT (Nirmala, 1999) can, though, lead to reductions in the hatching success of the embryos. Factors most likely to determine whether trans-generational effects occur include the length of the exposure period and the nature of the individual EACs (including the ability of the chemicals to bioaccumulate) and the mechanism(s) of effect (for example, EACs that alter DNA methylation are likely to result in trans-generational effects). Thus the potential of a chemical to induce effects in subsequent generations needs to be considered carefully case by case.

In conclusion, the results of this investigation clearly demonstrate that the fathead minnow pair-breeding test can be used to assess the effects of different classes of EACs on the reproductive axis. Measurements of reproduction were easy to do, highly consistent under control conditions and sensitive to the effects of each of the test chemicals. The studies on reproduction alone did not shed any light on the mechanism by which each chemical affected reproductive performance. But including selected biomarkers in the test design enabled identification of each chemical's mode of action. Further analysis of the data sets revealed strong correlations between alterations in the measured biomarkers (induction of VTG in oestrogen exposed males and females, reduction of VTG in females exposed to an aromatase inhibitor, and reduction of the FPI in anti-androgen exposed males) and a reduced reproductive success. Changes in plasma VTG concentrations and the prominence of the SSCs in the fathead minnow can, therefore, be used to signal subsequent adverse effects on reproductive success.

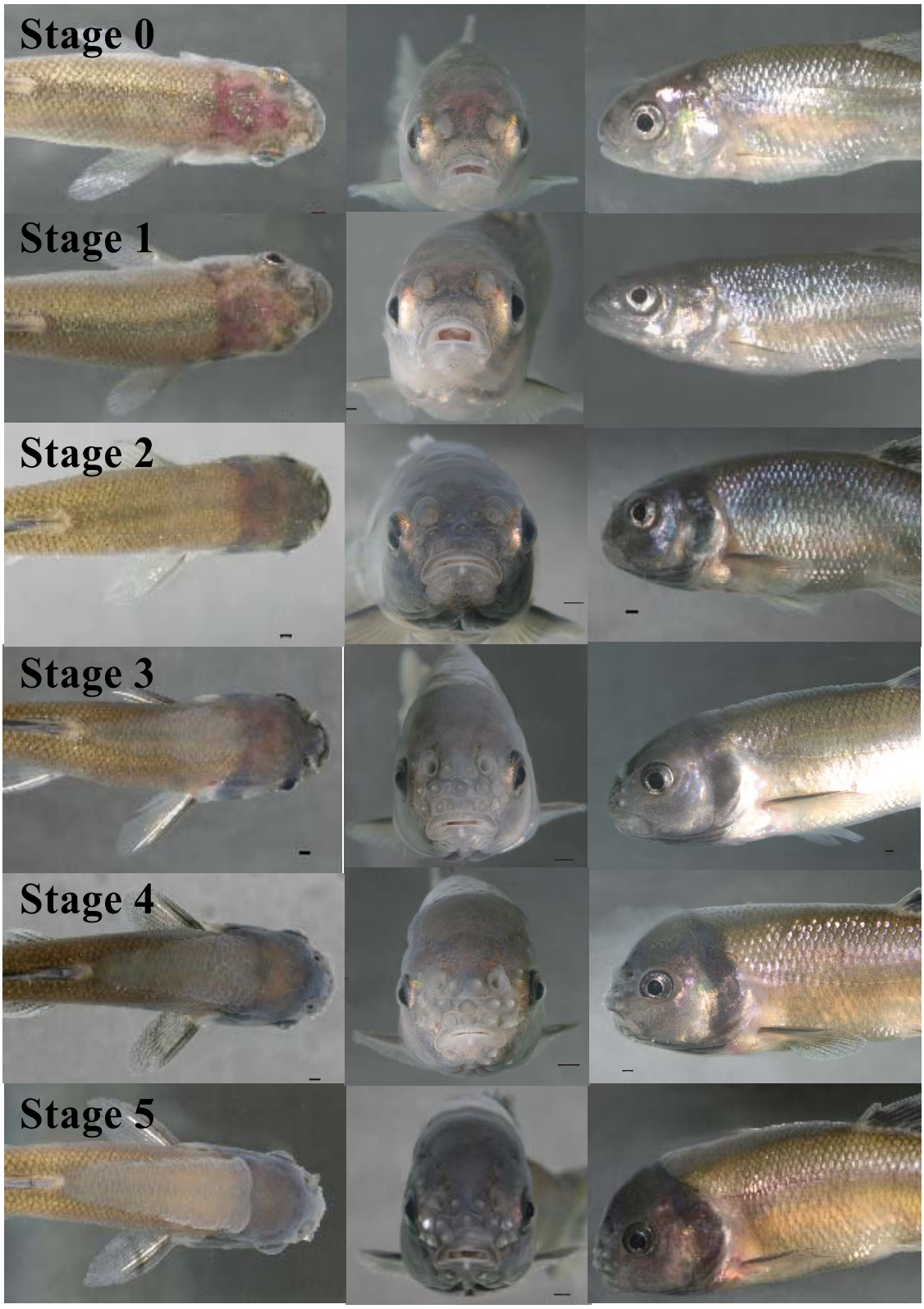


FIGURE 1. Photograph of the nuptial tubercle in maturing/mature according to the scoring system described by Smith, 1978. The corresponding appearance of the dorsal fatpad is also shown. Photographs provided by the Environmental and Molecular Fish Biology team at the University of Exeter.

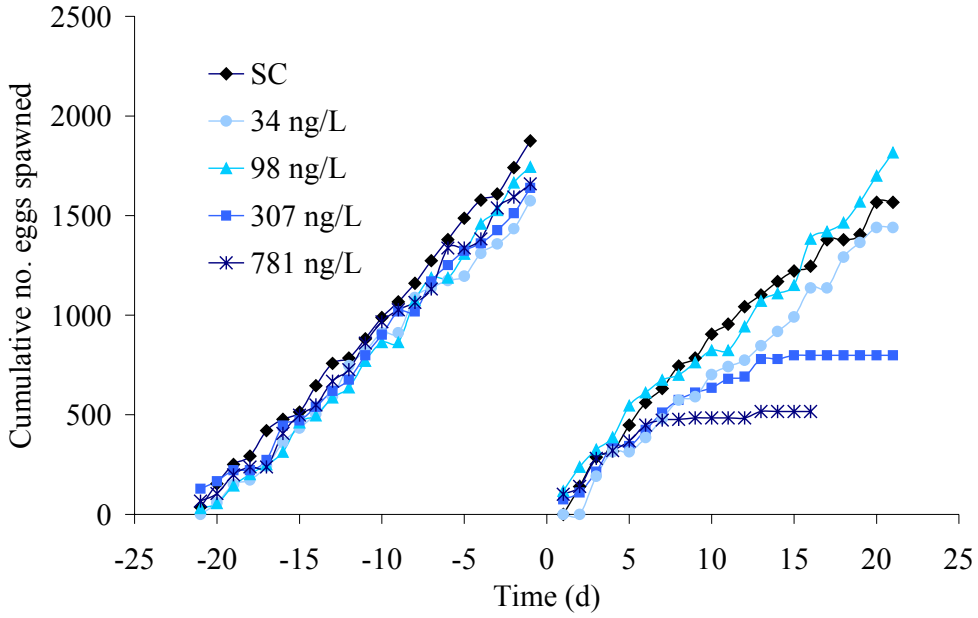


FIGURE 2. The effect of exposure to oestrone on the cumulative fecundity of fathead minnows. Each line shows the mean cumulative fecundity (as assessed daily) of six replicates, before and after exposure to oestrone for 21 days.

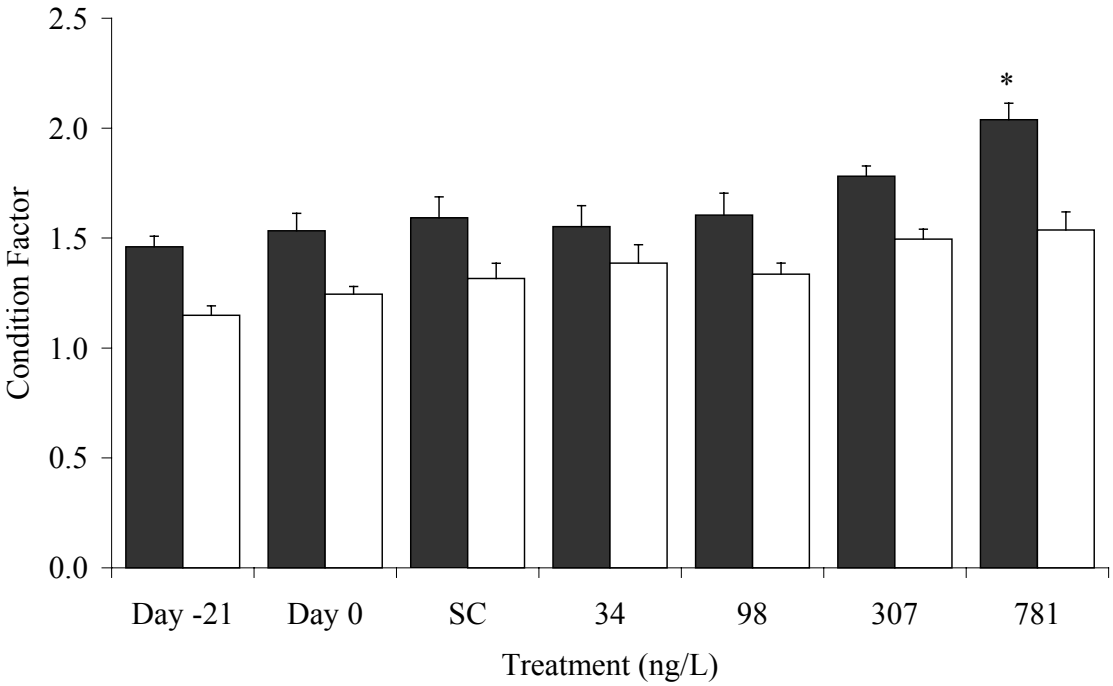


FIGURE 3. Condition factor in male and female fathead minnows exposed to oestrone for 21 days. Each column represents the mean (± SEM). Significant differences from solvent control values are denoted as *p < 0.05.

