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Long-term exposure to environmentally relevant concentrations of ethinyloestradiol affects sexual differentiation and development in roach, *Rutilus rutilus*

Science Report – SC030299/SR2

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Authors:
Dr Anke Lange, Mr Gregory C. Paull, Prof. Charles R. Tyler

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Research Contractor:
Prof. Charles Tyler
University of Exeter
Prince of Wales Road, Exeter

Environment Agency's Project Manager:
Dr Kerry Walsh, Ecotoxicology, Science Department, Wallingford, Oxon, OX10 8BD.

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Steve Killeen

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Executive summary

This report describes work undertaken in a research project co-funded by the UK Environment Agency and the Natural Environmental Research Council within the Environmental Genomics programme to investigate the impacts of exposure to environmental concentrations of the contraceptive pharmaceutical oestrogen, 17α-ethinyloestradiol (EE2), on sexual differentiation and development in the roach (*Rutilus rutilus*), a fish species native to UK rivers.

The feminising effects of EE2 were investigated for an exposure period spanning approximately 2 years (from fertilised eggs up to 720 days post hatch (dph)) and for a range of concentrations of EE2 (nominal concentrations of 0, 0.1, 1 and 10 ng EE2 L^{-1}) found in effluent discharges emanating from wastewater treatment works (WwTWs), and in some surface waters in the UK. The exposure period included early life, when the developmental processes defining sexual differentiation (gender assignment) occur in fish. In the long-term exposure to EE2, fish were sampled on days 56, 84, 112, 250, 518 and 720 of the exposure and analysed for gonadal sex development (via gonadal histology) and vitellogenin (VTG) induction, as a biomarker of oestrogen exposure.

Analysis of treatment effects during early life stages found that all fish exposed to 4 ng EE2 L^{-1} (measured concentration) developed a female-like gonad morphology, determined by a characteristic shape of the gonad and the presence of two points of attachment of the gonad to the peritoneal wall, forming an ovarian cavity. At later life stages, longer-term exposure to 4 ng EE2 L^{-1} was shown definitively to result in an all-female population (as assessed by gonad histology). In the 4 ng EE2 L^{-1}-treated fish, stages of ovarian development varied more widely compared with the control females (that were tightly synchronised in their development), which probably reflected the presence of both females and feminised (sex-reversed) males in this treatment group. At all life stages analysed, VTG levels (an indicator of oestrogen exposure) were significantly elevated in fish exposed to 4 ng EE2 L^{-1}, compared with the controls.

At lower exposure concentrations to EE2, over all the sampling periods as a whole, there appeared to be a higher proportion of females in the 0.3 ng EE2 L^{-1} (measured concentration) exposure group compared with controls. Two 'males' (out of a total of 52 histologically confirmed male fish sampled at 720 dph) were intersex, one derived from the exposure to non-detectable levels of EE2 (nominal exposure concentration of 0.1 ng EE2 L^{-1}) and one exposed to a measured concentration of 0.3 ng EE2 L^{-1}. This effect may have come about because of a bioconcentration of EE2 over time to reach a level sufficient to induce sex cell disruption. Alternatively, these intersex fish may have reflected the normal occurrence of this condition in a very small proportion of the roach population. Gonads of the other fish derived from these low level EE2 treatment regimes did not differ from controls in terms of the stage of sexual development (for either males or females). None of the control fish sampled throughout this study (n = 172) showed any signs of sex cell disruption.

To explore the possibility that gonadal effects induced by exposure to oestrogenic chemicals during early life persist in roach, embryos/fish were exposed to EE2 until 120 dph (when sexual differentiation was complete), then maintained in clean water for 400 days, and subsequently examined for effects on gonadal development (via histology). The range of EE2 exposure concentrations was the same as for the long-term exposure study described above. At the end of the depuration (clean water) phase, a high incidence of gonadal intersex was identified in the group of fish that had been exposed to 4 ng EE2 L^{-1} during early life, confirming that effects induced during early life persist for an extended period of time after withdrawal of the exogenous oestrogenic challenge.

In a further study the effect of exposure to the same range of EE2 concentrations during early life was assessed on the subsequent responsiveness of roach to oestrogen challenge in later life. After the initial EE2 exposure, fish were depurated in clean water for 400 days and then all treatment groups were challenged to a single measured concentration of 2.3 ± 0.2 ng EE2 L^{-1} and concentrations of VTG measured in the blood after 4 and 10 days of exposure. The results suggested an enhanced sensitivity (responsiveness) to oestrogen with previous exposure to EE2 during early life. For females especially, there was a strong suggestion for a concentration-related effect.

The data presented in this report demonstrate that roach are sensitive to sexual disruption on exposure to EE2 and that early life exposure to concentrations found in some UK WwTWs effluents can induce feminising effects in males, including gonadal duct disruption and germ cell disruption (oocytes in the testis). Long-term exposure to 4 ng EE2 L^{-1} induced complete gonadal sex reversal in roach, resulting in an all-female population. An especially novel finding from this work was that exposure to exogenous oestrogen during early life altered the subsequent responsiveness to oestrogen in later life (i.e. sensitised the fish to oestrogen). The findings presented further support the contention that EE2 is a significant contributor to the feminised responses seen in wild roach living in the vicinity of effluent discharges from WwTWs in UK rivers and heighten concern about the hazards associated with discharges of EE2 in UK rivers.

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1 Introduction

Extensive studies on wild populations of roach (*Rutilus rutilus*) living in UK rivers have shown that exposure to oestrogenic effluents emanating from wastewater treatment works (WwTWs) causes altered sexual development (e.g. Purdom et al. 1994, Harries et al. 1997). In a survey carried out on wild roach living downstream of WwTWs, fish were shown to have a high incidence of intersex (Jobling et al. 1998). Intersex is the term given to a range of effects on gonad development, including gonadal duct disruption where the male duct is feminised to form a female-like ovarian cavity, and/or the presence of both male and female germ cells within the same gonad (Nolan et al. 2001). This disruption in sexual development was subsequently shown to impact negatively on the reproductive success of affected fish (Jobling et al. 2002a, 2002b) with the potential for population-level effects. A further study on juvenile roach living in the vicinity of WwTW effluent discharges found an abnormally high proportion of 'females' and/or feminised males (these fish were identified by the presence of an ovarian cavity) at river sites receiving high effluent discharges from WwTWs (Beresford et al. 2004).

UK rivers can contain up to 80% treated wastewater effluent, and fish living in these rivers can be exposed continuously throughout their lives, including the period of sexual differentiation, which is the most sensitive period for chemical effects on gender assignment. Controlled exposures of roach to WwTWs effluents have confirmed the association between some of the feminised responses seen in wild fish (induction of the egg-yolk precursor protein vitellogenin (VTG) and duct disruption), but not induction of oocytes in the testis (Harries et al. 1999, Rodgers-Gray et al. 2000, 2001, Liney et al. 2005). It should be realised, however, that intersex in wild roach (oocytes in testes) is normally reported for fish that are 2+ years in age, and the studies cited above on roach used fish of a younger age.

It has been demonstrated that steroidal oestrogens, both natural and synthetic, present in effluents play a major role in the disruption of sexual function in wild roach in UK rivers. The pharmaceutical oestrogen 17α-ethinyloestradiol (EE2), used in the contraceptive pill, has been measured in effluents at concentrations ranging from non-detectable (< 0.5 ng L^{-1} for effluent samples) up to 42 ng L^{-1} (Desbrow et al. 1998, Ternes et al. 1999). EE2 is also present in surface waters from non-detectable levels (<0.1 ng L^{-1}) up to 5 ng L^{-1} (Aherne and Briggs 1989, Williams et al. 2003). Although EE2 is not the most prevalent steroidal oestrogen in the aquatic environment, it is the most potent (Segner et al. 2003b, Thorpe et al. 2003). In addition, EE2 is more persistent due to its longer half-life and it has a tendency to bioconcentrate in organisms (Larsson et al. 1999). For these reasons, EE2 has the potential to disrupt reproductive processes in fish at relatively low concentrations. Disruption of reproductive processes on exposure to EE2 have been demonstrated in the laboratory in a wide range of fish species, albeit often at concentrations exceeding those normally measured in the aquatic environment. These responses include effects on the normal sexual development and differentiation (Metcalfe et al. 2001, van Aerle et al. 2002, Andersen et al. 2003, Van den Belt et al. 2003, Weber et al. 2003, Fenske et al. 2005), reproductive behaviour (Balch et al. 2004, Brian et al. 2006), reduced fecundity (Scholz and Gutzeit 2000, Länge et al. 2001, Nash et al. 2004), and reduced fertilisation success or viability of embryos from exposed adults (Länge et al. 2001, Hill and Janz 2003, Segner et al. 2003a).

In fish, the period of sex determination and sexual differentiation is controlled by a delicate balance of genetic and environmental factors, and any imbalance created by other exogenous influences (including endocrine disrupting chemicals – EDCs) can ultimately impact on gender assignment, even in gonochoristic (single-sexed) species

(Patino 1997, Nakamura et al. 1998, Jalabert et al. 2000, Strüssmann and Nakamura 2002). Given the role of natural oestrogens and steroid hormones in the development of the gonads, sexual differentiation, and gametogenesis, it is necessary to explore whether exposure of fish to oestrogenic chemicals during critical periods of differentiation has consequences for their subsequent reproductive capabilities. Furthermore, the potential for an environmental oestrogen to produce permanent changes in function or 'imprint' on the endocrine system would have serious implications for the impact of WwTW effluent on wildlife populations (Foran et al. 2002). Early exposure (neonatally in mammals) has the potential to change the regulation of gene transcription, producing long-term and even epigenetic changes in response to excess hormonal signalling (McLachlan et al. 2001) and these changes in gene regulation, or imprinting, have been implicated in the susceptibility to environmentally related diseases, including cancer (Jirtle et al. 2000, Foran et al. 2002).

