CHAPTER 4

THE IMPACT OF 4-NONYLPHENOL ON THE NEUROENDOCRINE REGULATION OF REPRODUCTION IN MATURING FEMALE RAINBOW TROUT

4.1 *INTRODUCTION*

Reproduction in fish, as in all vertebrates, is ultimately controlled by the brain, via hormones from the pituitary gland (the gonadotropins) which control the gonads in both sexes. This axis, the so-called brain-pituitary-gonadal (BPG) axis, is kept in check by two feedback loops. Two groups of hormones, the sex steroid hormones (e.g. androgens and estrogens), and activin/inhibin, secreted from the gonads, act back at the level of the hypothalamus and pituitary gland to regulate synthesis and secretion of the gonadotropins. Relatively little is known in fish about activin and inhibin (but see a recent report by Yam et al, 1999), whereas the roles of the sex steroids are better known, although certainly not fully understood (Dickey and Swanson, 1998). It is now well established that xenoestrogens can mimic at least some of the effects of endogenous estrogens (such as stimulate vitellogenin synthesis) Therefore, they may also be able to mimic other effects, such as exert feedback (and hence affect the higher centres of the BPG axis), and hence disrupt the reproductive axis. This chapter aims to determine if they can indeed act in this manner.

4.1.1 The chosen model xenoestrogen: 4-Nonylphenol

4.1.1.1 Environmental occurrence

The test chemical chosen to address this question was 4-nonylphenol (4-NP). This choice was in part due to the fact that, over recent years, 4-NP has become well established as an 'environmental estrogen'. Another influential factor was that 4-NP is a widely employed industrial chemical which, due to the nature of its use, is commonly found in river systems, and at concentrations which have been shown to induce estrogenic effects in fish (e.g. Jobling et al, 1996).

4-NP is one of a class of industrial chemicals known as the alkylphenols. The structure of a typical alkylphenol is shown in figure 4.1. In the case of 4-NP (also known as para-nonylphenol), the alkyl chain has nine carbons (i.e. it is C_9H_{19}); the '4', or 'para,' refers to the position of the alkyl group relative to the hydroxyl group on the phenol ring.

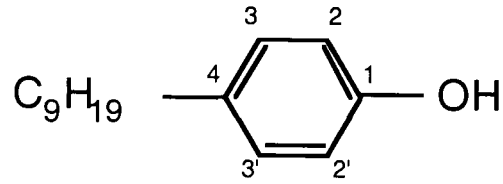


Figure 4.1

The structure of a typical alkylphenol (in this case, 4-nonylphenol)

There are also many different isomers of 4-NP itself (22 isomers were identified by Wheeler et al, 1997), due to branching of the alkyl chain. Alkylphenols as a group have been shown to have differing estrogenic potencies depending on their structural features (Routledge and Sumpter, 1997). Technical grade 4-NP, however, is sold as a mixture of isomers, in which the nonyl group is branched, and which is representative of the mixture employed industrially, and therefore the estrogenic activity of the mixture as a whole, and not the individual isomers, is the critical issue in this project. The estrogenic activity of alkylphenols also depends upon the length of the alkyl chain, whereby optimal estrogenic activity is achieved with an alkyl chain consisting of 8 carbons (octylphenol). However, nonylphenol (and its products) is the most prolifically used alkylphenol, and therefore of most relevance with respect to environmental (and particularly freshwater) pollution problems.

4-Nonylphenol has a variety of industrial uses. For example, it is used as a lubricant additive, a stabiliser in resins, an antioxidant, and a corrosion inhibitor (Heinis et al, 1999), and an emulsifier in pesticide formulations (Ahel and Giger 1985). The majority of 4-NP, however, is used in the production of nonylphenol polyethoxylates (NPEOs), which is achieved by reaction with ethylene oxide. Alkylphenol polyethoxylates (APEOs, the other major players being the octylphenol polyethoxylates) are widely used non-ionic surfactants, the general structure of which is shown in figure 4.2, and are a constituent of both domestic and industrial detergents, from where they find their way into the sewage system and subsequently the aquatic environment. It is for this reason that they are of major concern to fish toxicologists and freshwater ecologists in general.

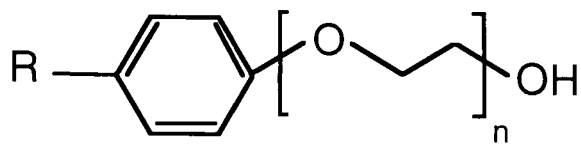


Figure 4.2

The structure of a typical alkylphenol ethoxylate, where n = 5 to 20, and R is typically nonyl (C_9H_{19}) or octyl (C_8H_{17}).

In the US, over 200,000 tonnes of APE surfactants were sold in 1988. Of these, 55% were used in industrial products, 30% in institutional cleaning products, and 15% in domestic formulations and personal care items. In the UK, the picture differs slightly, because domestic usage was phased out in 1976 (DoE, 1992), and hence the majority of end products are industrially based. This is not to say, however, that APEOs and their derivatives are not detected in domestic wastewaters in the UK, although it is not clear from where they originate. It has been suggested that it may be from car washes or laundries (ENDS, 1999b).

APEOs themselves are biodegraded during the wastewater treatment process. The accepted degradation pathway is depicted in figure 4.3, and involves the progressive microbial transformation of alkylphenol polyethoxylates (where n is usually 3 to 20), to alkylphenol mono- or diethoxylates (n =1 or 2, respectively). These metabolites are less water soluble than the parent compounds, due to the shortened ethoxy chain, and therefore can be more easily sorbed to lipophilic particles in the effluent, and less easily biodegraded. Alkylphenol carboxylic acids (APECs) are also a by-product of APEO metabolism during aerobic sewage treatment. During anaerobic sludge treatment, however, degradation of these short-chain APEOs occurs, resulting in the accumulation of alkylphenols (Giger et al, 1984). Alkylphenols are even more lipophilic than the mono- and diethoxylates, and are therefore more persistent in the environment.

Although much of the 4-NP accumulated in sewage sludge is disposed of with the sludge itself, for example through application to farm land, or incineration, there is also a significant proportion which leaves via effluent discharge into rivers and lakes. More indirectly, that portion of waste water-derived 4-NP which finds its way into soil through use of sludge as a fertiliser may subsequently reach water bodies via erosion or runoff processes, although the extent of this contamination has not, to my knowledge, been determined. Studies on the