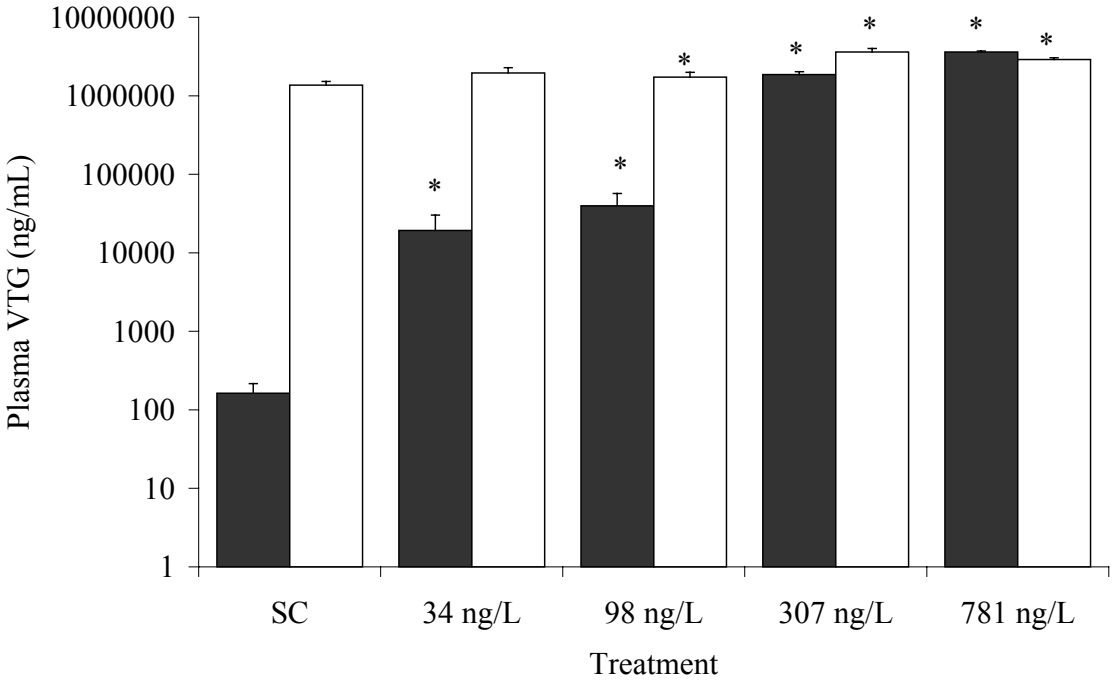


FIGURE 4. Plasma VTG concentrations in fathead minnows exposed to oestrone for 21 days. Each column represents the mean (± SEM). Significant differences from solvent control values are denoted as *p < 0.05.

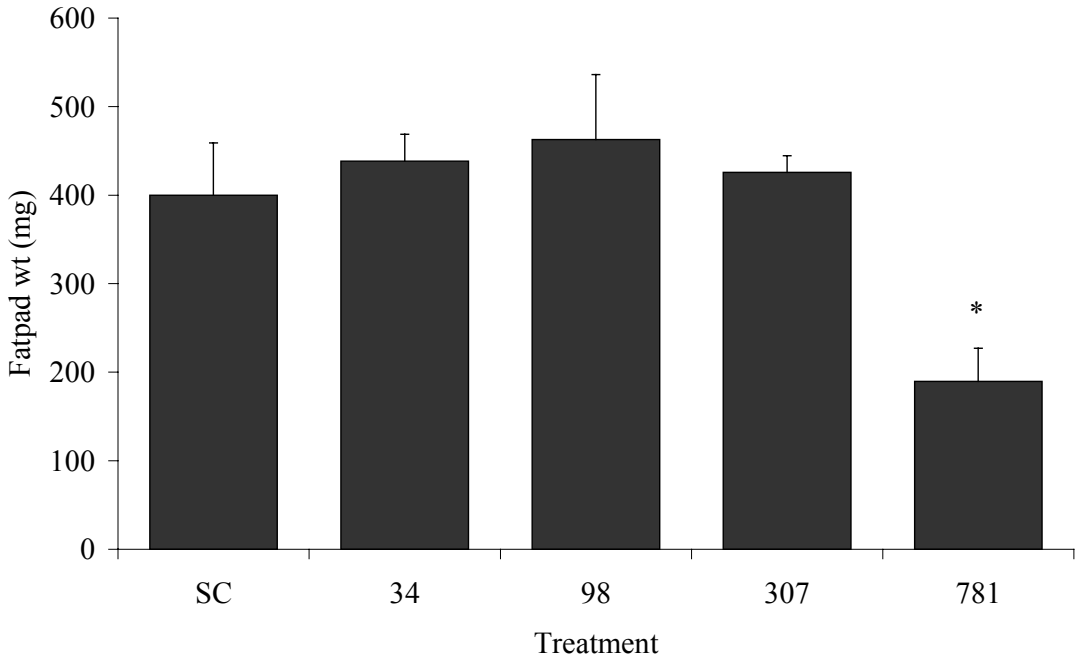
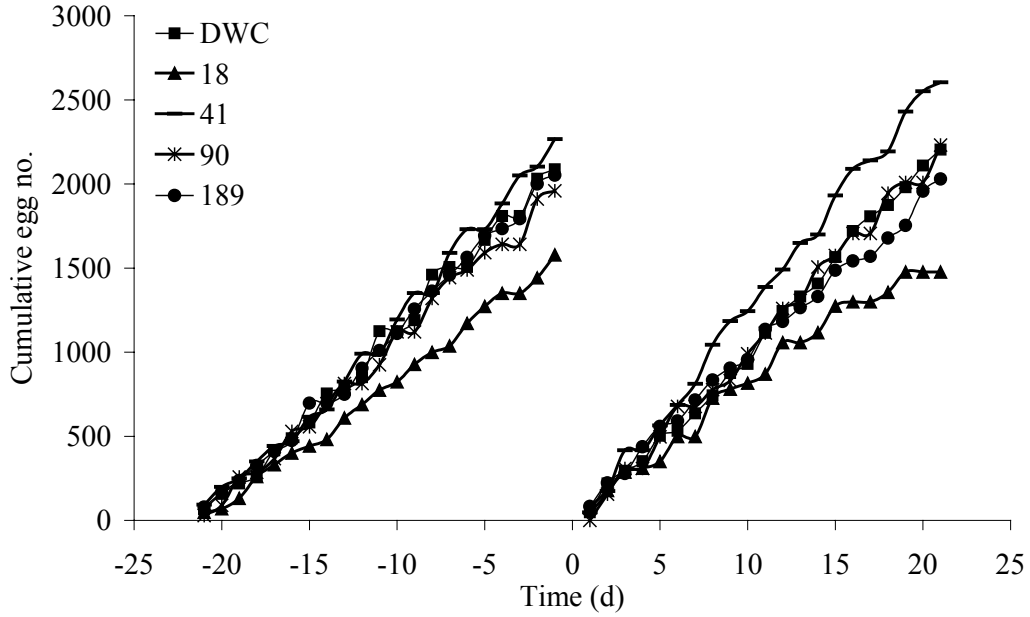


FIGURE 5. Fatpad weight in male fathead minnows exposed to oestrone for 21 days. Each column represents the mean (± SEM). Significant differences from solvent control values are denoted as *p < 0.05.

I.



II.

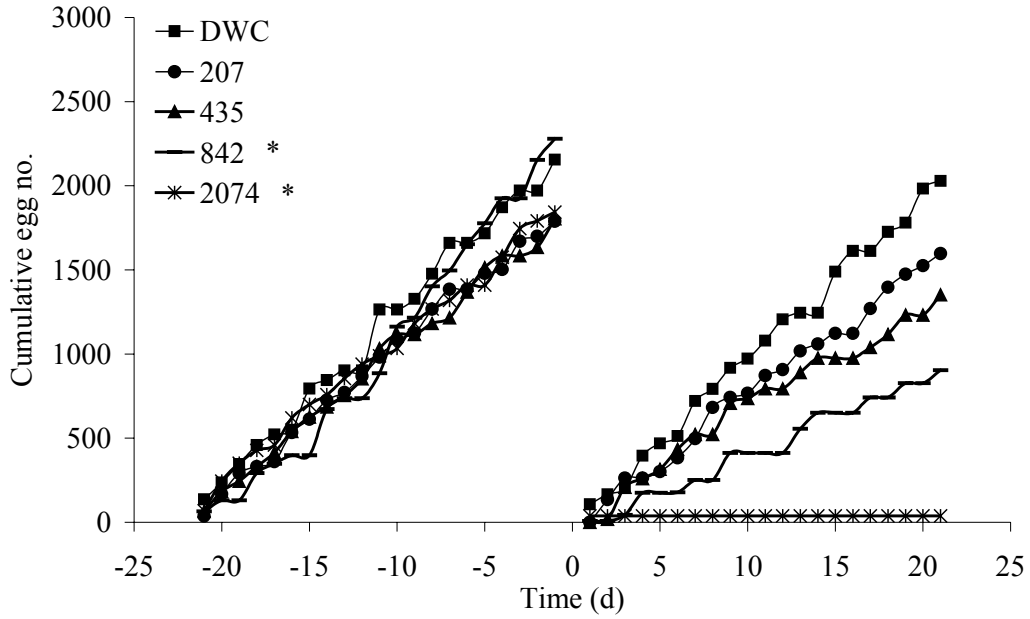


FIGURE 6. The effect of exposure to linuron on the fecundity of pair-breeding fathead minnows in experiments I and II. Each line shows the mean cumulative number of eggs spawned (as assessed daily) in each treatment group, before and after exposure to linuron for 21 days. Significant differences from control values are denoted as *$p < 0.05$.