Over the past decade, the roach has become established as a sentinel species for studies into disruption of sexual development in fish in UK rivers. The reasons for this are many and include:

- it is widespread, abundant and ecologically important in the UK (and in other parts of Europe), where it lives naturally in lowland rivers in a range of habitats;
- it is tolerant of poor water quality (and is, therefore, amenable for effluent exposures in which oxygen levels are typically low and sediment loads high);
- it is a gonochoristic species (developing as either a male or female), thus avoiding complications where gender changes occur as part of the natural process of sexual development;
- it belongs to the carp family Cyprinidae, one of the largest families of freshwater fish which also includes the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*), both model species that are commonly used for ecotoxicological studies;
- it is, like most other cyprinids, a group spawner, and, as such, the principles derived from work on roach are likely to be widely applicable to other group-spawning species;
- its normal reproductive development has been established (Environment Agency 2007) and analytical tools to determine oestrogenic effects in this species are available (e.g. for measuring roach VTG (Tyler et al. 1996, 1999)) and histological markers for gonadal feminisation);
- it can be easily cultured in the laboratory.

The key aim of this study was to investigate the impacts of long-term exposure to environmental concentrations of EE2 on the reproductive development and physiology of the roach. The study also aimed to determine whether EE2 effects on gonadal development during early life persisted in later life, after prolonged periods in clean water. Additionally, an investigation was made to determine how exposure to EE2 during early life subsequently impacted on the response of roach to EE2 exposure in later life.

2 Material and methods

2.1 Fish source, culture and husbandry

Pre-spawning, sexually mature male and female roach were obtained from the Environment Agency's Fish Farm (Calverton, Nottinghamshire, UK) in April 2003 and brought into the aquarium facility at the University of Exeter. Here they were induced artificially to spawn using established procedures with carp pituitary extract (see Jobling et al. 2002b). The resulting embryos were deployed into glass aquaria under flow-through conditions. Embryos hatched 7–10 days post fertilisation and the resulting fry were fed three times a day with Cyprico Crumble EX (Coppens International bv, Helmond, The Netherlands) dry food (0.01–0.2 mm until 35 dph, 0.2–0.3 mm until 64 dph followed by 0.5–0.8 mm until 250 dph) supplemented with freshly hatched *Artemia* sp. nauplii until satiation. Roach kept from 250 dph onwards were fed frozen gamma-irradiated brine shrimp (*Artemia* sp.) and bloodworm (*Chironomus* sp.) supplemented with Cyprico Start Premium EX (1 mm).

In their first year, roach were maintained at 18 ± 1 °C with a 16 hour light: 8 hour dark photoperiod. From December to February of their second year the temperature was gradually reduced to 12 ± 2 °C before being increased again (in a stepwise manner) up to 18 ± 2 °C by April. The photoperiod regime was also reduced during this same time period to 12 h:12 h light:dark.

2.2 Exposure system and experimental design

The experimental design for the exposures of roach to EE2 is shown in Figure 2.1. One group of roach were exposed to three environmental concentrations of EE2 (Sigma-Aldrich, Gillingham, Dorset, UK; nominal concentrations of 0.1, 1.0 and 10 ng L^{-1}) from fertilisation continuously up to 720 dph in flow-through conditions (experiment A). Fish from these treatments were sampled at regular intervals throughout the period of sexual differentiation and gonadogenesis to assess for effects on sexual development, as described below.

In a second experiment (experiment B) roach were exposed to the same range of EE2 concentrations until 120 dph, and then subsequently transferred into clean water and depurated for 400 days, when assessments were made on the status of their sexual development (described below). After the depuration period some fish from experiment B (at 518 dph) were then re-exposed to one concentration of EE2 at a nominal concentration of 5 ng L^{-1} for a period of up to 10 days.

Duplicate water control tanks were run under the same conditions, without the addition of EE2. Dilution water and EE2 dosing stock solution were both delivered to the tanks using peristaltic pumps. Water flow rates and EE2 dosing rates were monitored regularly during the course of the exposure and the EE2 dosing stock solution was renewed regularly (after a maximum interval of 7 days).

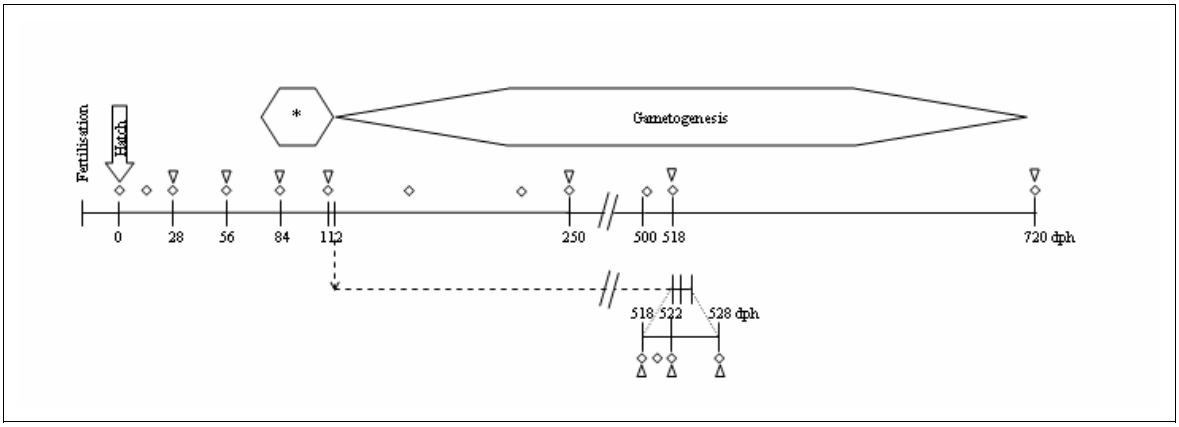

Figure 2.1 Experimental design of the long-term exposure of roach to EE2. Roach were exposed to three environmental concentrations of EE2 (nominal concentrations of 0.1, 1.0 and 10 ng L^{-1}) from fertilisation up to 720 dph (solid line). After gonadal sex differentiation at 120 dph, some fish from each treatment were transferred to clean water and allowed to depurate for 400 days (dashed line) . Some of these fish were then re-exposed to a single concentration of EE2 for 10 days. The symbols used represent: (*) gonadal sex differentiation, (▽) biological sampling, (◇) water collection.

2.3 Determination of EE2 exposure concentrations

Water samples were collected regularly from each tank to measure the concentrations of EE2. After the addition of 5% (v/v) methanol, the water samples were extracted onto preconditioned solid phase extraction columns (Sep-Pack C18 cartridges, Waters Ltd, Elstree, Hertsfordshire, UK) following the manufacturer's protocol. The extracts were subsequently eluted from the column with 100% methanol into solvent-cleaned glass vials and stored at 4 °C until required. At the time of analysis, the methanol was evaporated at 45 °C under a stream of nitrogen and the extracts were re-suspended in radioimmunoassay (RIA) buffer. A RIA was used to verify EE2 concentrations in water. The procedure was identical to that used for RIAs reported for other steroids by the CEFAS laboratory (Scott et al. 1982, Ellis et al. 2004). A polyclonal antiserum to EE2 was raised by injection of ethinyloestradiol 6-carboxymethyloxime: bovine serum albumin (Steraloids Inc. Ltd, London, UK) into rabbits. Tritiated EE2 was purchased from Perkin Elmer Life Sciences Inc., Boston, MA, USA. The antiserum had negligible (< 0.5%) cross-reaction with either oestradiol-17β or oestrone. The overall rate of recovery of EE2 during the extraction process is reported to be between 60 and 65% (Scott, personal communication).

2.4 Biological sampling

In experiment A (continuous EE2 exposure for 720 days) fish were sampled at random from the exposure populations at 56, 84, 112, 250, 518 and 720 dph and analysed for sexual development and VTG induction (see below). In experiment B, fish exposed to EE2 during early life and then maintained in clean water were sampled at 518 dph, and then at 522 and 528 dph (after 4 and 10 days of re-exposure to a single concentration of EE2).

In experiment A, 80 fish (40 from each duplicate tank) were sacrificed by terminal anaesthesia with benzocaine as approved by the UK Home Office (Animals (Scientific Procedures) Act 1986) after 56, 84 and 112 dph and analysed for gonadal development (via histology) and vitellogenin induction. At the later life stages (from 250 dph onwards) 12–60 fish were sampled from each treatment (fish were now large enough to measure both gonad development, via histology, and plasma VTG in the same fish). At the final sampling point (720 dph), the gender was determined macroscopically and the wet weight of the dissected gonad was determined before sections of the gonad were fixed for histological analysis. Determination of the gonadal wet weight allowed us to calculate the gonadosomatic index (GSI) by expressing the gonad weight as a percentage of the total body weight minus the gonad weight.

In experiment B, 72 fish were sampled at 518 dph (18 from each treatment group from the early life exposure) and 72 fish each at 522 and 528 dph (after 4 and 10 days of re-challenge to EE2; 18 from each treatment group from the early life exposure) and analysed for gonadal development (via histology) and vitellogenin induction.

At all sampling points in both experiments, total wet body weight and caudal peduncle length were determined for each fish and the condition factor calculated by expressing the cube of the fish length as a percentage of the body weight.