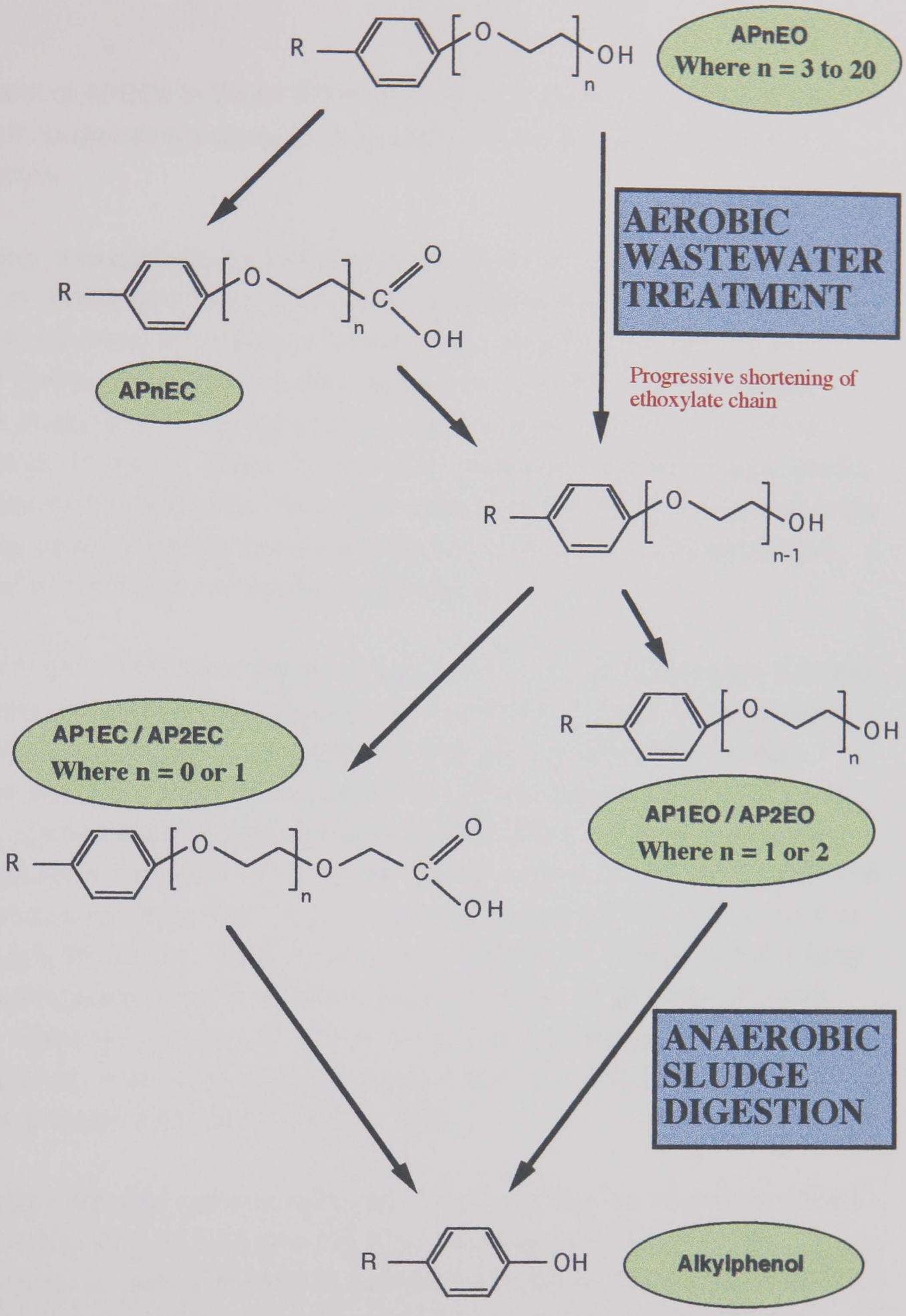


Figure 4.3

Degradation of APEOs during sewage treatment, leading to the formation of AP's. This scheme is based on behaviour studies by Ahel et al (1994a), but note that recently, another significant metabolic class of the APEOs has been recognised - the CAPECs (Di Corcia et al, 1998). These have a carboxylate group on both the alkyl and the ethoxylate side-chains, and their environmental significance is high as they appear to be extremely persistent.

degradation of APEOs in Swiss STWs led Ahel et al (1994a) to estimate that 60-65% of all nonylphenolic compounds entering STWs are discharged into the environment.

The pattern of degradation of APEOs described above is reflected in studies by Giger et al (1984), who found 4-NP concentrations in sewage effluent (pre-anaerobic digestion) to range from 36-202 μg/L, whereas in anaerobically digested sludge, concentrations rose markedly to 0.45-2.5 g/kg (dry weight). Likewise, Rudel et al (1998) reported the concentration of 4-NP in a treated effluent to be 15.9 μg/L, where the method of wastewater treatment was aerobic, with no secondary treatment. The same authors measured 4-NP concentrations in septage samples (where anaerobic digestion is the major treatment factor), and found concentrations in excess of 1000 μg/L.

Ahel and Giger (1985) also detected 8 μg 4-NP/L in treated wastewater samples. In this case, the authors also detected concentrations of 3 μg/L in the receiving waters. Closer to home, a study carried out by the Scottish Environmental Protection Agency (SEPA) measured 4-NP in STWs throughout Scotland, and reported concentrations in effluents from 'typical' STWs of 1-2 μg/L, whereas concentrations in effluents from 'industrial' STWs were in excess of 10 μg/L. The highest concentration of 4-NP from a major discharge in Scotland was found to be 36.7 μg/L (Pirie et al, 1996). Another type of effluent in which 4-NP has been detected at significant levels is vehicle wash effluents. In 'low volume' wash effluents monitored in Sweden, such as those used for cars as opposed to heavy goods vehicles, 4-NP was present at concentrations ranging from 10 to 4000 μg/L, with a mean of 600 μg/L (Paxeus, 1996).

Once treated effluents reach water bodies, however, they are invariably diluted, the extent of which is crucial, and dependent largely upon the size of the receiving body as well as the rate of discharge of effluent. Concentrations of 4-NP in American rivers have been reported to be extremely low (Naylor et al, 1992), and this may be a result of the higher dilution factor of U.S. effluents compared with the situation in the U.K., where a 50% dilution factor is not uncommon. The extent of effluent dilution also depends partly upon the season, and the location of the STW on the river. Other factors influencing ultimate concentrations of 4-NP in the river system are the temperature of the water, and the physico-chemical characteristics of the individual water body. This latter

point may affect how much 4-NP partitions into the particulate phase and/or how much is degraded by micro-organisms or photolytic processes.

The solubility of 4-NP in water has been calculated as being 5.43 mg/L at 20.5°C (Ahel and Giger, 1993a), and the log K_{ow} was determined to be 4.48 (Ahel and Giger, 1993b). These data suggest that, once in the aqueous environment, 4-NP is likely to partition into the more organic phases such as particulate matter, bed sediments, algae, and other aquatic organisms.

The above mentioned studies indicate that 4-NP is a persistent environmental contaminant. Conversely, studies have been carried out which indicate the degradative behaviour of 4-NP. For example, Marcomini et al (1989) reported an 80% loss of 4-NP in soil over 20 days. Tanghe et al (1998) concluded that the degradation of 4-NP in laboratory scale activated sludge units was temperature dependent. In these studies, 4-NP, applied to the system at 8.33 mg/L, was almost completely biodegraded at 28°C, whereas if the temperature was decreased to 10-15°C, the extent of degradation also decreased, to 13-86%. The latter temperature range is more likely to represent the situation in UK rivers, although many other factors will vary from those found in the activated sludge system. Varineau et al (1996) have also reported ultimate degradation of 4-NP in river die-away and semi-continuous activated sludge (SCAS) studies.

The concentration of 4-NP to which freshwater organisms are actually exposed is therefore influenced by a variety of factors, including the concentration in effluents entering the water system, the degree of dilution of the effluents, the temperature of the water body concerned, and the sediment characteristics (including suspended particulate matter) within the system.