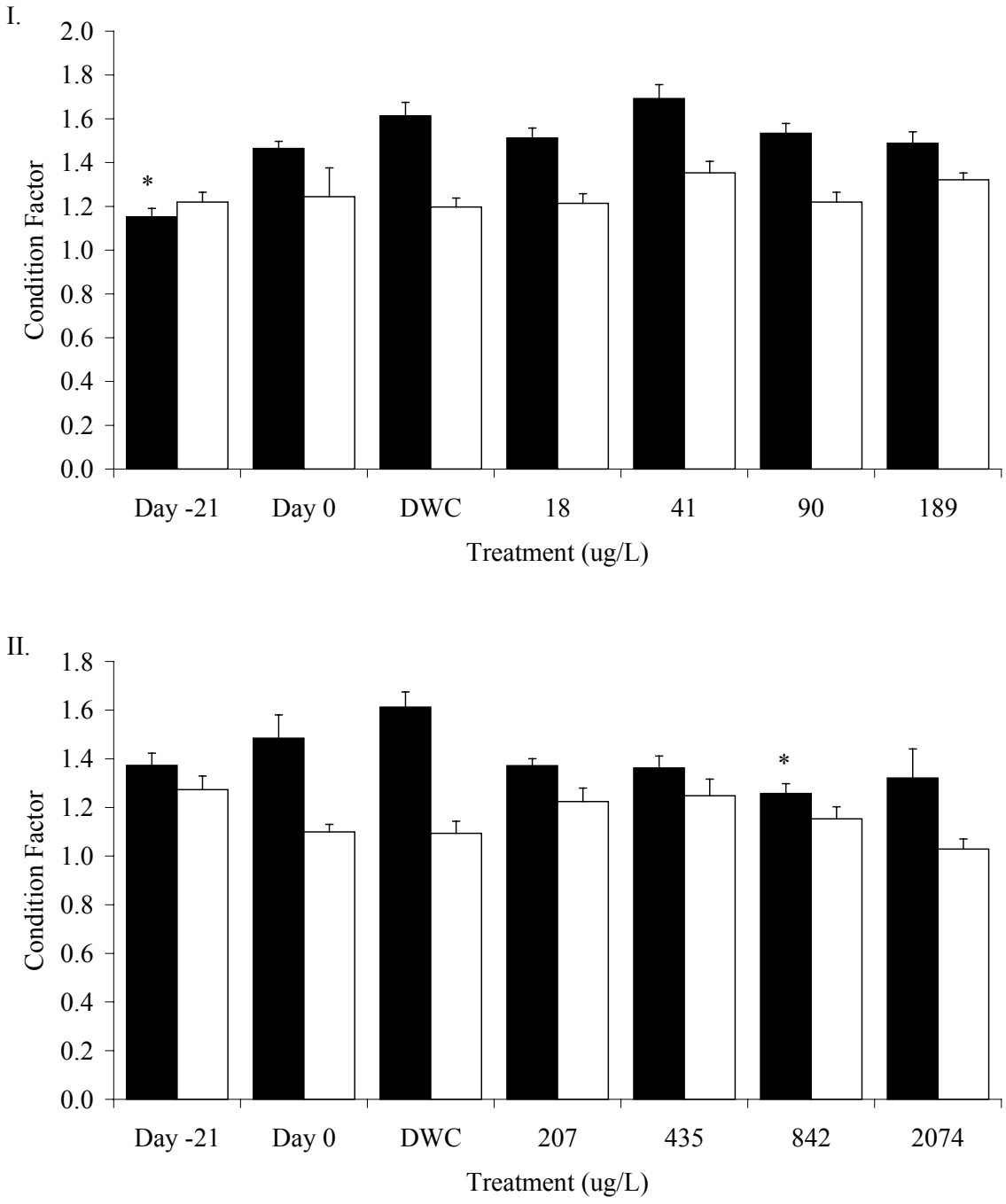
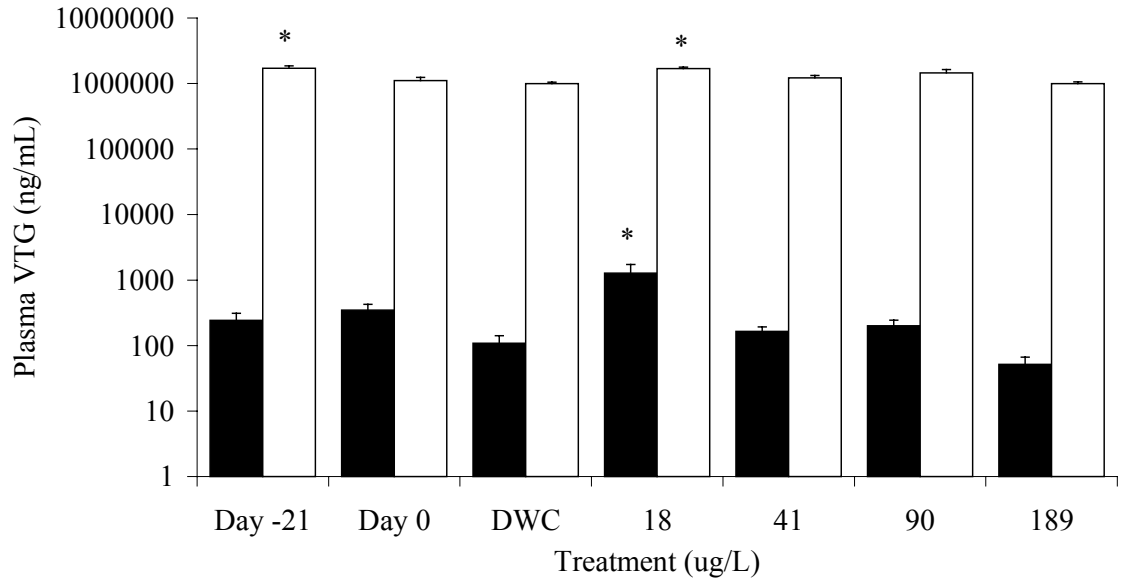


FIGURE 7. Condition factor in male and female fathead minnows exposed to linuron for 21 days. Each column represents the mean (± SEM). Significant differences from dilution water control values are denoted as *p < 0.05.

I.



II.

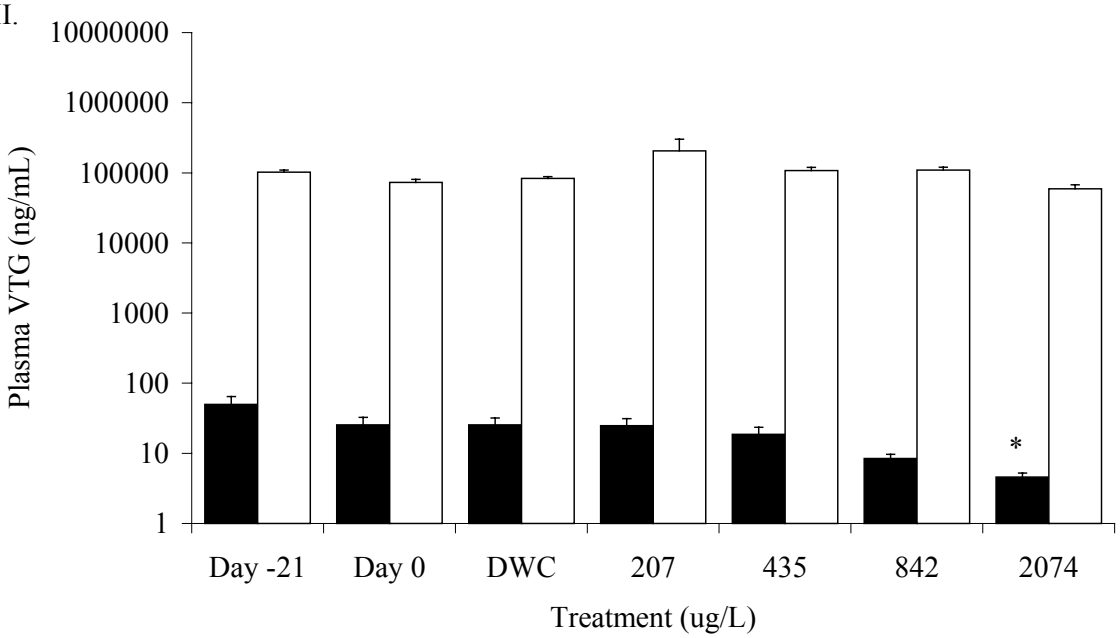


FIGURE 8. Plasma VTG (VTG) concentrations in male and female fathead minnows exposed to linuron for 21 days. Each column represents the mean (± SEM). Significant differences from dilution water control values are denoted as *p < 0.05.