Samples collected for histological analysis of the gonad were fixed in Bouin's fixative. Up to 112 dph fish were fixed *in toto*, whereas from 250 dph onwards the gonads were excised, each gonad was then divided in half and one half of each gonad fixed. After 4–24 hours of fixation, samples were transferred into 70% industrial methylated spirits (IMS) and stored until processed for sectioning. Specimens fixed for histological analysis were dehydrated and embedded in paraffin wax (Sigma-Aldrich). Sections for all fish were cut to 3–5 µm, collected on glass slides and stained with haematoxylin-eosin. Stained samples were mounted with Histomount (National Diagnostics) and analysed by light microscopy for the presence of primordial germ cells, for gonadal duct formation, and in later life stages (250 dph onwards) for staging of the sex cells.

For VTG analysis, fish up to 112 dph were snap-frozen in liquid nitrogen and stored at –80 °C until analysis. Whole body homogenates were prepared and assayed according to Tyler et al. (1999) with the exception that ice-cold PBS buffer was used for the homogenisation. From 250 dph onwards, fish were analysed for their blood content of VTG. Blood was collected from the caudal sinus using heparinised haematocrit tubes and stored at –20 °C until analysis. VTG was determined using an ELISA originally established for common carp (*Cyprinus carpio*) VTG which has subsequently been validated for measuring VTG in roach (Tyler et al. 1996, 1999).

2.5 Statistical analysis

Data were analysed for normality and homogeneity of variances and unless stated differently, statistical differences were determined by one-way ANOVA on ranks followed by Dunn's multiple comparison procedures. A probability level of $P < 0.05$ was considered to be statistically significant. Statistical analyses were carried out using SigmaStat® 3.01 (SPSS).

3 Results

3.1 Experiment A – Continuous EE2 exposure

3.1.1 Water chemistry

EE2 was not detected in control samples. The EE2 concentration in the nominal 0.1 ng L^{-1} treatment was below the detection limit (40 pg L^{-1} for the assay in this study) of the radioimmunoassay. The mean measured exposure concentrations of EE2 were 0.3 ± 0.1 and 4.0 ± 0.3 ng L^{-1} in the 1.0 and 10 ng L^{-1} test tanks, respectively.

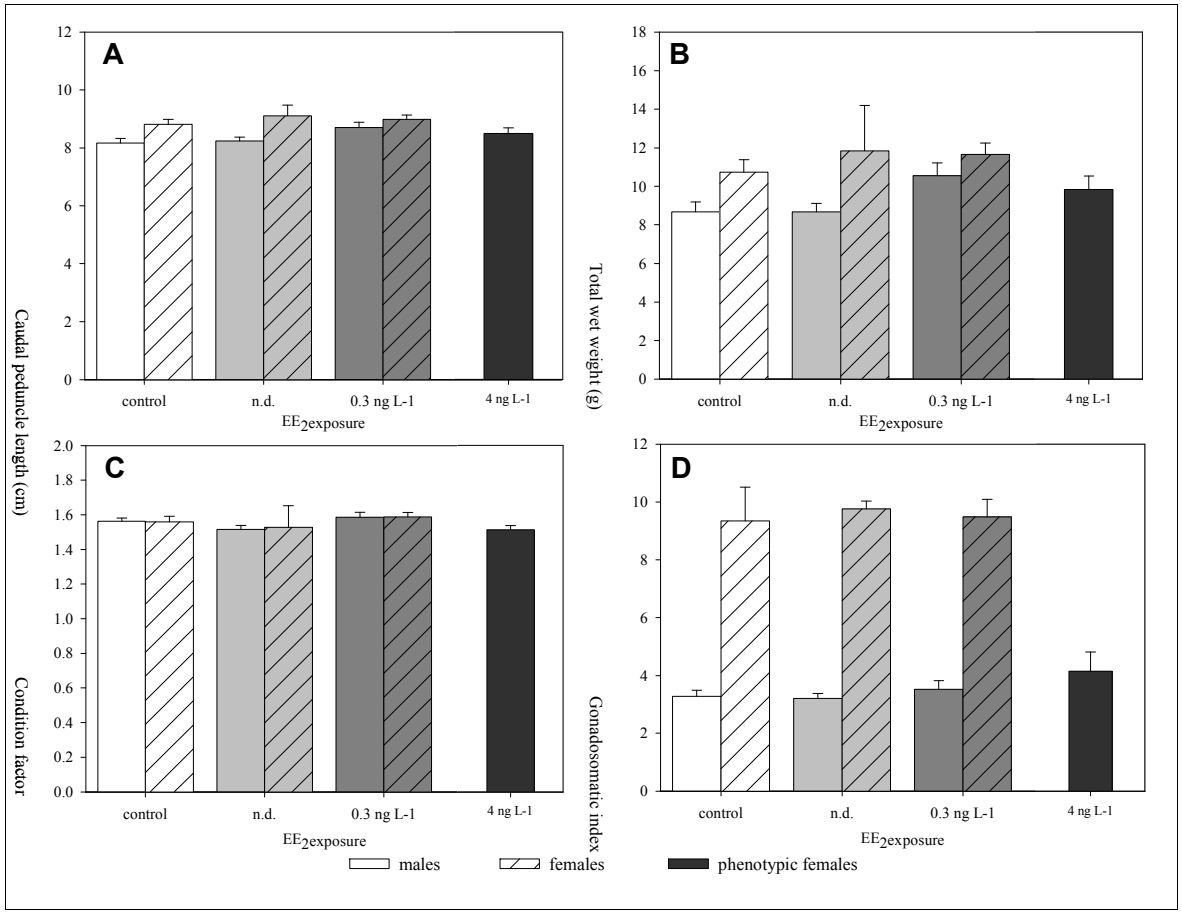

Figure 3.1 Morphometric measurements of male and female roach (sex confirmed by gonadal histology) exposed to EE2 (measured concentrations: non-detectable, 0.3 and 4 ng EE2 L^{-1}) for two years starting from fertilisation of the egg. (A) Length, (B) weight, (C) condition factor and (D) gonadosomatic index (GSI). Each column represents mean ± SEM. No significant differences were observed within genders ($P < 0.05$, Dunn's test). Fish exposed to 4 ng L^{-1} EE2 were excluded from statistical analysis since all fish were phenotypic females (i.e. they included both true females and sex reversed males).

3.1.2 Condition, somatic and gonadal growth

No mortalities occurred during the course of the experiment. There were no concentration-related effects of EE2 on length, weight or condition factor in male or female roach exposed to EE2 (Figure 3.1A–C, growth data are presented only for the fish sampled at the termination of the experiment, at 720 dph). The gonadosomatic index was also determined for all fish sampled at the end of the long-term exposure. At the lower two EE2 treatments the test chemical had no significant effects on the GSI of male and female roach compared to control fish (Figure 3.1D). All fish treated with 4 ng L^{-1} EE2 had an ovarian morphology (see below) and thus it was not possible to assign fish as females or (sex-reversed) males and consequently these fish were excluded from the analyses for effects of EE2 on growth.

3.1.3 Sexual development in control fish

The timing of sex cell differentiation and progression of gonadal development in the control fish (assessed by histology) in relation to age is presented in Figures 3.2–3.4. At 56 dph all of the roach sampled had undifferentiated gonads, and they were often still closely associated with the liver (Figure 3.2A). At 84 dph the gonads consisted of a few primordial germ cells covered by a thin layer of somatic cells (Figure 3.2B). Some fish at this life stage could be classified definitively as females and a few, less definitively, as presumptive males, with the remainder still undifferentiated. The female gonad was characterised by a large elongate structure with two points of attachment to the mesentery/peritoneal wall forming the ovarian cavity (female reproductive duct). These presumptive ovaries contained somatic cells on the periphery of the gonad and germ cells nearer to the centre (Figure 3.2C). The male gonad was distinguishable by a smaller and more oval structure with a single point of attachment to the peritoneal wall. These presumptive testes contained somatic cells dispersed among the germ cells (Figure 3.2D). At 112 dph, many more females could be distinguished with the ovaries containing sex cells of all stages from germ cells up to the Balbiani body stage (Figure 3.2G). Males were still difficult to definitively distinguish and only a few more presumptive testes were classified (Figure 3.2F).

From 250 dph onwards, histological analysis was carried out on dissected gonads and therefore the shape of the gonad and the number of points of attachment of the gonad to the peritoneal wall could no longer be used as a criterion to distinguish between genders. At these later sampling points, gonadal development had progressed further and gender could be determined by the sex cells alone. At 250 dph, the ovaries contained all sex cells up to primary oocytes at the Balbiani body stage (Figure 3.3B). Males at 250 dph were now clearly distinguishable and the testes observed had well-defined lobules with all stages of sex cells up to, and including spermatogonia A and spermatogonia B (Figure 3.3A). By 518 dph the ovaries contained predominantly primary oocytes, but also included early vitellogenic oocytes (Figure 3.3F). Male gonads now contained spermatocytes (Figure 3.3E). At 720 dph all females contained vitellogenic stage oocytes, while all males contained spermatocytes, with one male containing spermatozoa (Figure 3.3E).

3.1.4 Effects of EE2 on sexual development

Gender of the fish analysed at the early life stages (at 84 and 112 dph) was determined by means of the shape of the gonad and the presence of presumptive male and female

reproductive ducts (points of attachment to the peritoneal wall, as described for the controls) whereas from 250 dph onwards sex cells only were used for sex determination.