Reported actual concentrations detected in rivers have varied considerably. The majority of studies have reported freshwater concentrations of 4-NP to lie in the low µg/L range, but some rivers have been found to carry loads in excess of 100 µg/L in parts. For example, the River Aire in Northern England is notorious with respect to 4-NP contamination. Blackburn and Waldock (1995) found treated effluents discharged into the Aire to contain as much as 330 µg/L of total extractable 4-NP, and found water samples collected from this river to yield concentrations of up to 180 µg/L. 4-NP concentrations in the majority of other sites sampled, however, ranged from <0.2 to 12 µg/L (Blackburn and Waldock,

1995). Ahel et al (1994b) measured 4-NP in Swiss rivers and found concentrations ranging from <1 to 45 μg/L. In this case, only one value was found to exceed 10 μg/L, and more than 80% were in the range of 1 to 7 μg/L. Sheldon and Hites (1978) found concentrations of only 1 to 2 μg 4-NP/L in rivers in summer, and as little as 0.04 to 1 μg/L in samples collected in winter. Naylor et al (1992) found 70% of sites sampled to possess concentrations of 4-NP of less than 0.1 μg/L. Concentrations of 4-NP have also been determined in estuarine water samples (Blackburn et al, 1999), and found to lie in the range of 3.3 to 6.2 μg/L in the Mersey, and <0.2 to 5.8 μg/L in the Tees. In all other estuarine water samples tested, 4-NP concentrations were reported as being near or below the detection limit (Blackburn et al, 1999).

In essence, these data describe a pattern of 4-NP contamination which tends to be concentrated around urban or industrialised areas, and generally is detected at concentrations of less than 10 μg/L. Nonetheless, high concentrations such as those reported in the River Aire should not be discounted simply by virtue of their infrequent occurrence, and should be considered as realistic worst case scenarios.

The final aspect of exposure which is important with regards concentration of contaminants affecting freshwater organisms is that of bioaccumulation. 4-NP has been described as having a 'low to moderate' bioaccumulation potential by Staples et al (1998). Bioconcentration factors (BCFs) for NP were quoted in this review as ranging from <1 to 2500 in fish, and bioaccumulation factors (BAFs), which also take into account the uptake of contaminants through the food chain, varied from 6 to 487. BCFs vary widely between species, however, and even between tissues. For salmonids, BCFs have been reported as 280 in Atlantic salmon (McLeese et al, 1981), and 100 and 40 in the viscera and carcass, respectively, of the rainbow trout (Lewis and Lech, 1996). Higher concentrations of 4-NP have been reported in the liver and other digestive organs, as compared to the muscle, of exposed fish (Ahel et al, 1993; Lewis and Lech, 1996; Thibaut et al, 1998). In the latter article, the progress of tritiated 4-NP was followed after a single application, although the exact nature of the residues was not determined. Likewise, Lewis and Lech (1996) traced the distribution of radiolabelled 4-NP in rainbow trout, but by measuring only total radioactivity, they did not distinguish between metabolites and the parent compound in the majority of tissues analysed; they did report the presence of metabolites of 4-NP in the bile of the

fish, although these compounds were not identified. On the other hand, Coldham et al (1998) determined the presence of both tritiated 4-NP and its metabolites following a single i.v. dose in juvenile rainbow trout. The authors found that although total radioactivity was high in intestinal organs, metabolism was also extensive in these tissues. In muscle, however, there appeared to be an accumulation of the parent compound, which may be due to the lower potential for metabolism in this tissue compared to that found in intestinal organs. Ahel et al (1993) measured actual concentrations of 4-NP in wild fish, and although the BCF was not specified for the salmonid species sampled (*Salmo gairdneri*), BCFs in fish tissues as a whole were estimated as ranging from 13 to 410. The same study found that 4-NP concentrations in these fish were elevated in liver compared to muscle, and here it was actual 4-NP which was measured, and not the sum of its metabolites.

It would appear that the absolute figure for bioaccumulation is debatable, which may be largely due to the differing methods of application and analysis, and also the actual concentration of 4-NP used in experiments attempting to determine BCFs. Nonetheless, it is clear that NP does accumulate in fish tissues to a certain extent, and therefore over a period of time the fish may be exposed to a greater concentration than simply that which is found in the surrounding water body. The majority of studies covering this area have been short term, and have used radiolabelled compounds. In wild fish, it is likely that exposure will be at lower levels, but on a more chronic basis than those reported in the literature. If this is indeed the case, the examination of concentrations of 4-NP in fish tissues sampled from the wild may provide the most realistic idea of the extent and distribution of 4-NP contamination in fish, although close monitoring would be required to assess the concentration of 4-NP in the water to which the fish were exposed.

4.1.1.2 Estrogenic activity

Alkylphenols were first identified as estrogenically active as long ago as 1938 (Dodds and Lawson, 1938). This information, however, appears to have been laid to rest until very recently, when Soto et al (1991) 'rediscovered' the estrogenic activity of 4-NP by chance when conducting routine assays using the MCF-7 cell line. A maximal growth response (in the absence of E2) was observed when cells were supplemented with stripped serum which had been

stored in modified polystyrene, suggesting that something in the serum, originating from the polystyrene, was estrogenic to the cells. The component of this polystyrene which had induced this response was isolated by HPLC fractionation, and identified as 4-NP. Once identified, the estrogenicity of this chemical was further confirmed in an in vivo assay, whereby the endometrial mitotic index of ovariectomised rats was assessed. The purified 4-NP increased this index in a dose-dependent manner. This report appeared to open the floodgates for investigations of 4-NP, which has since been observed to be capable of inducing estrogenic effects in vitro in a wide variety of assays. These include rainbow trout hepatocyte cultures (Jobling and Sumpter, 1993), assays ranging from breast cancer cell lines to transfection and receptor binding assays (White et al, 1994), and a recombinant yeast estrogen assay (Routledge and Sumpter, 1996).

4-NP has also been shown to induce estrogenic effects in vivo in fish (Jobling et al, 1996; Lech et al, 1996), and in rats (Milligan et al, 1998). With regard to fish, exposure to 4-NP induced a dose-dependent increase in vitellogenin production in male rainbow trout (Jobling et al, 1996). A concentration of 20.3 μg 4-NP/L (or higher) was required to induce this response; the next lowest dose to which the fish were exposed (which did not induce a biological response) was 5.02 μg/L. At the highest concentration employed in these experiments (54.3 μg/L), testicular growth was impaired. The increase in vitellogenin production can be attributed to exposure of the fish to an estrogenic compound, whereas the mechanisms leading to inhibited testicular growth are likely to be less specific. Vitellogenin gene expression was also determined in rainbow trout exposed to 4-NP at concentrations ranging from 10 to 150 μg/L, and was found to occur at concentrations as low as 10 μg/L (Lech et al, 1996).

Other adverse reproductive effects induced by 4-NP in fish have been reported by Gray and Metcalfe (1997), who found intersex gonads in japanese medaka (*Oryzias latipes*) exposed to 4-NP, and by Ashfield et al (1998), who presented data showing an increased GSI in juvenile female rainbow trout exposed to 30 μg 4-NP/L. These effects, however, may not be estrogen-specific, and the mechanisms underlying them are unknown.