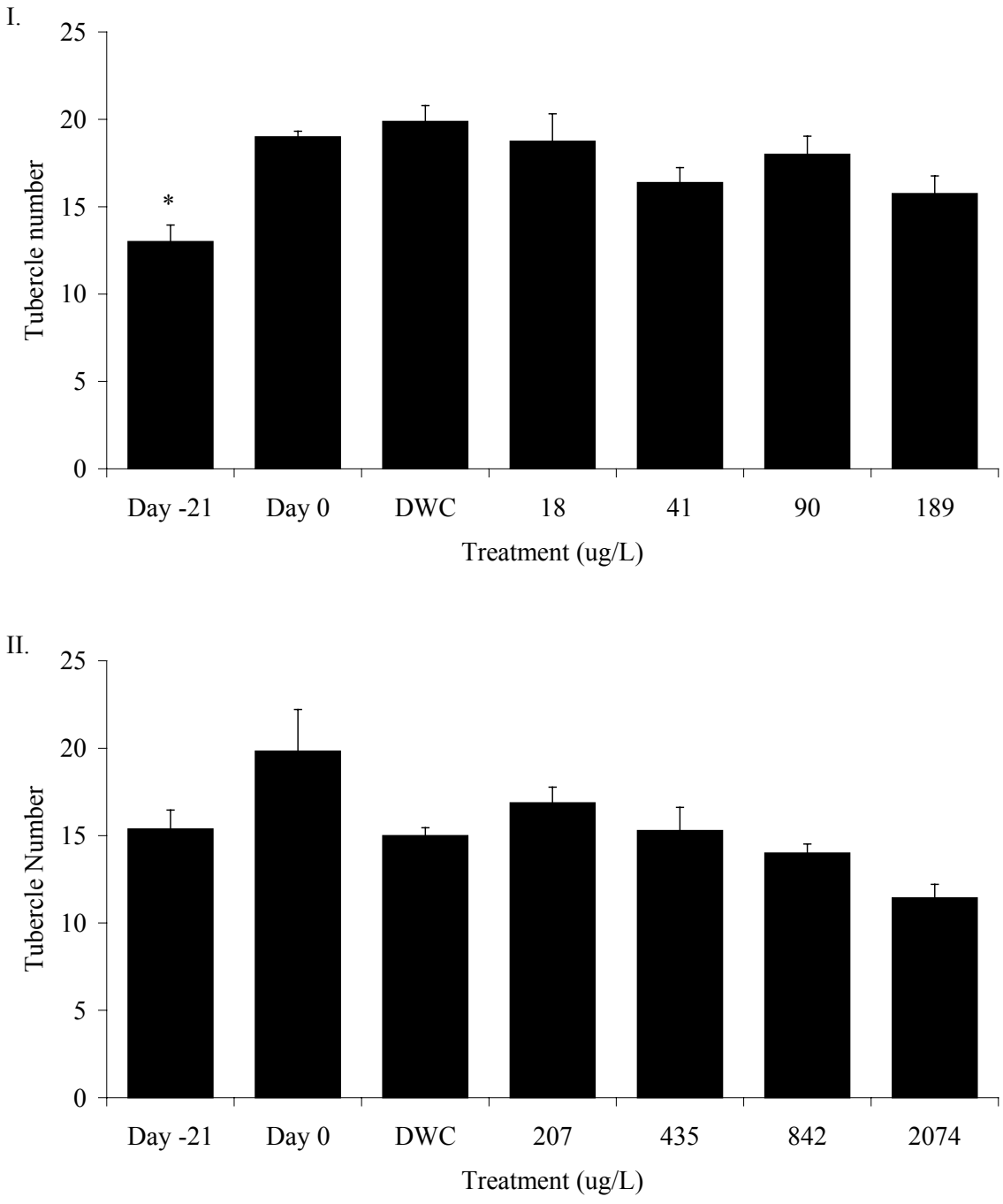


FIGURE 9. Tubercle number in male fathead minnows exposed to linuron for 21 days. Each column represents the mean (± SEM).

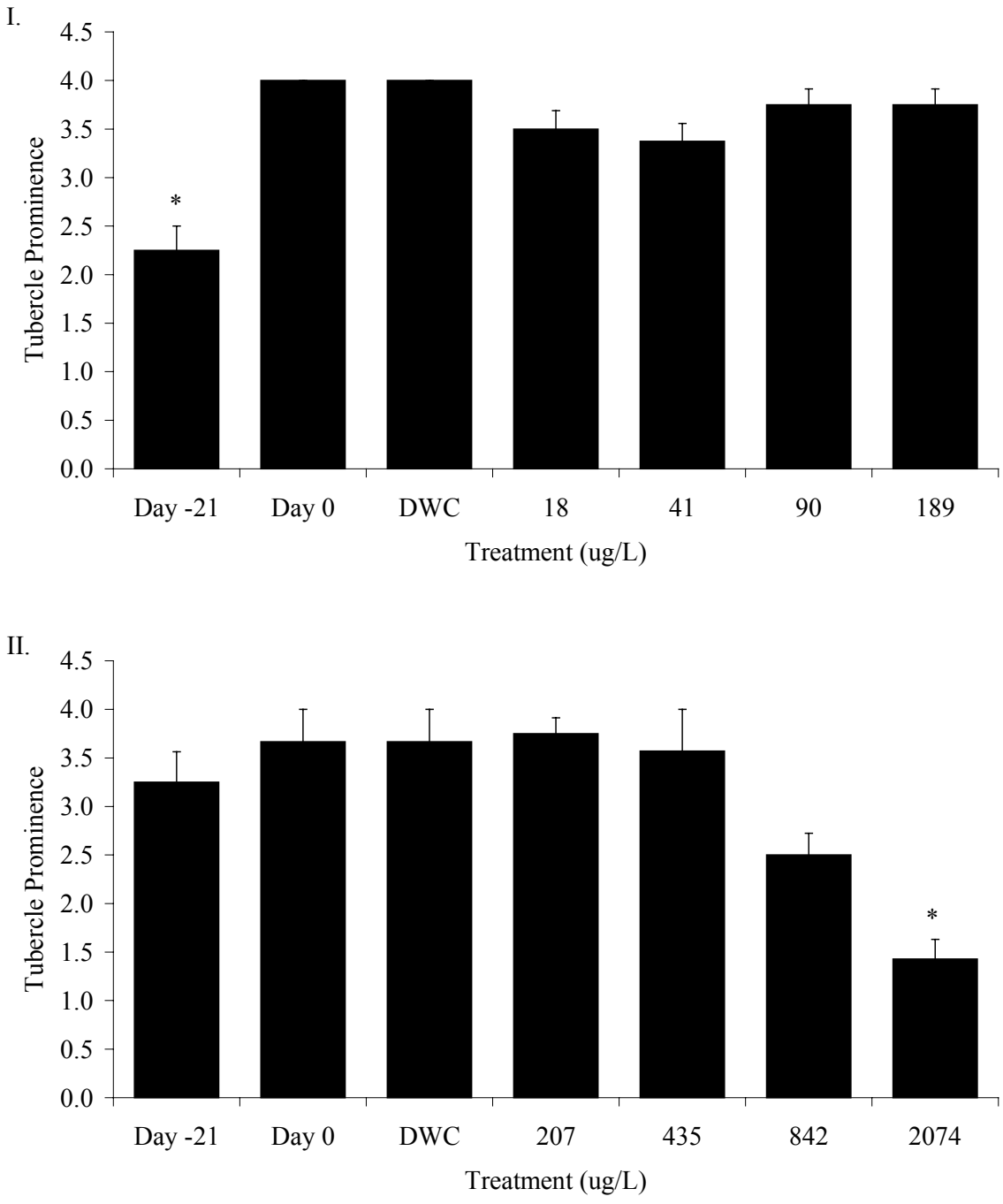


FIGURE 10. Tubercle prominence in male fathead minnows exposed to linuron for 21 days. Each column represents the mean (± SEM). Significant differences from dilution water control values are denoted as *p < 0.05.

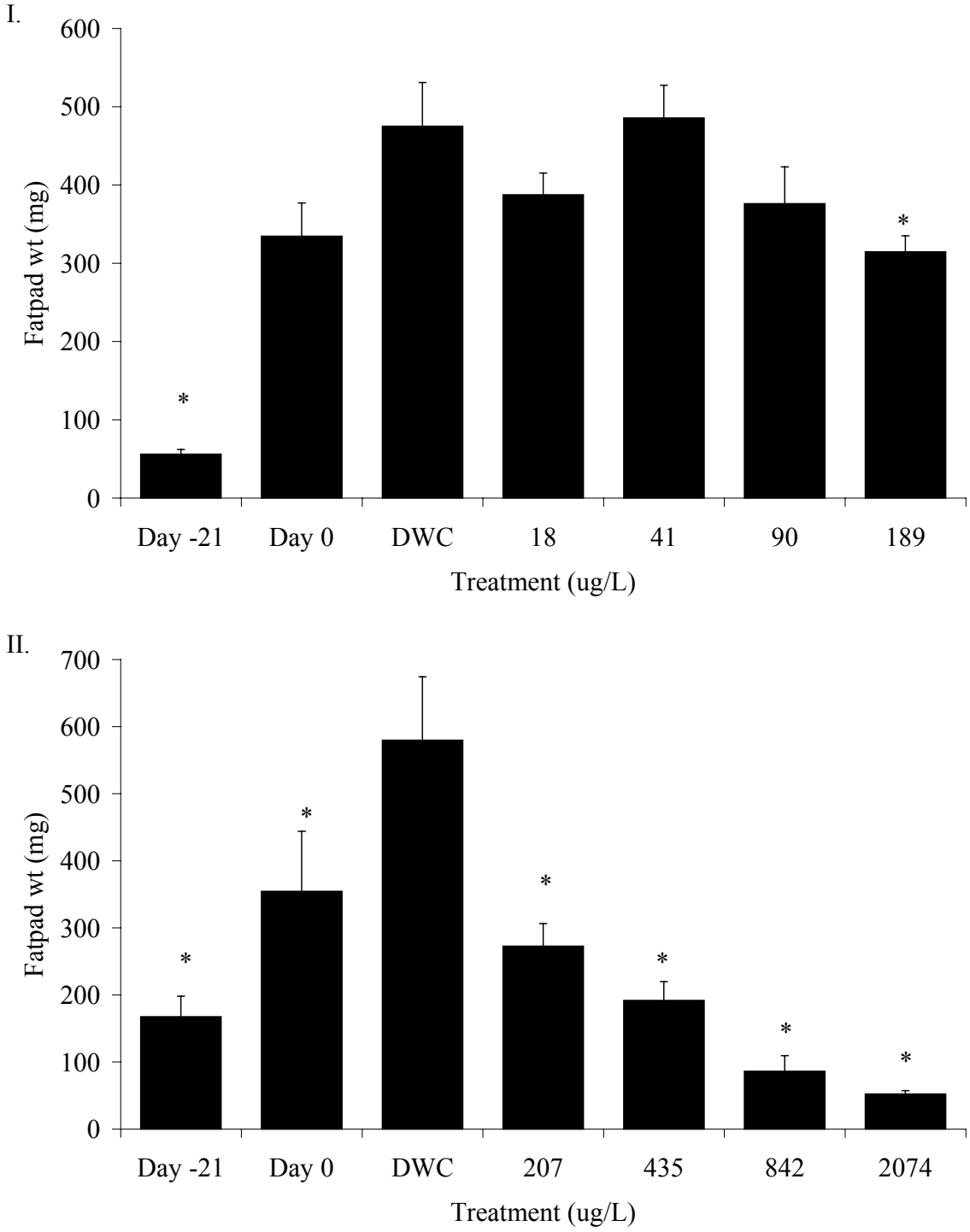


FIGURE 11. Fatpad weight in male fathead minnows exposed to linuron for 21 days. Each column represents the mean (± SEM). Significant differences from dilution water control values are denoted as *$p < 0.05$.