84 dph – Histological analyses of the gonads of fish exposed from embryos until 84 dph to nominal 0.1 ng EE2 L^{-1} (measured, non-detected) were found not to have observable difference in their gonadal status compared with control fish (both groups containing approximately 20% (n = 32) discernable females, fewer discernable males and the remainder undifferentiated). Indeed no observable differences in sexual development and gonadal status were found between these two treatment groups throughout the 720-day exposure. For fish exposed to 0.3 ng EE2 L^{-1} (measured concentration), at 84 dph there was a higher number of discernable female gonads (37%, n = 20) and no discernable males. In contrast, 95% (n = 20) of the fish exposed to the highest dose of EE2 (4.0 ng L^{-1}) were classified as presumptive females (which would have included feminised males), with all gonads showing a characteristic female morphology (Figure 3.2E).

112 dph – Histological analyses of the gonads of control fish at 112 dph showed that all of the fish had differentiated into either males or females and that there was a male bias in the population at this age (62% males versus 38% female; n = 26). In contrast, fish exposed to 0.3 ng EE2 L^{-1} showed a female bias with 64% females versus 36% males (n = 11). In the fish exposed to 4.0 ng EE2 L^{-1}, 30% could be classified as female (based on the presence of germ cells) and the remaining 70% all had a female-like morphology but were not advanced enough to distinguish them definitively as females (n = 10) (Figure 3.2H–I).

250 dph – At this time point there was again a male bias in the control population (60% males versus 40% females; n = 53). In contrast, fish exposed to 0.3 ng EE2 L^{-1} had a similar proportion of males and females (49% and 51%; n = 35). All fish that were exposed to 4.0 ng EE2 L^{-1} were female (n = 22). Gonads of fish exposed to non-detectable levels of EE2 or to 0.3 ng L^{-1} EE2 contained sex cells of the same developmental stage as control fish. Most fish sampled from the 4 ng L^{-1} EE2 exposure had female sex cells up to and including primary oocytes at the Balbiani body stage, comparable to the control fish at this time point. A few fish from this treatment group, however, had less developed gonads containing oogonia and perinuclear primary oocytes only (Figure 3.3C–D).

518 dph – In the control fish the male bias at this age persisted with 59% males versus 41% females (n = 17). At the highest EE2 dose (4.0 ng EE2 L^{-1}) all fish were female (n = 12). However, the ovaries of fish exposed to 4.0 ng EE2 L^{-1} contained primary oocytes only and therefore were not as advanced as female fish in the control population. Fish exposed to 0.3 ng EE2 L^{-1} were not sampled at this time point (Figure 3.3G–H).

720 dph – In the control fish sampled at this time point there was again a male bias with 69% males versus 31% females (n = 26). In contrast, fish exposed to 0.3 ng L^{-1} EE2 showed a bias towards females with 42% males and 58% females (n = 24). As observed in the previous life stages, all fish sampled from the highest treatment group (4.0 ng EE2 L^{-1}) were phenotypic females (n = 24). Again, ovaries and testes from fish exposed to the water control, non-detectable levels of EE2 and 0.3 ng L^{-1} EE2 did not differ in their stages of sex cells present. However, two males, one derived from the exposure to non-detectable levels of EE2 and one to 0.3 ng L^{-1} EE2, showed signs of sex cell disruption, that is they contained intersex gonads with only a few primary oocytes within the testicular tissue (no information is available on oviduct formation as the histological analyses was performed on dissected gonads). Gonads of fish exposed to 4 ng L^{-1} EE2, in contrast, varied in the stages of oocytes present. Ovaries from 58% of the fish analysed from this treatment group were comparable to the control females and contained all stages of sex cells, including vitellogenic oocytes, and 42% contained

predominantly primary oocytes with just a few more advanced female sex cells (Figure 3.4).

3.1.5 Vitellogenin induction

At early life stages (until 112 dph), VTG contents were determined in whole body homogenates. Analysis of whole body VTG at 56, 84 and 112 dph showed that there was a significant induction of VTG at all life stages analysed, but only at the highest exposure concentration of EE2 (4 ng L^{-1}; Figure 3.5A). At 56 dph, this treatment induced VTG concentrations 9-fold higher compared with control fish. The induction increased with longevity of exposure (and age) and was 12-fold higher at 84 dph and 53-fold at 112 dph. In the remaining treatments, the VTG concentrations were not elevated significantly relative to the VTG concentration of the corresponding control fish.

From 250 dph onwards, blood/plasma VTG concentrations were measured in control and exposed fish. The results for control and 4 ng EE2 L^{-1}-exposed roach at 250 and 518 dph are shown in Figure 3.5B. At 250 dph there were no significant differences in VTG concentrations between control males and control females. In contrast at 518 dph, the mean concentration of VTG in the plasma of control male roach was 20.3 ± 6.0 ng mL^{-1} compared with 5416 ± 4875 ng mL^{-1} in females. Due to the extremely high variability in VTG content in females at this life stage, this difference was not statistically significant. Significant increases in VTG concentrations compared with the control fish were observed in fish exposed to 4 ng EE2 L^{-1}. Discrimination between genders in the 4 ng EE2 L^{-1} exposed fish was, however, not possible as all were phenotypic females (gonadal histology). Exposure to 4 ng L^{-1} for 250 days resulted in 472- and 489-fold inductions of VTG compared with control male and female roach, respectively. At 518 dph, VTG induction was 103,581-fold and 388-fold, respectively.

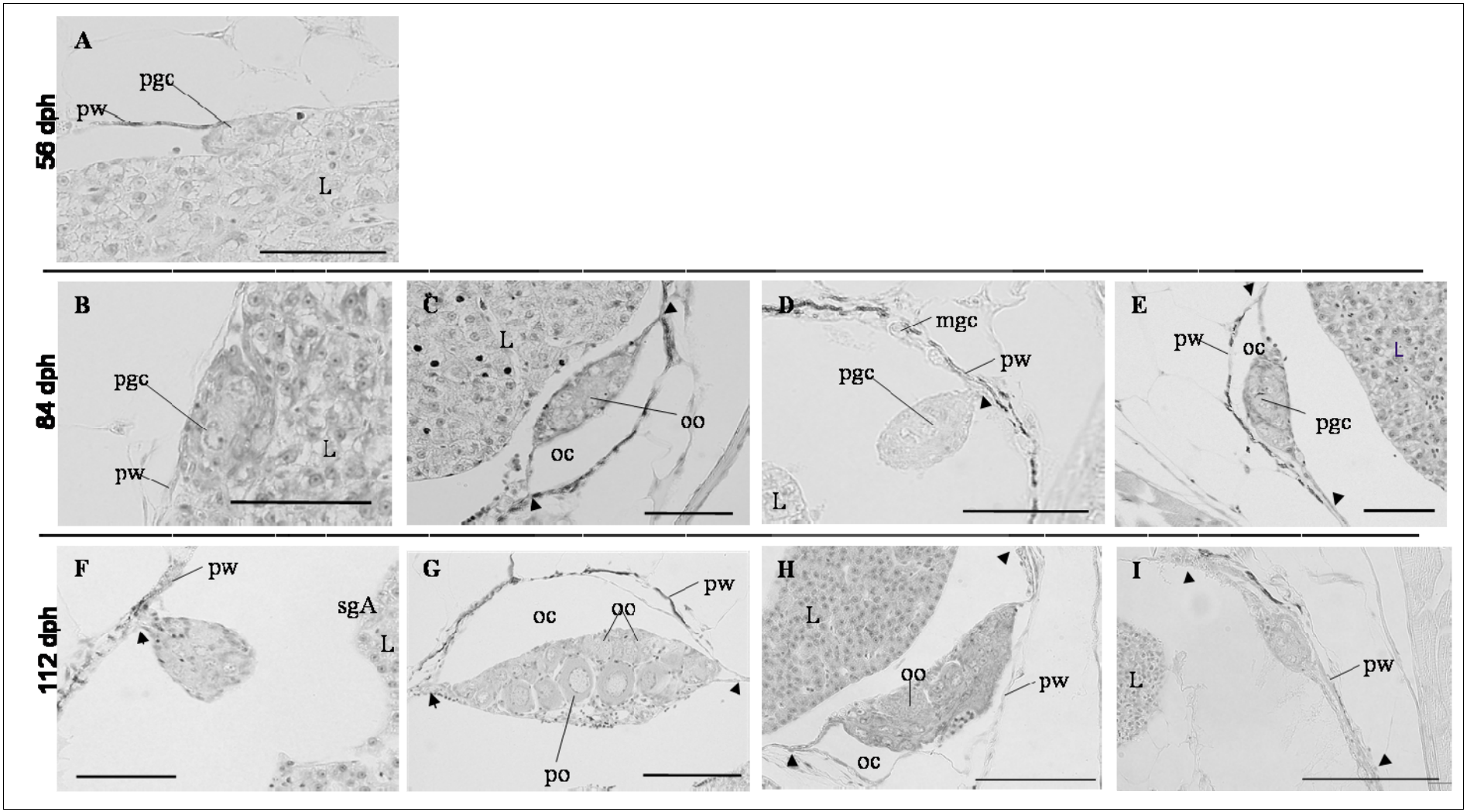

Figure 3.2 Transverse sections of roach gonads showing the normal progression of gonadal sex differentiation and development in the study population and the effects of 17α-ethinyloestradiol treatment. Images show undifferentiated gonads at 56 days post hatch (dph; A) and 84 dph (B), presumptive female gonad at 84 dph (C), presumptive male gonad at 84 dph (D), presumptive male gonad at 112 dph (F), more developed ovaries at 112 dph (G) and gonads showing female-like features after exposure to 4 ng L^{-1} EE2 at 84 dph (E) and at 112 dph (H and I). L, liver; mgc, migrating germ cell; oc, ovarian cavity; oo, nest of oogonia; pgc, primordial germ cell; po, primary oocyte; pw, peritoneal wall; sgA, spermatogonia A; ►, points of attachment to pw. Bar: 50 μm.