In conclusion, 4-NP, a chemical that can be found at varying concentrations in the freshwater environment, is capable of inducing both estrogenic responses,

and other less specific reproductive impairments, in fish, and can thus be considered a model aquatic xenoestrogen.

4.1.2 The brain-pituitary-gonadal (BPG) axis

The aspect of reproduction under investigation in this project was the BPG axis. This system regulates reproduction in all vertebrates, and sex hormones are crucial in maintaining a balance across the axis. Essentially, the hypothalamus in the brain sends signals (in the form of gonadotropin releasing hormone, GnRH) to gonadotrophs in the pituitary glands which are responsible for producing and releasing gonadotropins (GTH), and these in turn regulate development and maturation of the sex-hormone producing gonads. The whole system is controlled by a series of complex positive and negative feedback pathways between the three levels of the axis. A basic representation of the BPG axis is shown in figure 4.4.

Gonadotropins are glycoproteins, which consist of two distinct subunits. The first, the 'α' subunit, is common to both gonadotropins. The second, the 'ß' subunit, is hormone specific. The two subunits must combine (to form a heterodimer) to give the intact, biologically active hormone. In most vertebrates, there are two ß-subunits, forming two gonadotropins, namely follicle stimulating hormone (FSH) and luteinising hormone (LH). Until relatively recently, there was thought to be only one GTH in fish. This was thought to govern all gonadotropic-ovarian functioning (Breton, 1983; Le Menn and Burzawa-Gérard, 1985), yet plasma concentrations appeared to peak only for a short period prior to ovulation, remaining at basal levels for the majority of the reproductive cycle (Scott and Sumpter, 1983; Sumpter and Scott, 1989). In 1988, Suzuki et al reported the identification of two gonadotropins in chum salmon, *Oncorhynchus keta* (Suzuki et al, 1988a and 1988b), which are generally referred to in the literature as GTH I and GTH II, but more recently have been found to be similar to tetrapod FSH and LH, respectively (Swanson, 1991). It is now considered that GTH I plays the same developmental role in fish gonads as FSH does in those of mammals, and likewise GTH II in teleosts is primarily involved with regulation of gonadal maturation, as LH is involved with oocyte maturation and ovulation in mammals (GTH I and II will be therefore be referred to as FSH and LH, respectively, for the remainder of this chapter). This is reflected in the seasonal profiles of FSH and

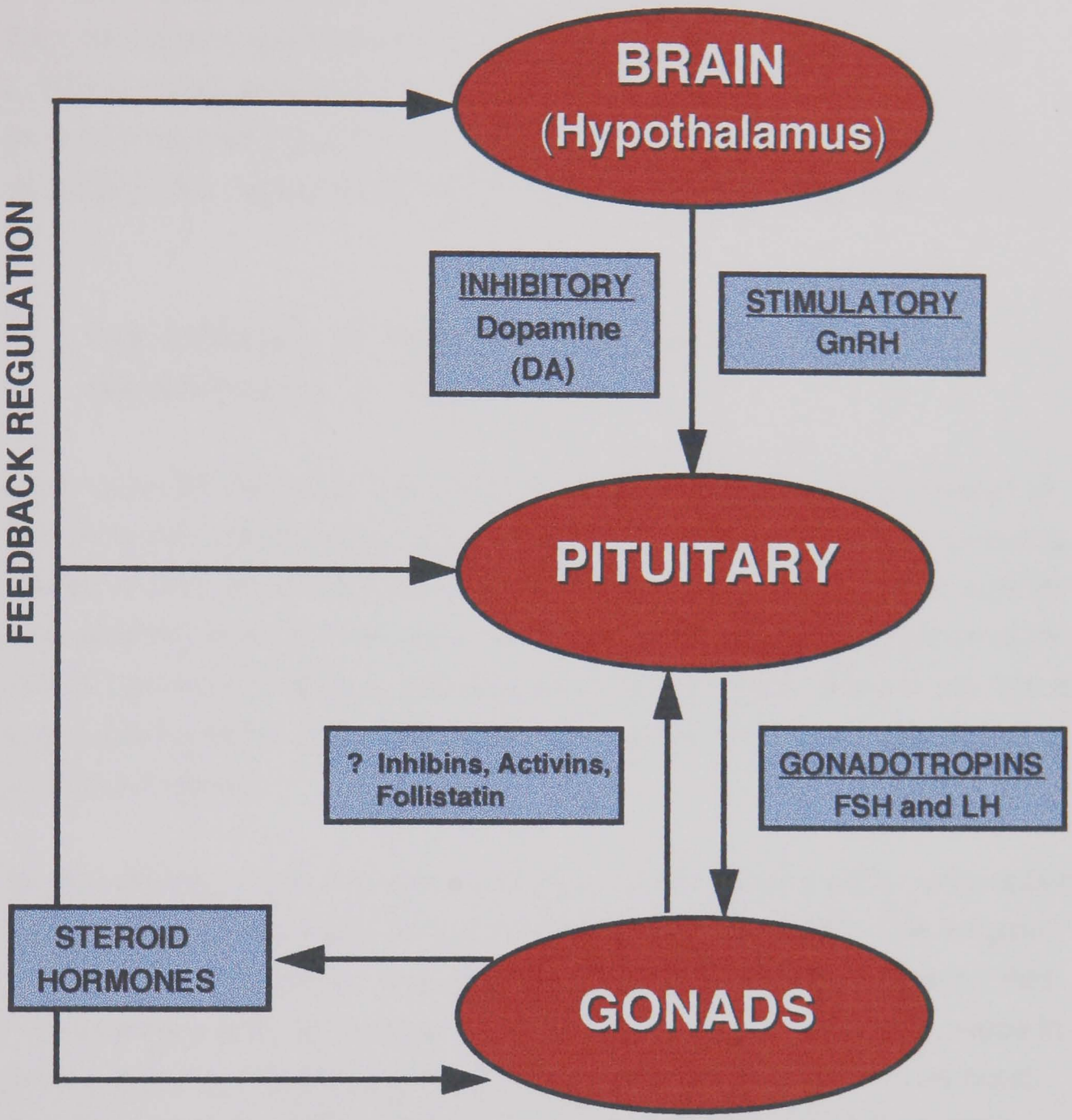


Figure 4.4
A simplified diagram of the brain-pituitary-gonadal (BPG) axis.

LH in the rainbow trout (Prat et al, 1996). In that study, the FSH concentration in plasma of maturing females was found to rise during early vitellogenesis, concomitant with a very gradual increase in GSI. Peak FSH levels occurred when the GSI reached around 0.4 to 1, following which FSH levels declined. Shortly prior to ovulation, concentrations of FSH, and also of LH, rose sharply (Prat et al, 1996). An illustration of this 'normal' hormonal profile can be seen in figure 4.5.

4.1.2.1 The influence of steroids on the regulation of gonadotropins in fish.

In mammals, gonadotropin synthesis and secretion is regulated by a number of factors, including the hypothalamic hormones (gonadotropin releasing hormone, GnRH - which is stimulatory, and Dopamine, DA - which is inhibitory), as well as gonadal sex steroids and peptides such as inhibin and activin. Regulation of the synthesis and secretion of teleost gonadotropins is not as well understood, but a summary of what is known of the impact of sex steroids on their synthesis and secretion is given below.