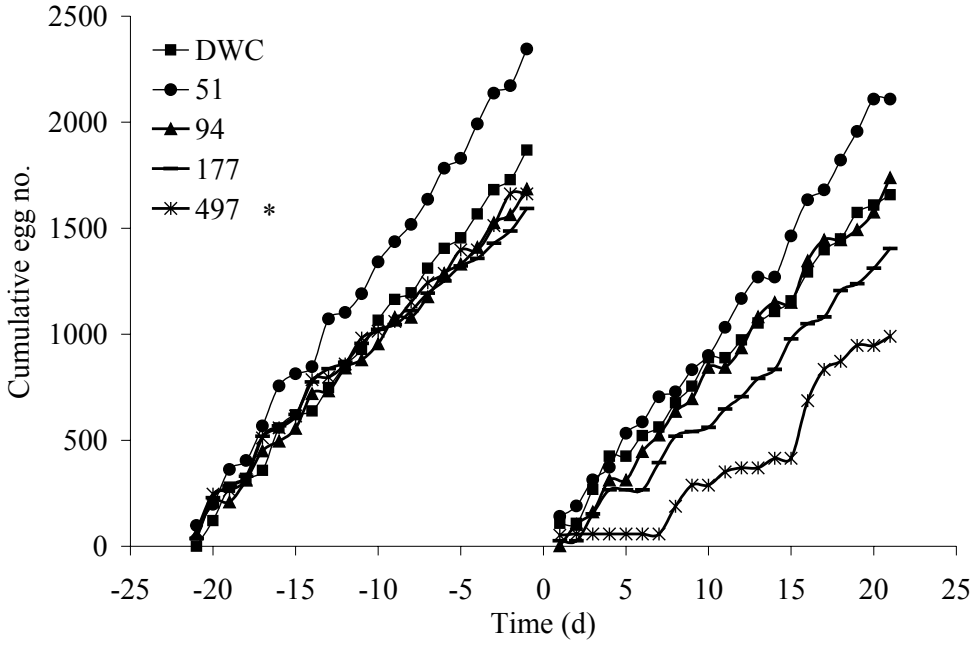


FIGURE 12.A The effect of exposure to fenarimol on the cumulative fecundity of fathead minnows. Each line depicts the mean cumulative fecundity (as assessed daily) of 8 replicates, prior to and after exposure to fenarimol for 21 days.

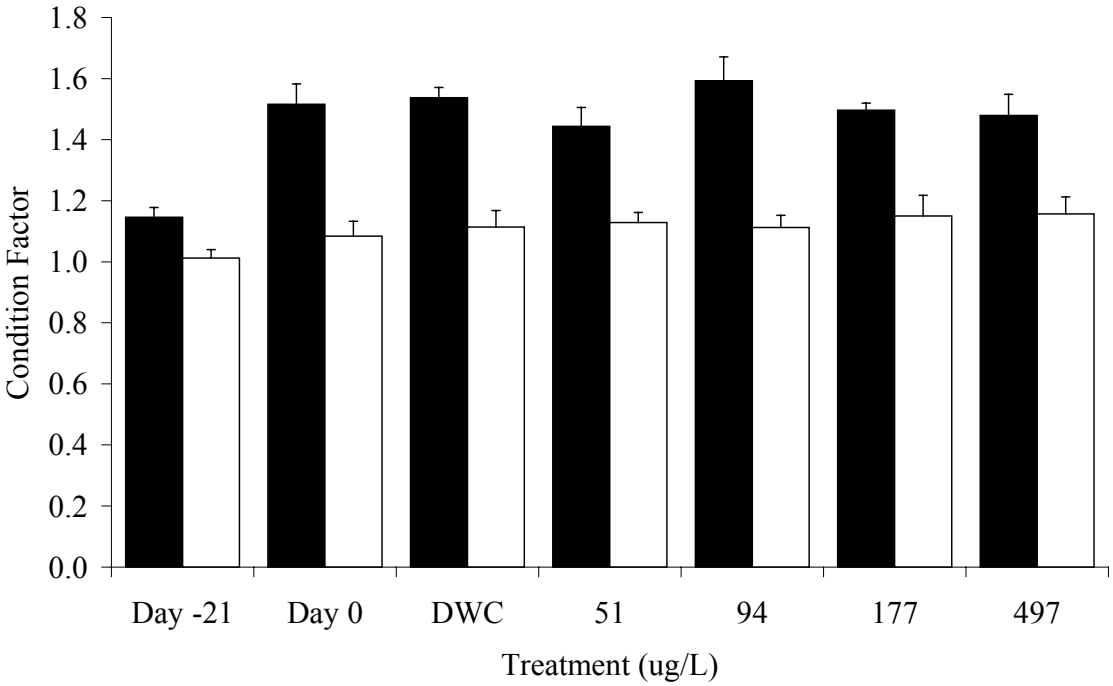


FIGURE 12.B Condition factor in male and female fathead minnows exposed to fenarimol for 21 days. Each column represents the mean (± SEM).

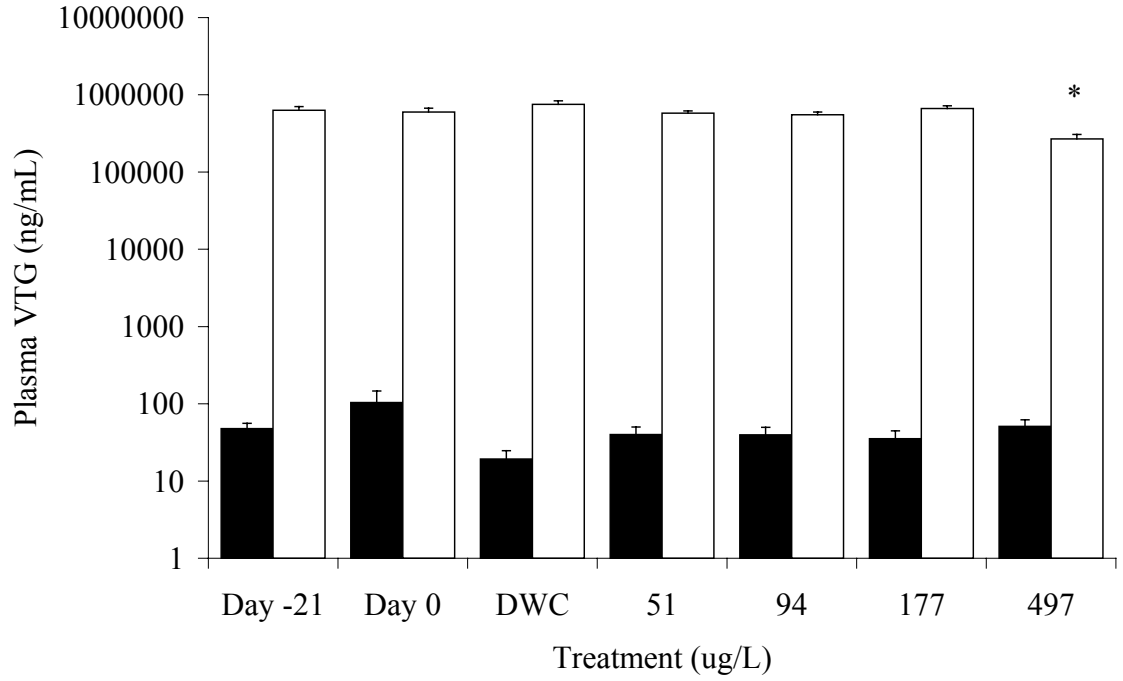


FIGURE 13. Plasma VTG concentrations in fathead minnows exposed to fenarimol for 21 days. Each column represents the mean (± SEM). Significant differences from control values are denoted as *p < 0.05.

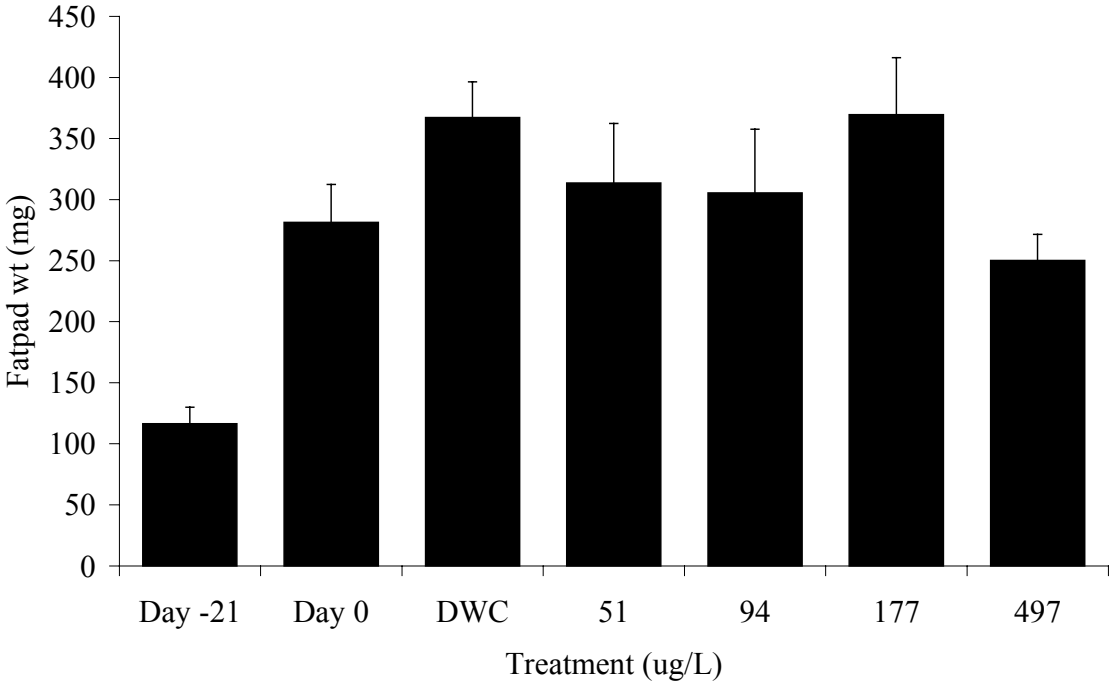


FIGURE 14. Fatpad weight in male fathead minnows exposed to fenarimol for 21 days. Each column represents the mean (± SEM).