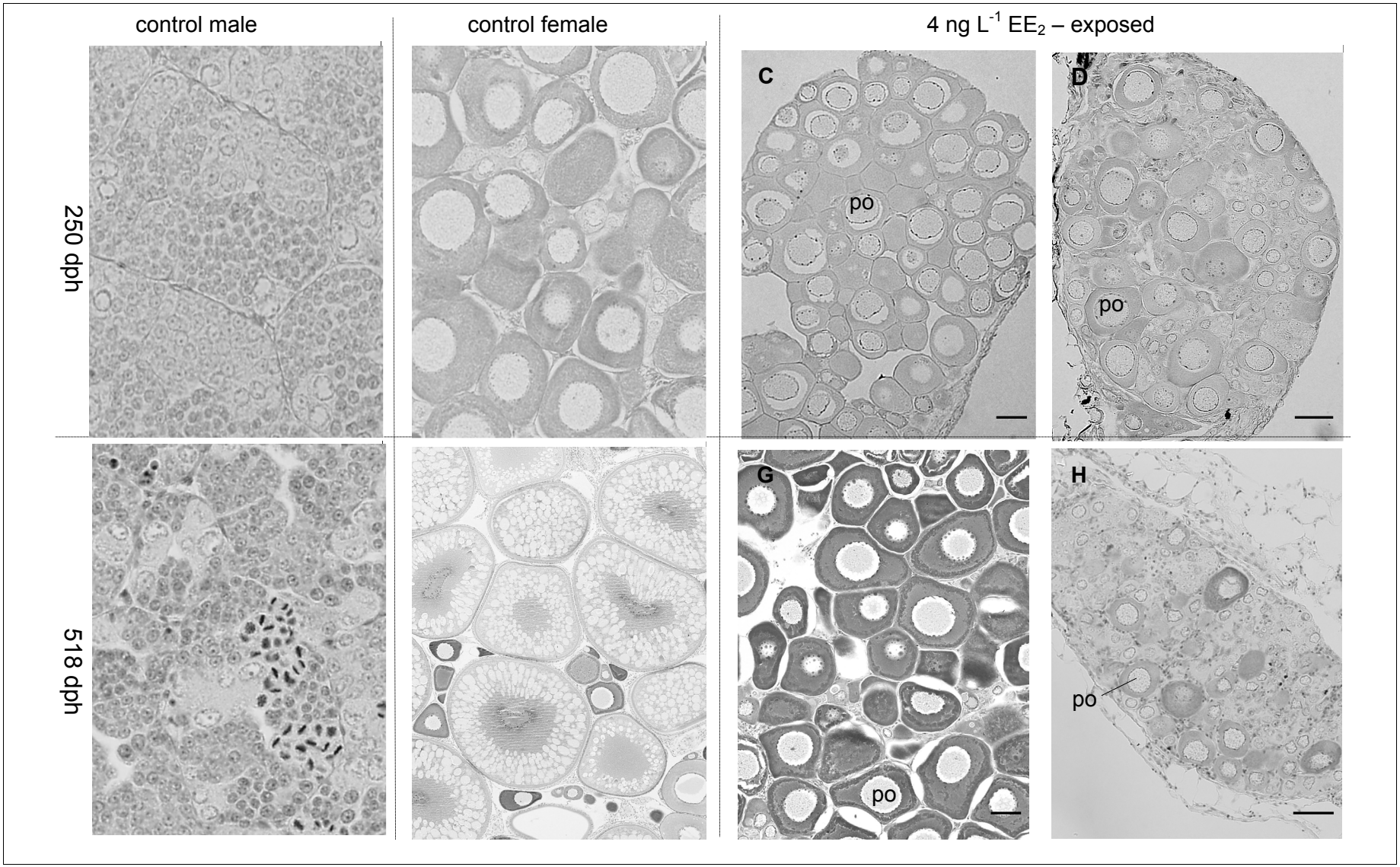

Figure 3.3 Histological sections of dissected roach gonads at 250 and 518 dph showing control testis (A and E), control ovary (B and F) and gonads of phenotypic females after exposure to 4 ng L^{-1} EE2 (C–D and G–H). po, primary oocyte; so, secondary oocyte; vo, vitellogenic oocyte; sgA, spermatogonia A; sgB, spermatogonia B; sy, spermatocytes; Bars 50 μm.

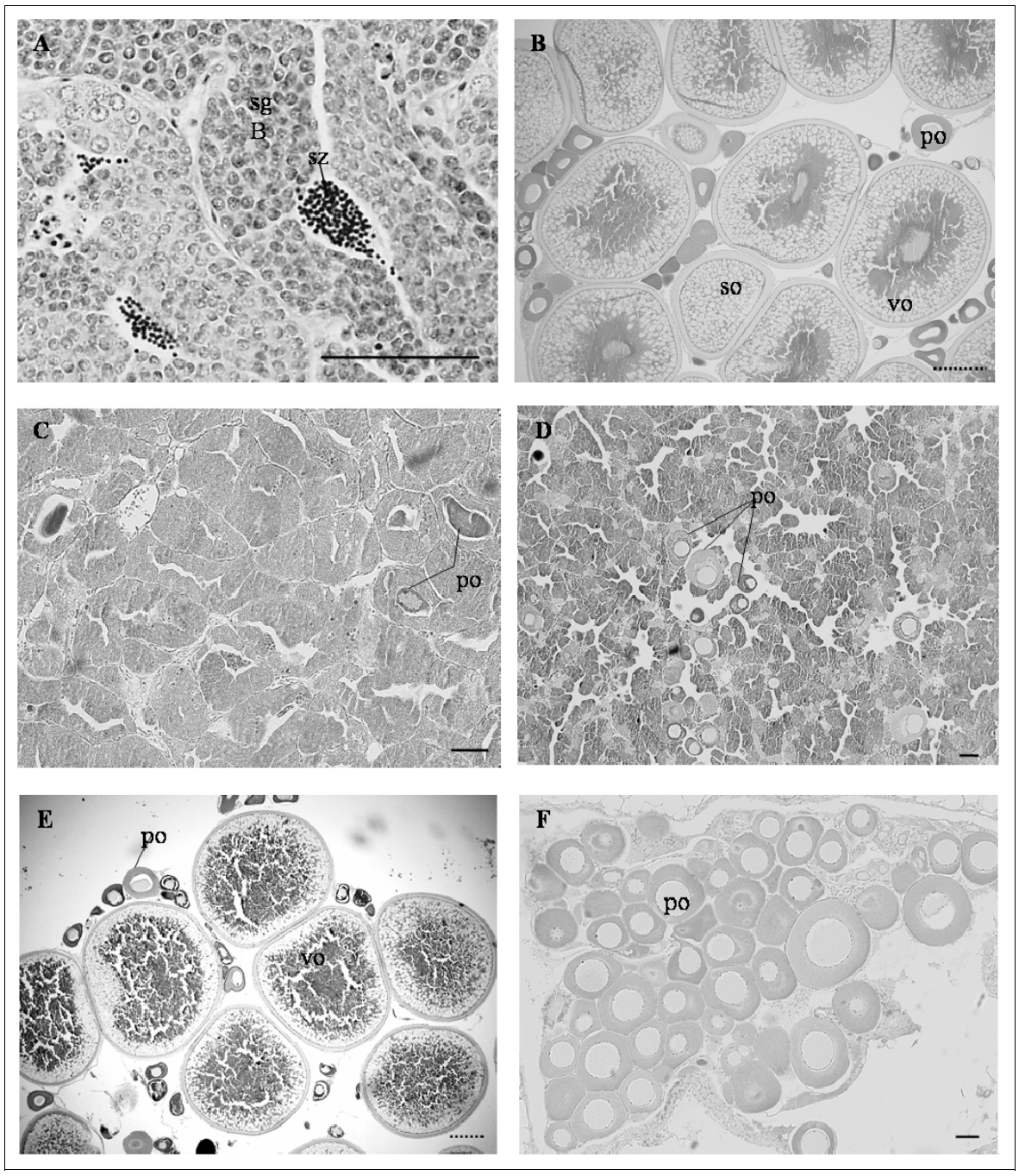

Figure 3.4 Histological sections of dissected roach gonads at 720 dph showing control testis (A), control ovary (B), intersex gonads of fish exposed to non-detectable levels of EE2 (C) and 0.3 ng L^{-1} EE2 (D), and gonads of phenotypic females after exposure to 4 ng L^{-1} EE2 (E and F). po, primary oocyte; so, secondary oocyte; vo, vitellogenic oocyte; sgB, spermatogonia B; sz, spermatozoa; solid bars 50 μm, dotted bars 250 μm.