In general, the effects of gonadal sex steroids on LH in fish are better understood than on FSH, as a result of the relatively recent 'discovery' of FSH (the 'original' gonadotropin is now considered to have been LH). It is possible, however, that some of the studies which have researched this subject are misleading, since in some cases, antibodies developed for 'GTH II' have been found to cross-react with 'GTH I' (Breton et al, 1997). Generally, LH and its antibodies have been found to be easier to purify / develop than FSH and its respective antibodies, so it is most likely that the 'GTH' assays used in the past were more specific for LH than for FSH, but there is the possibility that the standards, and therefore the antibodies developed against them, did not have a high LH purity.

It has been reported that the extent of synthesis and release of LH under the influence of steroids is dependent upon the reproductive status of the fish (Khan et al, 1999; Sohn et al, 1998). Khan et al (1999) demonstrated a positive feedback response, on both basal LH, and LHRH-analog induced LH secretion, to E2 or testosterone (an aromatizable androgen) in the early recrudescent phase of the reproductive cycle. This changed to a negative feedback response of LH secretion in the late recrudescent phase, i.e. once the gonads of the fish

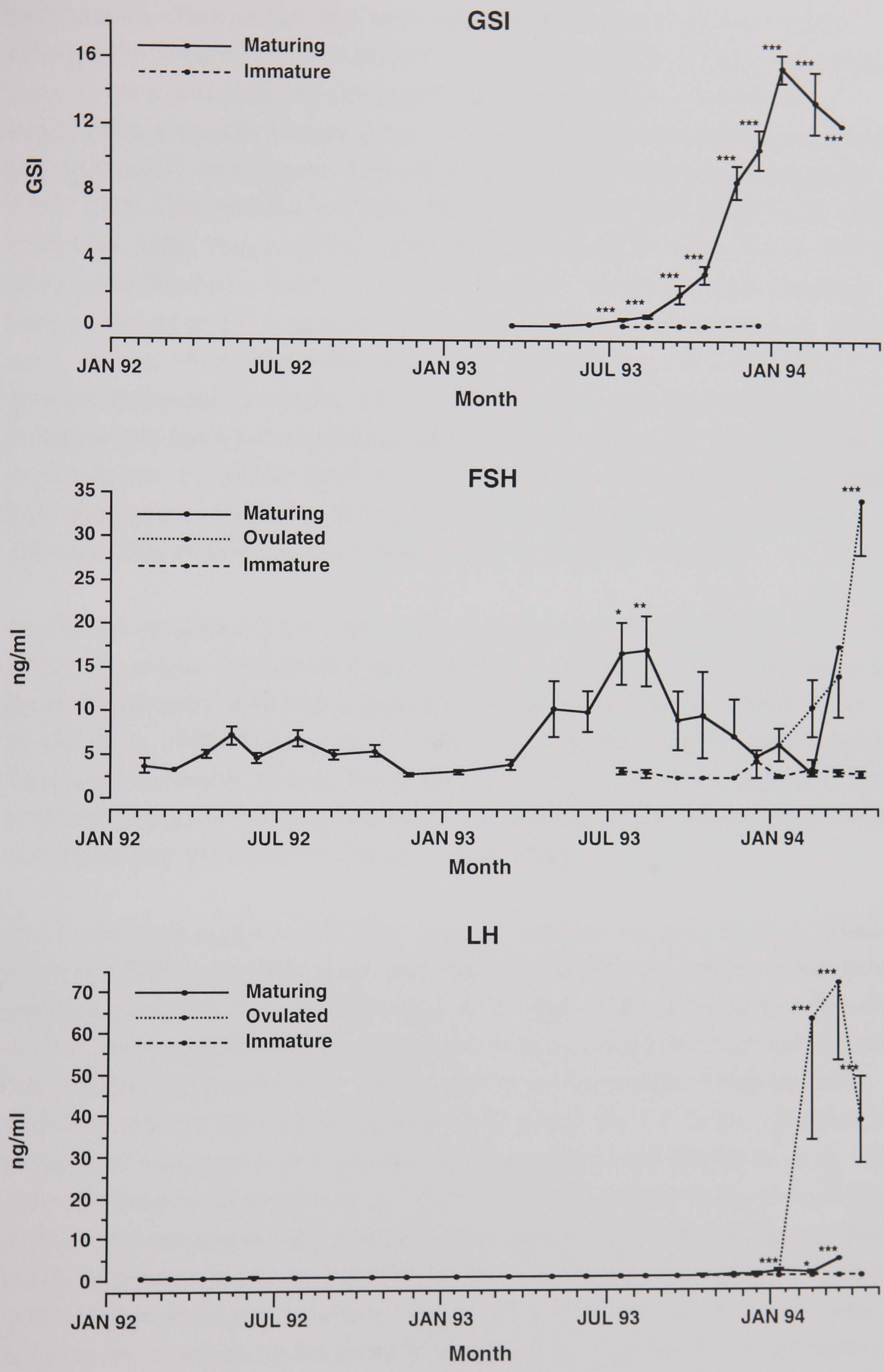


Figure 4.5
The annual profile of plasma FSH and LH concentrations relative to the GSI in female rainbow trout (Prat et al, 1996).

had matured. This pattern has been repeated in several separate studies, although the majority of these studies have concentrated on one or the other of these phases, and have not compared the feedback effect in different reproductive stages in a single study. A positive feedback response of LH to E2 or aromatizable androgens in immature fish has been reported in Crim and Peter, 1978, Crim and Evans, 1979, Gielen and Goos, 1983, Magri et al, 1985, Trinh et al, 1986, Huggard et al, 1996, Amano et al, 1997, Breton et al, 1997, and Dickey and Swanson, 1998. In contrast, negative feedback responses have been observed on LH in spawning or mature rainbow trout (Billard et al, 1976, 1977; Billard, 1978; Van Putten et al; 1981, Bommelaer et al 1981) and in goldfish (Kobayashi & Stacey, 1990). One explanation for this differential response has been put forward by Saligaut et al (1998), who suggested that in immature fish, E2 can upregulate LH, but at reproductive stages corresponding to high endogenous E2 levels, E2 may activate the dopamine (DA) inhibitory pathway, thus resulting in an indirect negative feedback response.

As mentioned earlier, fewer data are available concerning the response of FSH to steroids in fish. Those who have specifically researched this topic appear to have unanimously observed a negative feedback response in rainbow trout (Breton et al, 1997; Saligaut et al, 1998), in coho salmon (Dickey and Swanson, 1998), and in Atlantic salmon (Antonopoulou et al, 1999). It also appears that the inhibitory activity of DA on gonadotropin release is restricted to LH, and FSH is not affected by this hormone (Saligaut et al, 1998).