4. General conclusions

Substance risk-assessments should be able to measure the effects those substances have on reproduction. This project set out to establish a robust test system for assessing the effects of EACs on fish reproduction. A fathead minnow pair-breeding reproduction test, originally described by Harries et al. (2000), was adopted and validated for a selection of environmental EACs with different modes of action. The selected test chemicals included the natural steroid oestrogen, oestrone, the anti-androgenic pesticide, linuron, and the fungicide fenarimol that disrupts sex steroid biosynthesis. The reproductive effects of each of these chemicals had not been investigated in fish prior to these experiments and so this project also generated data for use in hazard and risk assessment. A major objective was to investigate whether specific biomarkers (VTG and SSC) could signal adverse reproductive health effects of EACs. The relationships between altered biomarker responses and reproductive performance at the level of the individual were investigated. It was demonstrated that both the oestrogenic induction of VTG and the anti-androgenic reduction of the SSCs act as signals of reduced reproductive success. This data set demonstrates that these selected biomarkers would be valuable endpoints for monitoring receiving waters for endocrine activity, and as indicators of the potential consequences for reproductive health.

The array of EACs that have been identified is diverse and expanding. Endocrine disruption is widespread in UK rivers. In addition, no two effluent discharges are likely to have the same chemical matrix. Given these facts, there is a need for cost-effective methods to assess the environmental risk of EACs and environmental mixtures. The most definitive test for assessing chronic effects is the full fish lifecycle test (FFLC). Investigating the effects of chemicals over the entire life cycle of a fish is extremely expensive, though, and such long-term exposures can be very difficult to maintain. Furthermore, applying a FFLC to studies on environmental samples routinely is not practical. Alternative and more cost-effective tests are therefore required. Ideally, such tests need to include endpoints that provide information on the consequences of any effects observed at the individual level for the population. This project demonstrated that the fathead minnow pair-breeding test is highly effective in detecting the adverse health effects of the selected EACs. It offers a cost-effective (though clearly not as inclusive, as exposure is only conducted on a single, albeit highly relevant, life stage of the fish) alternative to the FFLC.

The methodology developed for egg-collection in the pair-breeding assay ensured that as many of the eggs spawned were collected as possible. This resulted in a considerable reduction in the variation of fecundity estimates (chapter 2). The within assay coefficient of variation was less than 30 per cent in each of four experiments conducted, based on estimates obtained from between 36 and 48 pairs of fish within each experiment. The coefficient of variation between the four experiments conducted was less than 14 per cent. This indicates that fecundity estimates for the fathead minnow were highly consistent between different stocks of fish, irrespective of the starting age or the size of the fish. Each of the selected test chemicals reduced

reproductive success, despite their different modes of action on the endocrine system. This illustrates that reproductive output and the experimental test system are highly effective in detecting the adverse effects of different EACs. This means the test developed can be used to assess the effects of EACs with diverse modes of action. The test could therefore be applied to assess the effects of effluents containing a range of EACs that are likely to affect multiple targets within the reproductive axis. Reproduction is a key endpoint for risk assessment purposes, because of its direct relevance to the population. The potential for EACs and effluents to cause population-level impacts can, therefore, be conceptually extrapolated from their effects on reproductive performance. Reproductive performance (egg number) also offers value as an endpoint because it is determined on a daily basis. Potent effects of a chemical can, therefore, be detected within a few days of the initiation of exposure (e.g. high concentrations of linuron completely inhibited reproduction after only 24 hours of exposure). This offers considerable flexibility in the test design. The exposure period could be reduced for more potent chemicals or extended for less potent chemicals. Note, though, that in some cases, the fish may stop breeding for a while, but then acclimate to the exposure and resume reproduction. For example, fish exposed to fenarimol stopped reproducing during the first seven days of exposure, but then started again, spawning larger batches of eggs compared with the pre-exposure period, though the duration between spawns was increased.

Induction of plasma VTG in males was highly sensitive to the steroid oestrogens (oestradiol and oestrone) with a significant induction at environmentally relevant concentrations (29 and 34 ng/L, for E2 and E1, respectively). These effective concentrations were highly consistent with those previously reported in the fathead minnow (Panter et al., 1998; Kramer et al., 1998). Studies on the dynamics of the VTG response demonstrated that induction was rapid and that, once induced, VTG persisted for many weeks in the plasma. This supports the results of other investigations for other EACs (e.g. EE2; Schultz et al., 2001; Schmid et al., 2002). The speed and consistency of the VTG response to the effects of oestrogenic chemicals and the magnitude of induction (tens of thousands fold) show how robust VTG is as a biomarker to signal for the oestrogenic effects of EACs. Bioassays to measure plasma concentrations of VTG are now available for a number of fish species, including both laboratory test species and wild indigenous fish. This biomarker can, therefore, be applied very widely for assessing oestrogen exposure. Indeed, VTG has been, and continues to be, used with great success to monitor exposure to oestrogens in both caged fish *in situ* and in wild fish populations (Purdom et al., 1994; Harries et al., 1996; Rodgers-Grey et al., 2001; Pawlowski et al., 2003; Nakari, 2004). The data generated here, together with the extensive data now available in the literature, support VTG as the most developed biomarker for assessing oestrogenic exposure in fish. This is further reflected by the fact that OECD and US-EPA are currently validating VTG induction for use as an endpoint for quantifying the oestrogenic potency of EACs in fish tests. VTG induction is used principally as a biomarker of exposure to oestrogen receptor agonists. Mature females, though, have elevated plasma VTG concentrations and exposure of females to oestrogen receptor antagonists can lead to a reduction in plasma VTG concentrations (Panter et al., 2002). Furthermore, in this study, exposure to fenarimol, which is thought to interfere with sex steroid biosynthesis, reduced VTG synthesis in mature females. This is consistent with the effect reported for the reference aromatase inhibitor, fadrozole (Ankley et al., 2002; Panter et al., 2004).

These findings further demonstrate that VTG can also be used to signal for effects mediated at targets other than the oestrogen receptor(s) alone. The fact that both induction and suppression of VTG can be used to signal for EACs acting via different modes of action supports the use of both male and female fish together when assessing the possible effects of EACs and their mixtures.

Suppressing the androgen-dependent SSCs, (fatpad weight and nuptial tubercle number and prominence) in males was an effective biomarker of exposure to the anti-androgenic chemical, linuron. Exposure to linuron resulted in a concentration-dependent regression of the fatpad in the mature males after only 21 days of exposure. The response was highly consistent between the two linuron exposures. To date, no other investigation has demonstrated the effect of an anti-androgenic chemical on the weight of the fatpad. The connective tissue underlying the dorsal fatpad proliferates rapidly, and is accompanied by changes in the cellular structure of the epidermis covering the pad. This means that the fatpad is easily identified in male fathead minnows and can be dissected away, intact, from the head relatively easily. Measurements of the fatpad weight are therefore easy to perform and provide a simple and accurate quantification of (anti-)androgenic effects. The weight of the fatpad was influenced by the body weight of the fish, but the application of statistical procedures (analysis of covariance) allowed for this relationship to be accounted for in the subsequent chemical effects analyses. Though concentration-dependent effects of linuron on both tubercle number and prominence were observed, these endpoints were far less sensitive compared to reductions in the size of the fatpad. The induction of tubercle formation in immature male and female fathead minnows is, though, sensitive to the effects of potent androgenic chemicals (Smith, 1974; Ankley et al., 2003; Pawlowski et al., 2004). Exposure to the potent androgens methyltestosterone (Smith, 1974; Pawlowski et al., 2004) and trenbolone (Ankley et al., 2003) rapidly induced the formation of a dorsal fatpad (assessed visually) and nuptial tubercles. Changes in the prominence of the SSCs in male fathead minnows also respond to oestrogenic chemicals (Miles-Richardson et al., 1999; Harries et al., 2000; Pawlowski et al., 2003; Parrot & Blunt, 2005). This demonstrates that these features are also sensitive to the effects of chemicals that alter the endogenous sex steroid profile via mechanisms other than direct binding to the androgen receptor(s). A concentration-dependent suppression in the SSCs (fatpad weight and tubercle number) was observed herein male fish exposed to E2 for 14 days, but these effects were not significant compared with controls. Harries et al (2000) reported a lower sensitivity of the male SSCs, compared to the induction of VTG, in mature male fathead minnows exposed to NP. This indicates that, for short term exposures, the SSCs are less sensitive than VTG as biomarkers of oestrogen exposure. This is not surprising. VTG induction is a relatively ‘simple’ response. It involves transcription and translation of a hepatic gene(s) to produce a product (VTG) that is released into the blood. By contrast, changes in SSCs involve a more complex cascade of events that lead to changes in tissue structure. The concentration-dependent effects on both the fatpad weight and tubercle number, in this study, were still evident after several weeks of depuration, demonstrating that the effects of EACs on the SSCs are persistent. This is consistent with other reports in the literature for both the regression of the SSCs in response to an oestrogen (Miles-Richardson et al., 1999) and the induction of the SSCs in response to an androgen (Smith, 1974). The alterations in the expression of the SSCs in response to both masculinising and feminising chemicals are persistent. This fact, along with the speed of the response (three to six

days for induction, Smith, 1974; 10 days for reduction, Miles-Richardson et al., 1999), and the ease of measuring the SSCs, reveal that these features are valuable biomarkers for indicating exposure to EACs. Given that anti-androgenic activity has been demonstrated in UK STW effluents (Johnson et al., 2004), biomarkers sensitive to the effects of anti-androgenic chemicals are valuable additions to studies assessing the effects of effluents and for studies of effects in wild fish.