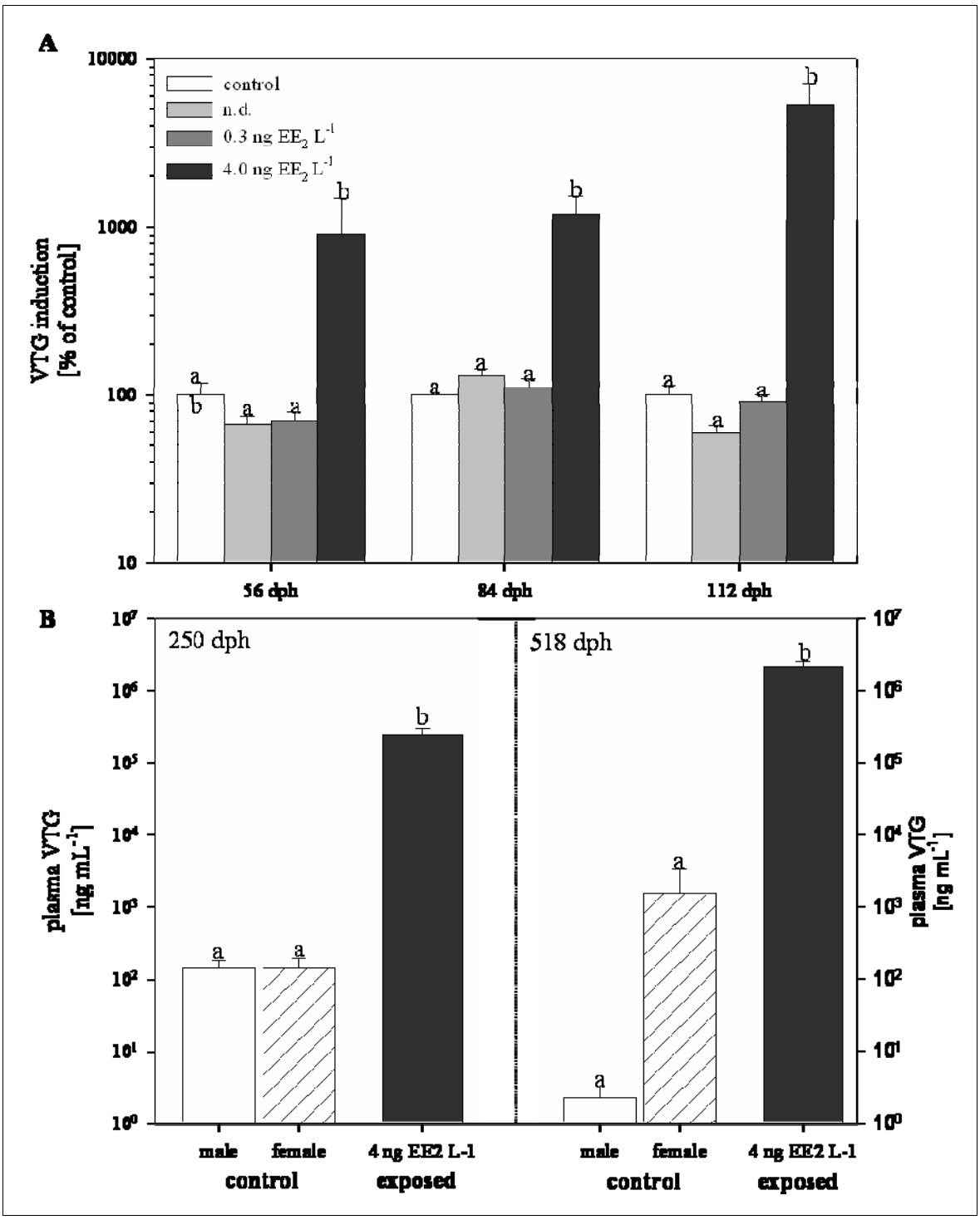

Figure 3.5 Vitellogenin concentrations in whole body homogenates of roach exposed to EE2 until 56, 84 and 112 dph (A) and in blood/plasma of roach at 250 dph and 518 dph after exposure to water control and 4 ng L^{-1} EE2 (B). Each column represents mean ± SEM. Different letters above bars indicate significant difference ($P < 0.05$, Dunn's test).

3.2 Experiment B – Depuration and re-challenging experiment

3.2.1 Water chemistry

No EE2 was detected in any of the fish maintenance tanks during the depuration phase or immediately prior to the start of the re-exposure to EE2 at 518 dph. During the course of the 10-day EE2 re-exposure study water samples were taken three times from each experimental tank (at days 2, 4 and 10) and the average EE2 exposure concentration was 2.3 ± 0.2 ng L^{-1} EE2 (n = 11, one sample was lost during processing), with no significant differences between tanks.

3.2.2 Effects of early life exposure to EE2 (with subsequent depuration) on sexual development

At 518 dph, following a period of 400 days of depuration, no obvious effects were observed on gonads in roach that had been exposed to the two lower levels of EE2 during the period of gonadal sex differentiation. All gonads contained germ cells at the same stages of development compared with the control fish from the continuous exposure experiment (A) (i.e. up to and including early vitellogenic oocytes in females and containing spermatocytes in males). Fish analysed from the group exposed to 4 ng EE2 L^{-1} during early life did not differ from the control group in terms of stages of germ cells present (Figure 3.6A–B). However, in this treatment group intersex fish occurred (germ cell disruption, Figure 3.6C). At 518 dph, four out of eleven (36%) males had intersex gonads, indicating that the effects of EE2 on gonadal development persisted even after prolonged periods of depuration. The gonads of intersex roach were characterised by the presence of a few primary oocytes, in most cases these were scattered throughout gonad sections of the testicular tissue in a multifocal arrangement. The primary oocytes occurred singly or in clusters. Since the gonads of these fish were dissected out prior to histological analysis it was not possible to observe any malformations of the reproductive ducts.

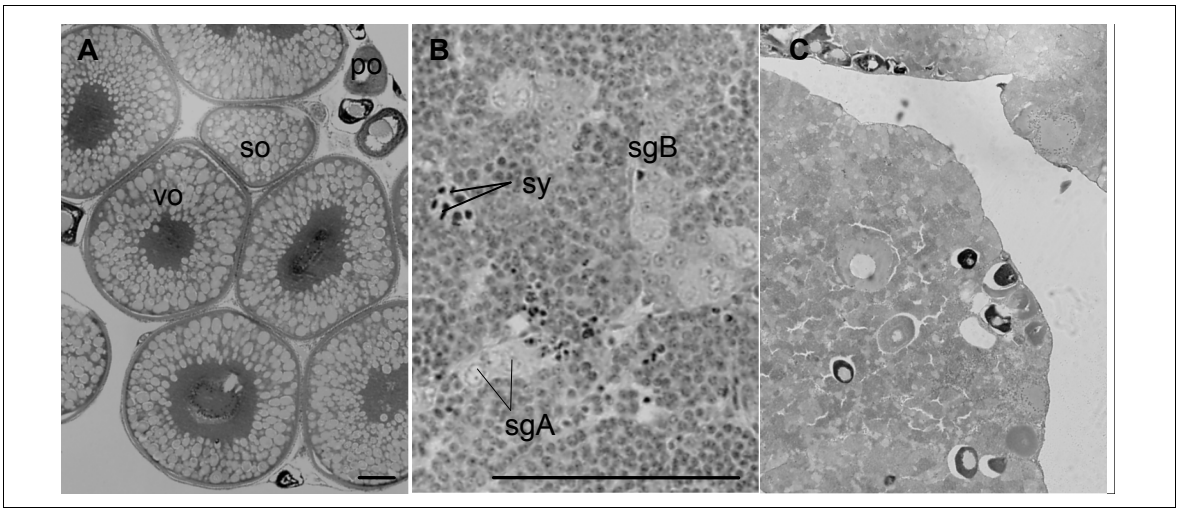

Figure 3.6 Histological sections through ovary (A), testis (B) and an intersex testis (C) from roach at 518 dph, exposed to 4 ng L^{-1} EE2 during early life (until 120 dph) and then kept in clean water for 400 days. po, primary oocyte; so, secondary oocyte; vo,

vitellogenic oocyte; sgA, spermatogonia A; sgB, spermatogonia B; sy, spermatocytes; Bars 50 μm.

3.2.3 Vitellogenin induction

As previously shown, plasma VTG levels were significantly induced in fish exposed continuously to 4 ng L^{-1} EE2 for 518 dph compared to control male and female roach (Figures 3.4B and 3.7A). At the same time point, plasma VTG levels were measured in depurated roach prior to re-exposure to EE2. There were no significant differences between the concentrations of VTG in the plasma of male and female fish exposed to 4 ng L^{-1} EE2 during early life and the control fish (Figure 3.7B).