It has also been suggested that the disparity between the nature of the responses of LH and FSH to steroidal treatment may be a result of the activity of the gonadal peptides, inhibin and activin (Melamed et al, 1998). The same review pointed out that the two gonadotropins have been found to be produced in two distinct cell populations (Nozaki et al, 1990a), unlike in mammalian pituitaries, and, therefore, that the differential responses may simply be due to the presence or absence of receptors to the relevant regulatory hormones (Melamed et al, 1998). Although there is little evidence as yet for regulation of gonadotropin levels by inhibins and activins in fish, a recent paper reported that recombinant goldfish activin stimulated expression of GTH Iß, but apparently inhibited the expression of GTH IIß in pituitary cell cultures (Yam et al, 1999). This contrasts with data from previous studies by the same group, in which mammalian preparations of both inhibin and activin stimulated the release of GTH II in vitro (Ge et al, 1992).

Clearly, a great deal more research is required before the differential regulation of gonadotropins in fish is understood.

To summarise, it would appear that the natural estrogen, E2, can exert positive feedback control over LH (GTH II) in immature fish, whilst a negative feedback response is observed in mature/spawning fish. E2 has also been demonstrated to inhibit the synthesis and/or release of FSH (GTH I). My experiment attempted to monitor FSH and LH concentrations in maturing female rainbow trout, exposed to a known xenoestrogen, in order to assess whether the above-mentioned profile of plasma gonadotropin concentrations (Prat et al, 1996) could be altered by an estrogen mimic. The study covered the time period when FSH concentrations are highest, during early ovarian recrudescence. Since this hormone is considered crucial to gonadal development, my hypothesis was that if the xenoestrogen was capable of suppressing FSH, it might also impair ovarian growth.

4.2 *METHODS*

4.2.1 Experimental design

4.2.1.1 Concentrations of 4-NP selected

I aimed to expose the fish to concentrations of 4-NP which were both environmentally relevant, and which covered a range of doses decreasing by a 10-fold differential at each step. The chosen concentrations were 100, 10, and 1 μg 4-NP/L. A solvent control tank was also set-up, whereby fish were exposed to methanol at the concentration present in the 4-NP treated tanks (0.002%). This was well within the level suggested by the U.S. EPA for use of solvents in aquatic toxicity test systems (0.01%, Zucker, 1985) . An absolute control tank, with a flow rate equal to that of the treatment tanks, was also set up.

4.2.1.2 Experimental protocol

The experiment was conducted at the NERC Centre for Ecology and Hydrology, based on the shore of Windermere, Cumbria. A photograph of the experimental facility can be seen in figure 4.6, which gives some idea of the scale of the experiment.

The following experimental design was employed:

Tank	Treatment
A	Absolute control
B	MeOH control
C	1 μg 4-NP/L
D	10 μg 4-NP/L
E	100 μg 4-NP/L

Thirty randomly selected fish were placed in each tank. In addition, thirty fish were sacrificed (hereafter called pre-exposure controls) at the beginning of the experiment. Treatments were initiated in early March, at a point prior to the expected increase in FSH concentrations (see figure 4.5). A tank of 'spare' fish was also maintained for the duration of the experiment. These were from the same batch as those used for the exposure study, but were not used as experimental fish (i.e. they were not exposed to 4-NP, and were not sampled prior to their sacrifice). At each timepoint, fifteen of these fish were terminally sampled in order to assess the stage of reproduction which the fish had reached. It was assumed that the relationship between the GSI and plasma FSH concentration of these fish would be similar to that described by Prat et al (1996), as shown in figure 4.5. The mean GSI of the 'spare' fish was, therefore, used to gauge when the peak of FSH concentration may occur, and the experiment was terminated at this estimated timepoint (mid-July).

Figure 4.6
The tank set-up at the NERC Centre for Ecology and Hydrology, Windermere.

An attempt was made to obtain a comprehensive profile of hormones during the exposure period, whilst preventing undue stress to the fish. Monthly sampling was considered as being potentially too frequent, and may itself have influenced hormone concentrations in the fish. Conversely, bi-monthly sampling may not have provided sufficient data to observe the progression of the reproductive development of the fish. A sampling interval of 6 weeks was therefore chosen as a suitable compromise.

A water sample was collected from each tank every six weeks, immediately prior to commencing the sampling of the fish. Samples were also analysed from each tank prior to initiation of the 4-NP dosing, to obtain background levels of 4-NP and/or estrogenicity of the tank water. During the initial stage of the experiment, tanks were equilibrated with 4-NP, in the absence of fish, for three weeks, after which 'pre-dose' water samples were collected (t=0). Water samples were extracted and analysed as discussed in section 2.5.

4.2.1.3 Selection of fish

Female rainbow trout (Oncorhynchus mykiss) of the 2+ age group were obtained from New Mills Trout Farm (Brampton, Cumbria). We attempted to obtain fish which would be maturing for the first time in the following spawning season, and therefore were all at the same stage of maturity (i.e. the ovaries of all of the fish were at the primary oocyte growth stage at the beginning of the experiment).

4.2.1.4 Dosing of experimental tanks

4-NP (99% purity, and consisting of a mixture of isomers) was purchased from Acros Organics, Loughborough, Leics. Stock solutions were prepared in 4 litre amber glass bottles using methanol (MeOH; BDH, Poole, Dorset) as a carrier solvent. Concentrations of stocks were 5 g/L, 0.5 g/L, and 0.05 g/L for the dosing of 100 μg/L, 10 μg/L, and 1 μg/L 4-NP treatments, respectively. The 4-NP solutions were added to the tanks using a multichannel peristaltic pump (Watson-Marlow, Falmouth, Cornwall) at a rate of 0.4 ml/min. Water was abstracted from the depths of Windermere, and flowed into the 1500 litre tanks at a rate of 20 L/min. Delivery tubing was made of silicon, and pump tubing was replaced each month.

4.2.1.5 Sub-lethal sampling

Fish were sampled sub-lethally at the start of the experiment and at intermediate timepoints. Approximately three fish were netted from a tank at a time. Fish were anaesthetised using 1:2000 2-phenoxyethanol. Blood was sampled from the caudal sinus using heparinised syringes, treated with aprotinin, and kept on ice prior to centrifugation. Blood plasma was then drawn off and frozen at -20°C until use. Whilst anaesthetised, electronic 'passive integrated transponder' (PIT) tags (14 mm, Avid, Uckfield, UK) were implanted into the dorsal musculature of the fish at the first timepoint, and these were read (using an Avid Powertracker II) at each timepoint subsequently. This allowed the fish to be tracked individually throughout the experiment. Weight and forklength were also recorded prior to returning the fish to the appropriate tank.

4.2.1.6 Terminal sampling

Fish were anaesthetised, blood was collected, and weight and length were measured, all as described above. In addition, pituitaries were collected and frozen in liquid nitrogen, and transferred to -80°C on returning to the laboratory. The ovaries and livers of the fish were removed and weighed, for calculation of gonadosomatic and hepatosomatic indices.

4.2.2 Extraction of pituitary glands

Pituitaries were extracted using a method described by Hassin et al (1998), developed so that a single pituitary could be used to provide samples for both GTH protein assay by RIA, and GTH subunit mRNA analysis.

Frozen pituitaries were homogenized, using 1.5 ml eppendorf tubes with fitted pestles, in 200 µl of LiCl (3 M) / Urea (6 M). 10 µl aliquots were removed and diluted in 990 µl PAB (see section 2.3.1) prior to analysis by RIA (see 2.3.4 for details of the FSH assay, and 2.3.5 for the LH assay). To the remaining homogenate, one tenth it's volume of 2 M sodium acetate, and 2.5 times its volume of ethanol were added.