Further analyses of the datasets demonstrated that the oestrogen-dependent induction of VTG and the anti-androgenic reduction of fatpad weight correlated with changes in reproductive success. There was a strong inverse correlation between inductions of plasma VTG concentrations in oestrogen-exposed males and females and reproductive success (total egg number and frequency of spawning). This relationship was exponential. Effects on reproduction were observed when plasma VTG concentrations in both the males and females exceeded concentrations of approximately 1 mg/ml, demonstrating that VTG at this level in males is a signal of reproductive inhibition. These data further support studies on wild fish (Jobling et al 1998) and laboratory fish (Kramer et al., 1998; Harries et al., 2000; Kang et al., 2002, 2003; Mills et al., 2003; Bringolf et al., 2004) where a reduced reproductive success was found in oestrogen-exposed males and females that had plasma VTG concentrations that exceeded those normally measured in mature females. There was also a strong correlation between reductions in VTG in mature females exposed to fenarimol and reductions in the frequency of spawning. This demonstrates that any changes in the circulating VTG concentrations in both males and females could signal for a potential impact on the reproductive axis. This further demonstrates the value of VTG as a bioindicator of reproductive health. In the pair-breeding males, there was a strong correlation between fatpad weight and reproductive success (total egg number and spawning frequency). This is the first investigation to demonstrate such a relationship. It illustrates the importance of these features as an indicator of the reproductive status of the male fish and the potential impact for reproduction of alterations in the prominence of the SSCs. Effects on reproduction, though, were observed only when the fatpad was highly regressed (85 per cent compared to controls). It's worth pointing out, though, that the females in this study did not choose their mates. In the wild, changes in SSCs as a consequence of EAC exposure may affect mate choice and in turn reproductive success.

In summary, we have developed and validated a highly robust experimental test for assessing the effects of EACs on reproductive performance using pair-breeding fathead minnow. The reproduction test provides quantitative data (on reproductive capabilities) that is relevant for the risk assessment of individual EACs and chemical mixtures at the population level. This project tested individual chemicals with clearly defined mechanisms of action. But the reproduction test can be easily applied to assess the effects of chemical mixtures, both defined and more complex undefined mixtures, such as effluent discharges. Mechanism specific biomarkers (e.g. VTG induction and SSCs) are easily incorporated in the reproduction test design. They provide powerful information on the chemical(s) mode of action, and their inclusion is thus recommended in the fish reproduction test, particularly when determining the effects of chemical mixtures and effluent discharges. With this in mind, it is proposed that the fathead minnow pair-breeding assay, including the adaptations developed during this project and the proven biomarkers of reproductive effects that signal for specific mechanisms, be considered for wider use in assessing the endocrine activity

of chemicals mixtures in effluents and environmental samples.

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References

Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20: 1276-1290.

Ankley GT, Jensen KM, Makynen EA, Kahl MD, Korte JJ, Hornung MW, Henry TR, Denny JS, Leino RL, Wilson VS, Cardon MC, Hartig PC, Gray LE. 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22 (6): 1350-1360.

Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol. Sci. 67 (1): 121-130.

Bauer ERS, Meyer HHD, Stahlschmidt-Allner P, Sauerwein H. 1998. Application of an androgen receptor assay for the characterisation of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives. Analyst 123 (12): 2485-2487.

Bayley M, Junge M, Baatrup E. 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat. Toxicol. 56: 227-239.

Bayley M, Larsen PF, Baekgaard H, Baatrup E. 2003. The effects of vinclozolin, an anti-androgenic fungicide, on male guppy secondary sex characters and reproductive success. Biol. Reprod. 69 (6): 1951-1956.

Bettin C, Oehlmann J, Stroben E. 1996. TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgolander Meeresuntersuchungen 50 (3): 299-317.

Bjerselius R, Lundstedt-Enkel K, Olsen H, Mayer I, Dimberg K. 2001. Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17 beta-estradiol. Aquatic Toxicol. 53 (2): 139-152.

Borg B. 1994. Androgens in teleosts fishes. Comp. Biochem. Physiol. C. 109 (3): 219-245.

Bringolf RB, Belden JB, Summerfelt RC. 2004. Effects of atrazine on fathead minnow in a short-term reproduction assay. Environ. Toxicol. Chem. 23: 1019-1025.

Brion F, Tyler CR, Palazzi X, Laillet B, Porcher JM, Garric J, Flammarion P. 2004. Impacts of 17 beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). Aquatic Toxicol. 68 (3): 193-217.

Cook JC, Mullin LS, Frame SR, Biegel LB. 1993. Investigation of a mechanism for leydig cell tumorigenesis by linuron in rats. Toxicol. Appl. Pharmacol. 119 (2): 195-204.

Degani G, Boker R, Jackson K. 1998. Growth hormone, sexual maturity and steroids in male carp (*Cyprinus carpio*). Comp. Biochem. Physiol. C. 120: 433-440.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environ. Sci. Technol. 32 (11): 1549-1558.

Dreze V, Monod G, Cravedi JP, Biagianti-Risbourg S, Le Gac F. 2000. Effects of 4-nonylphenol on sex differentiation and puberty in mosquitofish (*Gambusia holbrooki*). Ecotox. 9 (1-2): 93-103.

Ensenbach U, Nagel R. 1997. Toxicity of binary chemical mixtures: Effects on reproduction of zebrafish (*Brachydanio rerio*). Arch. Environ. Contam. Toxicol. 32 (2): 204-210.

Fisk AT, Johnston TA. 1998. Maternal transfer of organochlorines to eggs of walleye (*Stizostedion vitreum*) in Lake Manitoba and western Lake Superior. J. Great Lakes Res. 24 (4): 917-928.

Folmar LC, Hemmer M, Hemmer R, Bowman C, Kroll K, Denslow ND. 2000. Comparative estrogenicity of estradiol, ethynylestradiol and diethylstilbestrol in an *in vivo*, male sheepshead minnow (*Cyprinodon variaegatus*), VTG bioassay. Aquat. Toxicol. 49: 77-88.

Gale WF, Buynak GL. 1982. Fecundity and spawning frequency of the fathead minnow – A fractional spawner. Trans. Am. Fish. Soc. 111: 35-40.

Gibson R, Smith MD, Spary CJ, Tyler CR, Hill EM. 2005. Mixtures of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents. Environ. Sci. Technol. 39 (8): 2461-2471.

Hammerschmidt CR, Sandheinrich MB. 2005. Maternal diet during oogenesis is the major source of methylmercury in fish embryos. Environ. Sci. Technol. 39 (10): 3580-3584.

Harries JE, Runnalls T, Hill E, Harris CA, Maddix S, Sumpter JP, Tyler CR. 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 34: 3003-3011.

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, Rycroft R, Sumpter JP, Tylor T. 1996. A survey of estrogenic activity in United Kingdom inland waters. Environ. Toxicol. Chem. 15 (11): 1993-2002.

Harris CA, Santos EM, Janbakhsh A, Pottinger TG, Tyler CR, Sumpter, JP. 2001. Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female

rainbow trout. Environ. Sci. Technol. 35 (14): 2909-2916.

Herman RL, Kincaid HL. 1988. Pathological Effects of Orally-Administered Estradiol to Rainbow-Trout. Aquaculture 72: 165-172.

Jensen KM, Kahl MD, Makynen EA, Korte JJ, Leino RL, Butterworth BC, Ankley GT. 2004. Characterisation of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. Aquat. Toxicol. 70: 99-110.

Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). Comp. Biochem. Physiol. C. 128: 127-141.

Jobling S, Coey S, Whitmore JG, Kime DE, Van Look KJW, McAllister BG, Beresford N, Henshaw AC, Brighty G, Tyler CR, Sumpter JP. 2002a. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Repro. 67 (2): 515-524.

Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty GC, Sumpter JP, Tyler CR. 2002b. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Biol. Repro. 66 (2): 272-281.

Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32 (17): 2498-2506.

Johnson I, Hetheridge M, Tyler CR. 2004. Assessment of the (anti-)oestrogenic and (anti-)androgenic activity of sewage treatment works effluents. R&D Technical Report, Environment Agency.

Kahl MD, Russom CL, DeFoe DL, Hammermeister DE. 1999. Saturation units for use in aquatic bioassays. Chemosphere 39 (3): 539-551.

Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Hano T, Maeda M, Imada N, Tadokoro H, Honjot T. 2003. Effects of 4-nonylphenol on reproduction of Japanese medaka, *Oryzias latipes*. Environ. Toxicol. Chem. 22 (10): 2438-2445.

Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H, Honjo T. 2002. Effect of 17 beta-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). Chemosphere 47 (1): 71-80.

Kirby MF, Allen YT, Dyer RA, Feist SW, Katsiadaki I, Matthiessen P, Scott AP, Smith A, Stentiford GD, Thain JE, Thomas KV, Tolhurst L and Waldock MJ. 2004. Surveys of plasma vitellogenin and intersex in male flounder (Platicythys flesus) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001. Environ. Toxicol. Chem. 23: 748-758.

Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17 beta-estradiol. Aquat. Toxicol. 40: 335-360.

Lahnsteiner F, Berger B, Grubinger F, Weismann T. 2005. The effect of 4-nonylphenol on semen quality, viability of gametes, fertilization success, and embryo and larvae survival in rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 71 (4): 297-306.

Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20: 1216-1227.

Lazier CB, MacKay ME. 1993. Vitellogenin gene expression in teleost fish. In: Biochemistry and Molecular Biology of Fishes, Vol.2 (Hochachka PW, Mommeson TP, eds.). Elsevier, pp.391.

LeGuellec K, Lawless K, Valotaire Y, Kress M, Tenniswood M. 1988. Vitellogenin gene expression in male rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 71: 359-371.

McAllister BG, Kime DE. 2004. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*) (vol 65, pg 309, 2003). Aquat. Toxicol. 67 (3): 301-302.

McMillan V. 1972. Mating of the fathead. Nat. Hist. 81: 73-78.

McMillan VE, Smith RJF. 1974. Agonistic and reproductive behaviour of the fathead minnow (*Pimephales promelas* Rafinesque). *Z. Tierpsychol*. 34: 25-58.

Metcalfe TL, Metcalfe CD, Kiparissis Y, Niimi AJ, Foran CM, Benson WH. 2000. Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to o,p '-DDT in water or through maternal transfer. Environ. Toxicol. Chem. 19 (7): 1893-1900.

Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. 1999. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 47: 129-145.

Miles-Richardson SR, Pierens SL, Nichols KM, Kramer VJ, Snyder EM, Snyder SA, Render JA, Fitzgerald SD, Giesy JP. 1999. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Environ. Res. 80 (2): S122-S137.