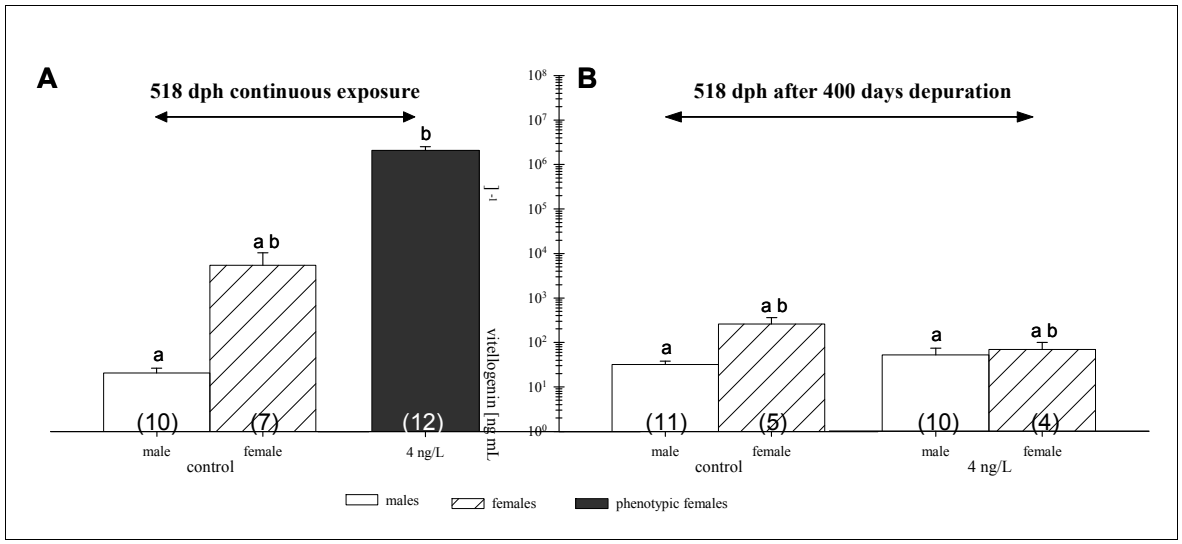

Figure 3.7 Plasma vitellogenin concentrations in maturing male and female roach at 518 dph exposed to water control or 4 ng L^{-1} EE2 continuously from fertilisation (A) and during early life until 120 dph followed by a depuration phase for 400 days (B). Each column represents mean ± SEM and the numbers in brackets indicate the number of samples analysed. Different letters above bars indicate significant difference ($P < 0.05$, Dunn's test).

The EE2 re-exposure part of this experiment was carried out to determine whether early life exposure to increasing environmental concentrations of oestrogen resulted in a sensitisation or de-sensitisation to oestrogen exposure in later life. Roach exposed to EE2 during gonadal sex differentiation, then kept in clean water, and subsequently re-challenged with EE2 in later life, showed an enhanced vitellogenic response. This response was more pronounced for fish exposed to progressively higher EE2 exposure concentration during early life (Figure 3.8). This response was observed after 4 days of re-challenging and the pattern persisted at 10 days, but with further increases in the levels of VTG. The observed trend appeared to be more pronounced in females compared with males. Prior to re-challenging, plasma VTG levels were 2932.2 ± 1805.8 ng mL^{-1} in females and 33.5 ± 6.6 ng mL^{-1} in males.

In females a 4-day re-exposure to 2.3 ± 0.2 ng L^{-1} EE2 resulted in a 1.6-fold, 3.8-fold and 7.5-fold induction of plasma VTG in fish exposed to non-detectable levels of EE2, 0.3 ng L^{-1} EE2 and 4.0 ng L^{-1} EE2, respectively, during early life. Females maintained

in clean water during early life (control) had slightly lower levels of VTG compared to the start of the experiment. After 10 days re-exposure to EE2, these effects were more pronounced and the changes in induction of VTG were 36-fold, 37-fold, 65-fold and 120-fold in fish from the early life control, non-detectable EE2, 0.3 and 4.0 ng L^{-1} EE2 treatments, respectively. The differences in plasma VTG levels were significantly elevated after 10 days compared to the start of the experiment , whereas VTG levels at day 4 were not significantly different from either the start of the experiment or day 10.

In males, 4 days of re-exposure to 2.3 ± 0.2 ng L^{-1} EE2 resulted in 58-fold, 63-fold and 111-fold induction of plasma VTG in fish exposed to water control, non-detectable levels of EE2 and 0.3 ng L^{-1} EE2, respectively, during early life. In males exposed to 4.0 ng L^{-1} EE2 during early life the VTG response was lower after 4 days compared to the other three treatments (29-fold induction). After 10 days, higher fold-changes were observed in males compared with females. VTG was 1212-fold, 1116-fold, 4016-fold and 4264-fold higher in fish from the early life control, non-detectable EE2, 0.3 and 4.0 ng L^{-1} EE2 treatments, respectively. With the exception of males at day 4 in the re-exposure that had been exposed to 4.0 ng L^{-1} EE2 during early life, the changes in plasma VTG were significantly elevated in all re-exposure groups after 4 and 10 days of oestrogenic re-challenge. The VTG levels after day 4 did not differ statistically from those on day 10 for the re-exposure to EE2, but this was as a reflection of the high variation in the responses between the individuals in each group.

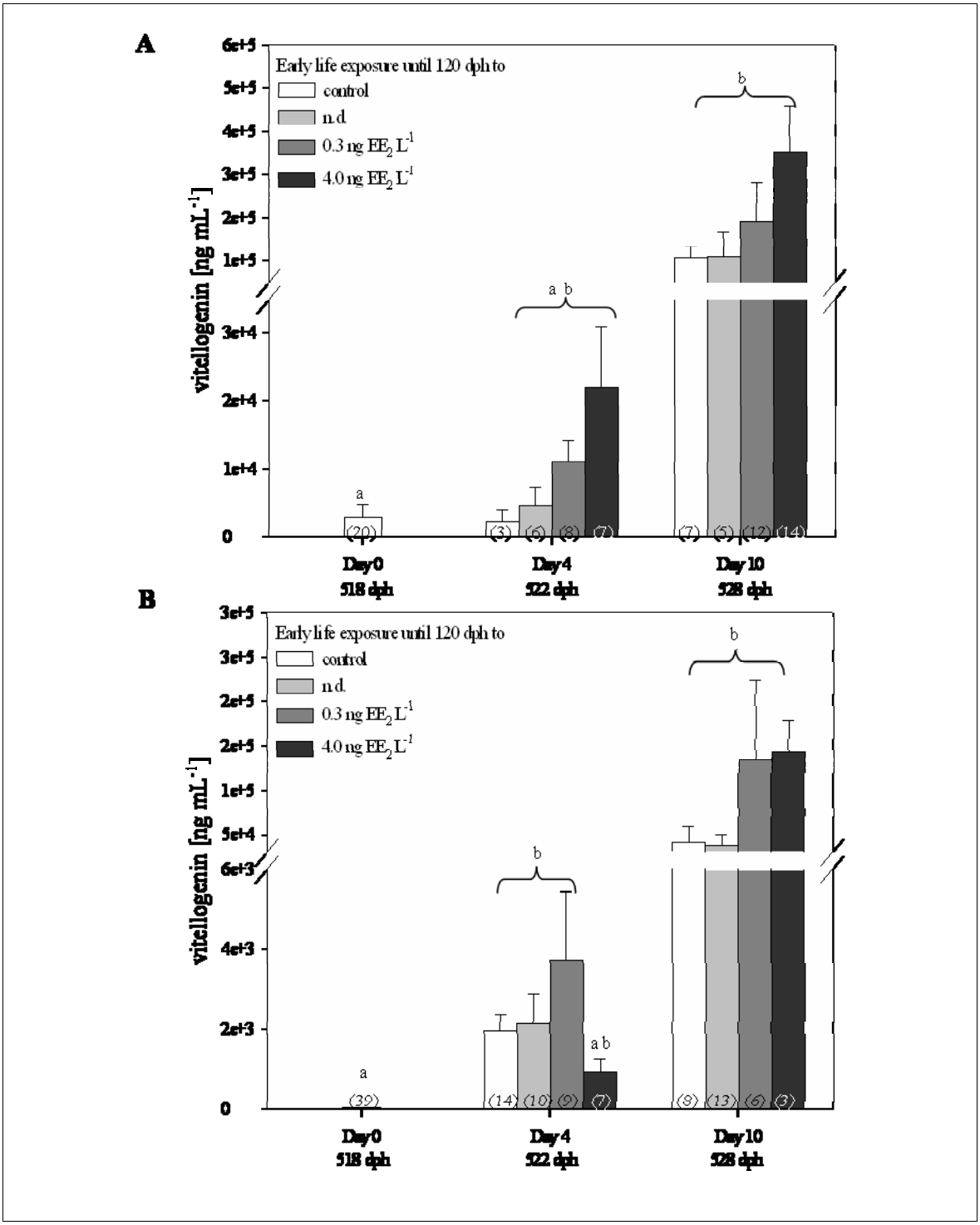

Figure 3.8 Plasma vitellogenin concentrations in female (A) and male (B) roach exposed to 2.3 ng L^{-1} EE2 for 10 days following early life exposure to different concentrations of EE2 until 120 dph and a subsequent depuration period for 400 days. Each column represents mean ± SEM and the numbers in brackets indicate the number of samples analysed. Different letters above bars indicate significant difference ($P < 0.05$, Dunn's test).

4 Discussion

Histological analysis of gonadal development and the measurement of VTG in fish have been key endpoints in establishing the fact that effluent discharges from WwTWs are oestrogenic to fish and induce sexual disruption (Rodgers-Gray et al. 2001, Liney et al. 2005), and in identifying sexual disruption in wild fish populations in UK rivers (Jobling et al. 1998, 2006). Histology of the gonad has also been used to determine the capacity of individual environmental oestrogens to induce sexual disruption in fish in the laboratory (e.g. van Aerle et al. 2002, Brion et al. 2004).

The most comprehensive details on sexual disruption in wild fish have come from studies on roach living in UK rivers. Nolan et al. (2001) described the intersex condition in wild roach. Furthermore, recently a detailed histological description of the ontogeny of sexual development in the roach has provided the basic information against which the effects of EDCs (including EE2) on gonadal development can be examined effectively in this species (Environment Agency 2007).