The samples were stored at -70°C for at least 2 hours. A total of 12 pituitaries from each treatment were extracted for GTH subunit mRNA analysis. Precipitated samples were centrifuged at 14,000 rpm for 10 minutes. The resulting pellet was resuspended in 700 µl TES (10 mM TRIS, 1 mM EDTA, 0.5% SDS) and subsequently extracted twice with 700 µl acid-phenol / chloroform (50:50). The final aqueous phase was transferred to a fresh eppendorf, the total RNA in the sample was ethanol precipitated as described above, and the sample was stored at -70°C for at least 2 hours. Following centrifugation (as above), this precipitate was resuspended in 40 µl DEPC treated (i.e. RNase-free) water, and stored at -70°C until analysis.

4.2.2.1 Dot blot hybridisation

Chum salmon LH cDNA (Sekine et al, 1989) was a gift from Dr. F. Le Gac (Laboratoire de Physiologie des Poissons, INRA, France), and its use had been previously validated for detection of rainbow trout mRNA by Weil et al (1995) and Gomez et al (1999). Trout ß-actin cDNA (Pakdel et al, 1989) was also a gift from Dr. F. Le Gac. A fragment of rainbow trout FSH cDNA containing the coding region was isolated and cloned from rainbow trout pituitaries. I designed primers to lie either side of the coding region, and obtained a fragment of just over 500 base pairs (bp) in size; the coding region of FSH consists of 426 bp. Of these, 185 nucleotides were sequenced, and compared to the nucleotide sequence of chum salmon FSH. The two sequences were found to be 88% homologous (see figure 4.7). Probes were labelled using a random primer system (Amersham Pharmacia Biotech, Bucks, UK) with 5'-[α-^{32}P]dCTP (3000 Ci/mM), just prior to hybridisation.

Levels of total RNA in the extracted samples were quantified by 'Gene Quant' (Pharmacia), and samples were loaded onto nylon membranes (Hybond N$^+$, Amersham Pharmacia Biotech, Bucks, UK) at 10- and 5 µg total RNA. Membranes were hybridised with three successive cDNA probes: FSH, LH and ß-actin. Membranes were prehybridised at 42°C for 3 hours in hybridisation buffer (50% formamide, 5 x SSC, 5 x Denhardts reagent (Denhardt, 1966), 1% SDS, 0.1 g/ml dextran sulphate, 0.2 M phosphate buffer), and 100 µg calf-thymus DNA/ml buffer; hybridisation was carried out overnight, using the same buffer. Membranes were washed at 42°C for 1 x 10 minutes and 1 x 5 minutes in 2 x SSC, 0.1% SDS, then for 2 x 5 minutes in 1 x SSC, 0.1% SDS, then for 2 x 5

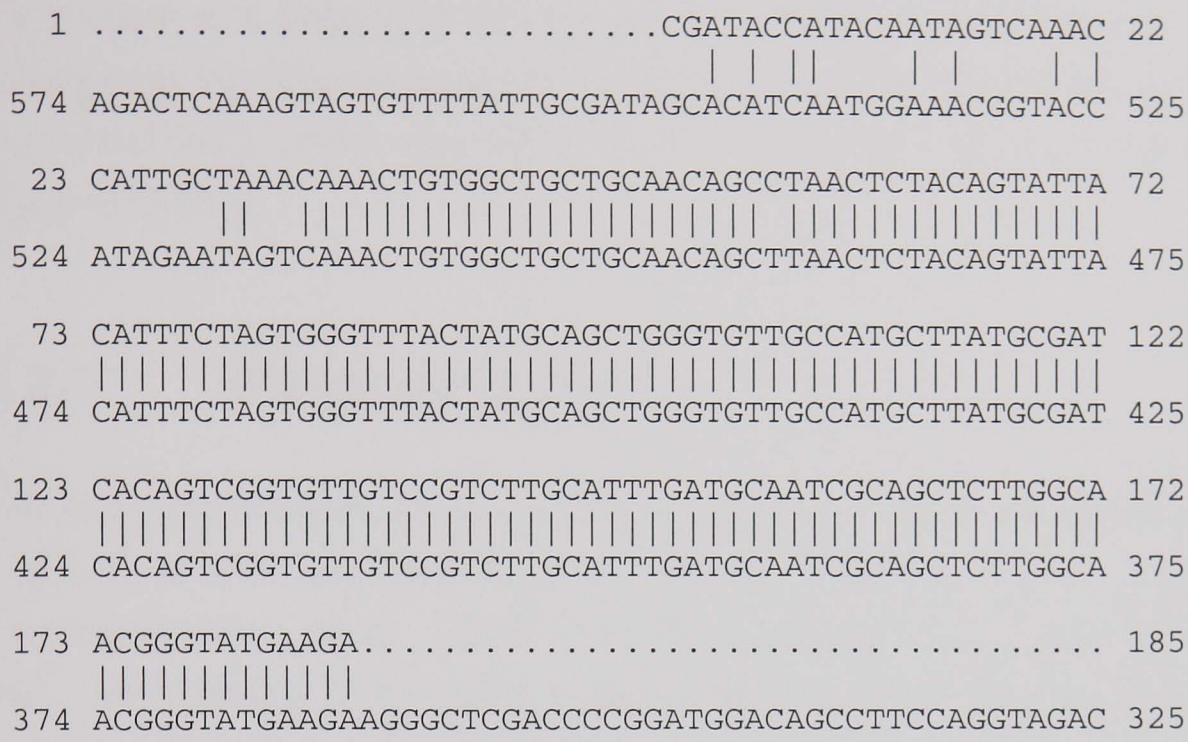


Figure 4.7
Comparison between the sequenced region of the isolated fragment of rainbow trout FSH cDNA (top) and the corresponding region of chum salmon FSH cDNA (bottom). The high homology between the two nucleotide sequences can be clearly seen.

minutes in 0.5 x SSC, 0.1% SDS, and finally for 5 minutes in 0.1 x SSC, 0.1% SDS. The probes were stripped from the membranes between hybridisations using boiling 0.1% SDS, which was then allowed to cool to 42°C and the membrane was shaken in this solution for approximately 3 hours. The radioactive signal was quantified using a 'Storm' phosphorimager (Molecular Dynamics Inc.), which was linked to an ImageQuant software package (Molecular Dynamics Inc.).

4.2.3 Statistical analysis

The data were log-transformed where necessary, and analysed by multi-factorial ANOVA, or one-way ANOVA, as applicable. Fisher's PLSD was used as a post-hoc test in most cases. The Bonferroni-Dunn (control) - a more stringent post-hoc test - was applied to the plasma FSH data, due to the number of outliers in this dataset. The statistical package used was SUPERANOVA (Abacus Concepts, Berkeley, CA). Significant differences between treatments (in most cases, compared to the MeOH control fish) are shown on the figures presented here according to their probabilities (p-values); i.e. the smaller the p-value, the less likely the difference observed is to have occurred by chance.