Miller MA. 1993. Maternal transfer of organochlorine compounds in salmonines to their eggs. Can. J. Fisheries Aquat. Sci. 50 (7): 1405-1413.

Nakayama K, Oshima Y, Nagafuchi K, Hano T, Shimasaki Y, Nakayamaj K. 2005. Early-life-stage toxicity in offspring from exposed parent medaka, *Oryzias latipes*, to mixtures of tributyltin and polychlorinated biphenyls. Environ. Toxicol. Chem. 24 (3):

591-596.

Nakari T. 2004. Estrogenicity of municipal effluents assessed in vivo and in vitro. Environ. Toxicol. 19 (3): 207-215.

Nash JP, Kime DE, Van der Ven LTM, Wester PW, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Perspect. 112 (17): 1725-1733.

Ng TB, Idler DR. 1983. Yolk formation and differentiation in teleost fishes. In: Fish Physiology (Hoar WS, Randall DJ, Donaldson EM, eds.) Academic Press, Orlando, FL, pp.373.

Nirmala K, Oshima Y, Lee R, Imada N, Honjo T, Kobayashi K. 1999. Transgenerational toxicity of tributyltin and its combined effects with polychlorinated biphenyls on reproductive processes in Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 18: 717-721.

OECD. 1992. Test Guideline 210 – Fish, early-life stage toxicity test. Adopted 17th July 1992. OECD Environment, Health and Safety Division, 2 rue André-Pascal, Paris.

Ogino Y, Katoh H, Yamada G. 2004. Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. FEBS Letters 575 (1-3): 119-126.

Oshima Y, Kang IJ, Kobayashi M, Nakayama K, Imada N, Honjo T. 2003. Suppression of sexual behavior in male Japanese medaka (*Oryzias latipes*) exposed to 17 beta-estradiol. Chemosphere 50 (3): 429-436.

Panter GH, Hutchinson TH, Hurd KS, Bamforth J, Stanley RD, Duffell S, Hargreaves A, Gimeno S, Tyler CR. 2005. Development of chronic tests for endocrine active chemicals. Part 1. An extended fish early-life stage test for oestrogenically active chemicals in the fathead minnow (*Pimephales promelas*). Submitted to Aquatic Toxicol.

Panter GH, Hutchinson TH, Hurd KS, Sherren A, Stanley RD, Tyler CR. 2004. Successful detection of (anti-) androgenic and aromatase inhibitors in pre-spawning adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. Aquatic Toxicol. 70 (1): 11-21.

Panter GH, Hutchinson TH, Lange R, Lye CM, Sumpter JP, Zerulla M, Tyler CR. 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. Environ. Toxicol. Chem. 21 (2): 319-326.

Panter GH, Thompson RS, Sumpter JP. 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquatic Toxicol.

42 (4): 243-253.

Parrott JL, Blunt BR. 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20 (2): 131-141.

Pawlowski S, Sauer A, Shears JA, Tyler CR, Braunbeck T. 2004. Androgenic and estrogenic effects of the synthetic androgen 17α-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. Aquat. Toxicol. 68: 277-291.

Pawlowski S, van Aerle R, Tyler CR, Braunbeck, T. 2004. Effects of 17 alpha-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57 (3): 330-345.

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8:275-285.

Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP, Tyler CR. 2001. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environ. Sci. Technol. 35 (3): 462-470.

Rodgers-Gray TP, Jobling S, Morris S, Kelly C, Kirby S, Janbakhsh A, Harries JE, Waldock MJ, Sumpter JP, Tyler CR. 2000. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environ. Sci. Technol. 34 (8): 1521-1528.

Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environ. Sci. Technol. 32 (11): 1559-1565.

Routledge EJ, Sumpter JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15 (3): 241-248.

Schmid T, Gonzalez-Valero J, Rufli H, Dietrich DR. 2002. Determination of vitellogenin kinetics in male fathead minnows (*Pimephales promelas*). Toxicol. Letters 131 (1-2): 65-74.

Schultz IR, Orner G, Merdink JL, Skillman A. 2001. Dose-response relationships and pharmacokinetics of VTG in rainbow trout after intravascular administration of 17α-ethynylestradiol. Aquat. Toxicol. 51: 305-318.

Schwaiger J, Mallow U, Ferling H, Knoerr S, Braunbeck T, Kalbfus W, Negele RD. 2002. How estrogenic is nonylphenol? A transgenerational study using rainbow trout (Oncorhynchus mykiss) as a test organism. Aquat. Toxicol. 59 (3-4): 177-189.

Seki M, Yokota H, Matsubara H, Tsuruda Y, Maeda N, Tadokoro H, Kobayashi K. 2002. Effect of ethinylestradiol on the reproduction and induction of VTG and testis-ova in medaka (Oryzias latipes). Environ. Toxicol. and Chem. 21: 1692-1698.

Shimasaki Y, Kitano T, Oshima Y, Inoue S, Imada N, Honjo T. 2003. Tributyltin causes masculinization in fish. Environ. Toxicol. Chem. 22 (1): 141-144.

Shioda T, Wakabayashi M. 2000. Evaluation of reproductivity of medaka (*Oryzias latipes*) exposed to chemicals using a 2-week reproduction test. Water Sci. Technol. 42: 53-60.

Smith RJF. 1973. Testosterone eliminates alarm substance in male fathead minnows. Can. J. Zool. 51: 875-876.

Smith RJF. 1974. Effects of 17α-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). Can. J. Zool. 52: 1031-1038.

Smith RJF. 1978. Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow *Pimephales promelas*. Can. J. Zool. 56: 2103-2109.

Smith RJF, Murphy BD. 1974. Functional morphology of the dorsal pad in fathead minnows (*Pimephales promelas*). Trans. Amer. Fish Soc. 103: 65-72.

Sohoni P, Sumpter JP. 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158 (3): 327-339.

Tinwell H, Ashby J. 2004. Sensitivity of the immature rat uterotrophic assay to mixtures of estrogens. Environ. Health Perspec. 112: 575-582.

Thibaut R, Monod G, Cravedi JP. 2002. Residues of C-14-4n-nonylphenol in mosquitofish (*Gambusia holbrooki*) oocytes and embryos during dietary exposure of mature females to this xenohormone. Mar. Environ. Res. 54 (3-5): 685-689.

Thorpe KL, Cummings RI, Hutchinson TH, Scholze M, Brighty G, Sumpter JP, Tyler CR. 2003. Relative potencies and combination effects of steroidal oestrogens in fish. Environ. Sci. Toxicol. 37: 1142-1149.

Thorpe KL, Hetheridge MJ, Hutchinson TH, Scholze M, Sumpter JP, Tyler CR. 2001. Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (*Oncorhynchus mykiss*). Environ. Sci. Toxicol. 35: 2476-2481.

Thorpe KL, Hetheridge MJ, Hutchinson TH, Sumpter JP, Tyler CR. 2000. Development of an in vivo screening assay for estrogenic effects using juvenile rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 19: 2812-2820.

Tyler CR 1991. Vitellogenesis in salmonids. In: Reproductive Physiology of Fish (Scott AP, Sumpter JP, Kime DA, Rolfe MS, eds.). Fish Symposium 91, Sheffield, pp.297-301.

Tyler CR, van Aerle R, Hutchinson TH, Maddix S, Trip H. 1999. An *in vivo* testing system for endocrine disrupters in fish early life-stages using induction of VTG. Environ. Toxicol. Chem. 18: 337-347.

Thomas-Jones E, Thorpe K, Harrison N, Thomas G, Morris C, Hutchinson T, Woodhead S, Tyler C. 2003. Dynamics of estrogen biomarker responses in rainbow trout exposed to 17 beta-estradiol and 17 alpha-ethinylestradiol. Environ. Toxicol. Chem. 22 (12): 3001-3008.

U.S. Environmental Protection Agency (1982). User's guide for conducting life-cycle chronic toxicity tests with fathead minnows (*Pimephales promelas*). EPA/600/8-81-011. Duluth, MN, USA.

U.S. Environmental Protection Agency (1986). Hazard Evaluation Division Standard Evaluation Procedure. Fish life-cycle toxicity tests. EPA 540/9-86-137, Office of Pesticides Programs, Washington, DC 20460.

U.S. Environmental Protection Agency (1987). Guidelines for the culture of fathead minnows (*Pimephales promelas*) for use in toxicity tests. EPA/600/3-87/011. Environmental Research Laboratory, Duluth, MN, USA.

U.S. Environmental Protection Agency (1994). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 3rd ed, Lewis P A, D O Klemm, J M Lazorchak, T J Norberg-King, W H Peltier and M A Heber (eds). EPA-600-4-91-002. Office of Research and Development, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio, USA.

van Aerle R, Nolan M, Jobling S, Christiansen LB, Sumpter JP, Tyler CR. 2001. Sexual disruption in a second species of wild cyprinid fish (the Gudgeon, *Gobio gobio*) in United Kingdom freshwaters. Environ. Toxicol. Chem. 20 (12): 2841-2847.

van Aerle R, Pounds N, Hutchinson TH, Maddix S, Tyler CR. 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. Ecotoxicol. 11 (6): 423-434.

Van Bohemen CG, Lambert JGD, Goos HJT, Van Oordt PGWJ. 1982. Estrone and estradiol participation during exogenous vitellogenesis in the female rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 46: 81-92.

Van Hoeven-Arentzen. 1995. Fenarimol. Report for the National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands.

Williams RJ, Johnson AC, Smith JJL, Kanda R. 2003. Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. Environ. Sci. Technol. 37 (9): 1744-1750.

Yokota H, Seki M, Maeda M, Oshima Y, Tadokoro H, Honjo T, Kobayashi K. 2001. Life-cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 20 (11): 2552-2560.

Zar JH. Biostatistical Analysis. 4th Edition. Prentice Hall Inc., New Jersey.

Zillioux EJ, Johnson IC, Kiparissis Y, Metcalfe CD, Wheat JV, Ward SG, Liu H. 2001. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: A partial life-cycle test with 17 alpha-ethynylestradiol. Environ. Toxicol. Chem. 20: 1968-1978.

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