The histological analyses conducted in this study confirmed that exposure of roach to environmental concentrations of EE2, notably during early life, and encompassing the period of gonadal sex differentiation, induces alterations in the subsequent gonadal phenotype, including disruptions of the gonadal duct and altered germ cell development. At the highest exposure concentration studied (4.0 ng EE2 L^{-1}) complete feminisation of the roach population occurred. At this exposure concentration, 95% of the fish exposed continuously from embryos to 84 dph had feminised ducts and at 250 dph, with continued exposure, all of the fish were phenotypic females (the gonads were all clearly recognisable as ovaries). Other studies have induced similar feminising effects in males of laboratory test species of fish (including VTG induction and feminisation of gonadal ducts) after exposure during early life to similar concentrations of EE2 (van Aerle et al. 2002, Maack and Segner 2004, Fenske et al. 2005). Some of these studies have also induced complete gonadal feminisation of males (Länge et al. 2001, Örn et al. 2003, Fenske et al. 2005).

In roach, the pattern of normal ovarian development is a synchronous development of oocytes. In the ovary in maturing females two 'populations' of developing oocytes occur at any one time: primary oocytes from which the later phases are recruited and a batch of synchronously maturing oocytes which form the season's batch of eggs and are all released in the span of a few days (Scott and Canario 1987, Tyler and Sumpter 1996, Rinchard et al. 1997). At 720 dph, control females showed the normal pattern of ovarian development expected at this stage (i.e. a batch of large vitellogenic oocytes and smaller primary oocytes). In contrast with this pattern of development, after exposure to 4 ng EE2 L^{-1} for 720 dph, the stages of oocytes present between individuals was wide ranging in the 'phenotypic female group'. In this group, the ovaries of 58% of the fish were comparable with ovaries of the control females, but ovaries of the remaining 42% were much less developed and contained predominantly primary oocytes with just a few oocytes at more advanced stages. This finding would suggest that this group of 'all phenotypic females' contained both true genetic females, but also feminised males.

Interestingly, no intersex fish (males with oocytes in testis) were found in the roach exposed continuously to 4.0 ng EE2 L^{-1}. In a study on the fathead minnow, exposure to only 3.2 ng EE2 L^{-1} induced intersex after 56 dph and complete feminisation after 172 dph (Länge et al. 2001). It is possible that in this study on roach a transient intersex condition in the process of the gonadal feminisation of males was missed because of the long interval between the samples taken at 112 dph (before completion of sex differentiation for all fish) and the next sampling time point at 250 dph.

In roach from the low (nominal, 0.1 ng EE2 L^{-1}, but non-detectable measured) and medium (0.3 ng EE2 L^{-1}) treatment groups, neither duct disruption (at 84 and 112 dph) or other signs of gonadal feminisation were observed in later life stages, with the exception at 720 dph (the end of the continuous exposure) when intersex gonads occurred in one male fish from each of these treatment groups. This effect on the gonad at such low EE2 exposure concentrations may be due to the fact that EE2 is known to bioconcentrate in fish (Larsson et al. 1999, Skillman et al. 2006) and over this extensive period of time may have accumulated sufficiently to induce germ cell disruption in these two fish. Alternatively, the intersex condition seen may have occurred naturally in these two fish. A very low incidence of 'natural' intersex, has been reported (less than 0.5%) in roach, even when kept in spring (contaminant-free) water throughout their life (our own unpublished data).

There was no difference in the gonad size (mass) relative to the body weight (GSI) in fish at 720 dph between males in the control, low and medium dose treatments (no males were present in the high dose treatment group) or between females across the same treatments. Interestingly, the GSI of the females from the high dose group were more closely aligned with the GSI of the males across the other treatment groups (a relatively smaller gonad weight compared with 'normal' females). A suppressive effect of EE2 on gonad size has been shown for both male and female fathead minnows, albeit at much higher concentrations than used in the present study on roach (Pawlowski et al. 2004). This finding for the roach suggests that 4 ng EE2 L^{-1} had detrimental effects for reproductive development in females, as well as for the males.

Exposure to the highest concentration of EE2 (4 ng EE2 L^{-1}) induced significant elevations in VTG in juvenile (56, 84 and 112 dph) and maturing (250 and 518 dph) roach compared with controls. This effective concentration for EE2 corresponds well with previous studies on VTG induction in other fish species (Länge et al. 2001, Panter et al. 2002, Fenske et al. 2005), showing a comparable sensitivity of roach to this synthetic steroidal oestrogen. VTG induction was also shown to be a transient response (in the depuration study); however, some fish living in UK rivers are exposed constantly to mixtures of oestrogenic chemicals sufficient to induce VTG synthesis. In these cases there are likely to be health consequences for continuously elevated levels of blood VTG. In contrast to the transient nature of the vitellogenic response, altered sexual development (intersex/germ cell disruption) induced during early life persisted in some fish even after a very long period of depuration (400 days), resulting in a high incidence of intersex males. Therefore, exposure to EE2 during early life has the potential to impact on subsequent reproductive health in adult fish. The intersex condition would also probably have been observed in roach exposed continually to EE2, but no samplings for this group were conducted between 112 and 250 dph.

Interestingly, exposure to EE2 during early life appeared to alter the subsequent responsiveness and sensitivity of roach to oestrogen (EE2) in later life. There was also a sensitised response to EE2 in sub-adult fish when they have been exposed to EE2 in early life and subsequently maintained in clean water (for 400 days). In females there was an enhanced vitellogenic response (on re-challenge to EE2) for fish exposed to the higher concentrations versus lower concentrations of EE2 during early life. All of these findings strongly indicate that multiple exposures to oestrogen, even with considerable time intervals between the successive exposures, can markedly affect the dynamics of the response to an environmental oestrogen such as EE2. These findings clearly have implications for assessing the hazards and health risks associated with exposure to this synthetic steroidal oestrogen. As to why exposure to EE2 during early life changes the subsequent responsiveness of roach to oestrogen in later life is not known, but potentially the molecular mechanisms might include a priming effect, where the sensitivity of oestrogen receptors (ERs) is enhanced and/or leads to an increase in the number of ERs.

The findings presented from the research undertaken in this project further support the contention that EE2 is a significant contributor to the feminised responses seen in wild roach living in the vicinity of effluent discharges from WwTWs in UK rivers and further heighten concern about the hazards associated with discharge of EE2 into UK rivers. EE2 has been measured in effluents from WwTWs at concentrations from non-detectable (< 0.5 ng L^{-1} for effluent samples) up to 42 ng L^{-1} (Desbrow et al. 1998, Larsson et al. 1999, Ternes et al. 1999, Johnson et al. 2000) and in surface waters from non-detectable (< 0.1 ng L^{-1}) up to 5 ng L^{-1} (Aherne and Briggs 1989, Belfroid et al. 1999, Williams et al. 2003) and therefore the present exposure concentrations of up to 4 ng EE2 L^{-1} can be considered as environmentally relevant. Generally, however, roach will experience exposure EE2 concentrations below the highest exposure concentration adopted in this study (4 ng L^{-1}) that caused complete feminisation of the population. When considering the causation of intersex in wild fish living in UK rivers, however, we need to be mindful of the fact that the fish are not exposed to EE2 alone, but rather a cocktail of environmental oestrogens that have been shown to have interactive (additive) effects (Thorpe et al. 2003).

5 Conclusions

- Exposure of roach, *Rutilus rutilus*, during early life to the contraceptive pharmaceutical oestrogen, 17α-ethinyloestradiol (EE2), including environmentally relevant concentrations, alters sexual differentiation and sexual development, inducing feminisation of the reproductive duct and oocytes in the testis in males, and induces the oestrogen biomarker vitellogenin.
- Effects on sexual development induced during early life can persist for extended periods of time (more than 1 year) thus potentially affecting the subsequent reproductive capability of adult fish.
- Long-term exposure of roach to EE2, at a concentration found in some of the more polluted effluents (at 4 ng L^{-1}) can alter gender assignment in roach, inducing all-female populations of fish.
- Exposure to exogenous oestrogen (EE2) during early life can result in an enhanced responsiveness to oestrogen on re-exposure in later life, with the potential for an enhanced disruptive effect on reproduction on re-exposure.
- The findings presented further support the contention that EE2 is a significant contributor to the feminised responses seen in wild roach living in UK rivers.

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List of abbreviations

ANOVA – analysis of variance

dph – days post hatch

EDC – endocrine disrupting chemical

EE2 – 17α-ethinyloestradiol

ELISA – enzyme linked immunosorbant assay

ER – oestrogen receptor

GSI – gonadosomatic index

RIA – radioimmunoassay

VTG – vitellogenin

WwTW – Wastewater treatment works

Glossary

Depuration – The process of depurating or freeing from foreign or impure matter.

Endocrine disruptors – Exogenous substances that interfere with the endocrine system and disrupt the physiologic function of hormones.

Ethinyloestradiol – A synthetic derivative of oestradiol and the oestrogen in almost all contraceptive pills.

Germ cell – A reproductive cell; gamete or cell giving rise to a gamete.

Gonadogenesis – development of the gonad.

Gonochorists – Single sexed species.

Intersex – Condition where both male and female features occur in the same gonad.

Mesogonium – Primordial reproductive duct.

Oestrogen receptor – A nuclear receptor for oestrogens such as oestradiol.

Oocyte – Female ovarian cell in which meiosis occurs to form the egg. Cells undergoing first meiotic division are often termed primary oocytes, after which they become secondary oocytes which undergo the second meiotic division to become mature eggs.

Oviduct, ovarian cavity – female reproductive duct, carries eggs from the ovary to the exterior.

Spermatogonium – Primordial male germ cell, gives rise to spermatocytes.

Undifferentiated – Fish that have not undergone the process of translation of genetic into phenotypic sex.

Vitellogenin (VTG) – A female-specific yolk protein produced in the liver in response to oestrogens

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Rio House
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