4.3 *RESULTS*

In the figures presented here, data obtained from the fish exposed to 4-NP have been compared to those from the fish maintained in the solvent (MeOH) control tank, as this was considered to be the correct scientific control. As such, in the following text and figures, the solvent control is simply referred to as the 'control'. Where the absolute (dilution water) control is discussed, it will be specified as the 'absolute control'.

4.3.1 Actual concentrations of 4-NP to which the fish were exposed

The actual concentrations of 4-NP detected in the tank water are shown in table 4.1.

TIME (weeks)	B MeOH CONTROL	C 1 µg/L	D 10 µg/L	E 100 µg/L
0	<0.2	1.9	7.8	79.3
6	<0.2	0.8	10.1	83.5
12	<0.2	<0.2	9.2	87.8
18	<0.2	<0.2	6.1	91.8

Table 4.1

Concentrations of 4-NP (µg/L) over the course of the experiment.

The Limit of Detection (L.O.D.) was calculated according to the Manual on Analytical Quality Control for the Water industry (Cheesman and Wilson, 1989), and was found to be 0.242 µg/L (see Janbakhsh, 1996, for details). In most cases the actual concentrations of 4-NP in the tanks were maintained at close to nominal concentrations, and an approximately 10-fold differential was maintained between treatments, as can be observed in figure 4.8. The actual concentrations were, as had been expected, slightly lower than nominal concentrations, with the mean measured concentrations (± standard error) being 0.7 ± 0.4, 8.3 ± 0.9, and 85.6 ± 2.7 µg 4-NP/L for tanks C, D, and E, respectively. The concentration of 4-NP in the tank containing the lowest nominal concentration (1 µg/L) fell progressively during the exposure period, particularly so in the latter half of the experiment, and was below the detection limit at the end of the experiment.

The estrogenicity of the tank water samples was subsequently confirmed using the recombinant yeast estrogen assay, as described in section 2.1.1. An

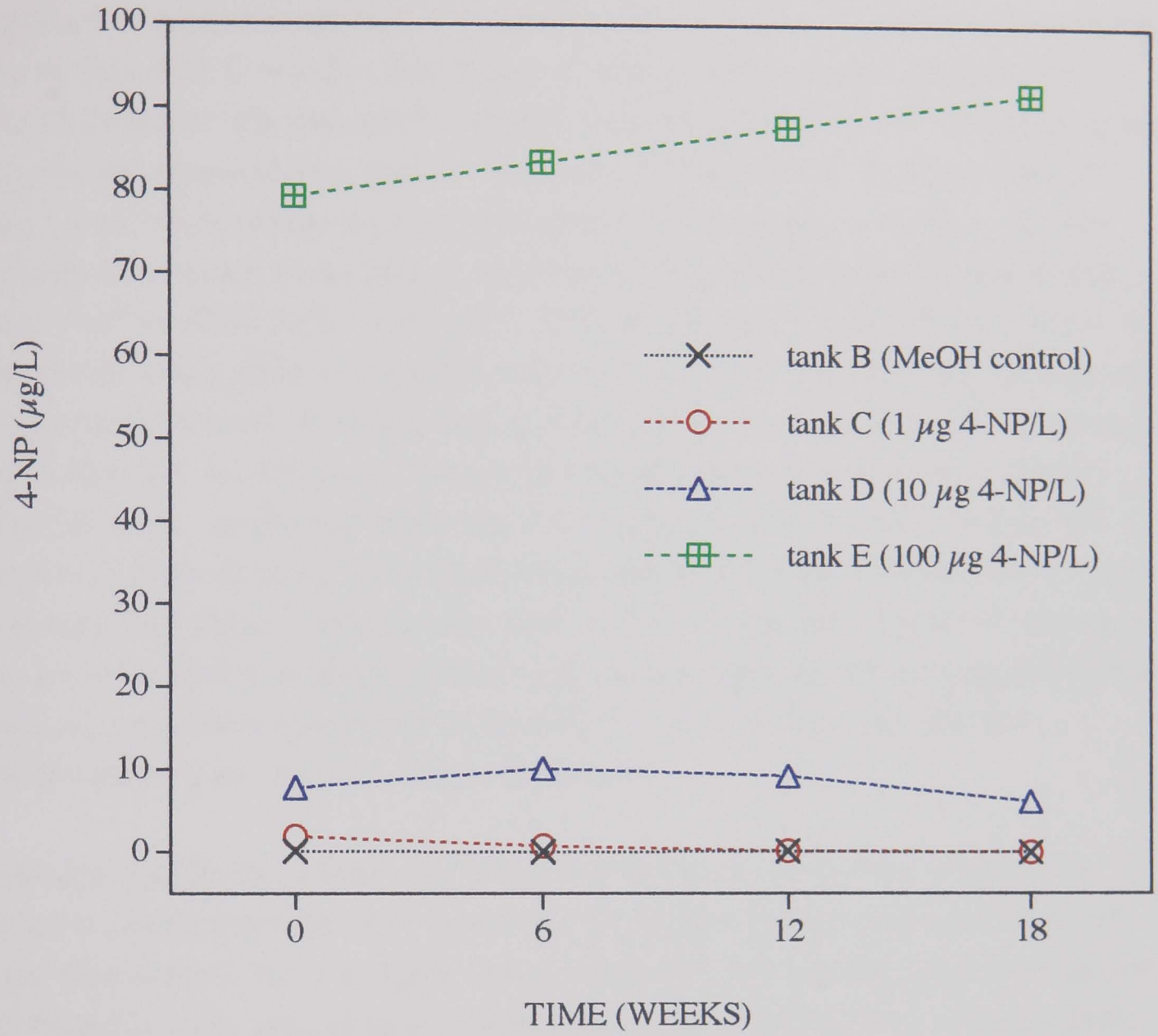


Figure 4.8
Actual concentrations of 4-NP in experimental tanks over the period of the study.

example of the data obtained using this method (in this case, using the samples collected at t=0) is shown in figure 4.9. This indicates clearly that there was estrogenic activity in tanks C, D, and E, whereas in tank B there was no significant estrogenic activity. The water samples were concentrated by varying factors (E x 400; C and D x 2000) prior to assay. The results here have been plotted based on the expected (nominal) concentrations of 4-NP, after taking into account the concentration factor. For example, from tank C, I took 500 ml of water, and concentrated it up x 2000 using a C18 sep-pak cartridge; the final volume of the extract was 250 µl, and the nominal concentration of this extract was therefore 2000 µg/L, (≡ 2 mg/L). The sample was diluted 40 times in the first well of the assay plate in the yeast screen, and hence the curve was plotted with the highest (nominal) concentration at 0.05 mg/L. This means that the curves for these samples should, were the actual concentrations equal to the nominal concentrations, overly that of the standard curve shown for 4-NP. In fact, the curves for tanks D and E are slightly displaced to the right of the standard curve, indicating that there is slightly less 4-NP in the sample than expected, whereas the curve for tank C is displaced to the left of the standard curve, suggesting that there was a greater concentration of 4-NP in this tank than the nominal concentration (see table 4.1 for confirmation).

Since the results have been discussed in relation to the solvent control, the actual concentrations of 4-NP measured in the absolute control tank have not been shown here, but it is worth noting that 4-NP was undetectable in the water sampled from this tank at all timepoints, and also that there was no significant estrogenic activity in the water in this tank.

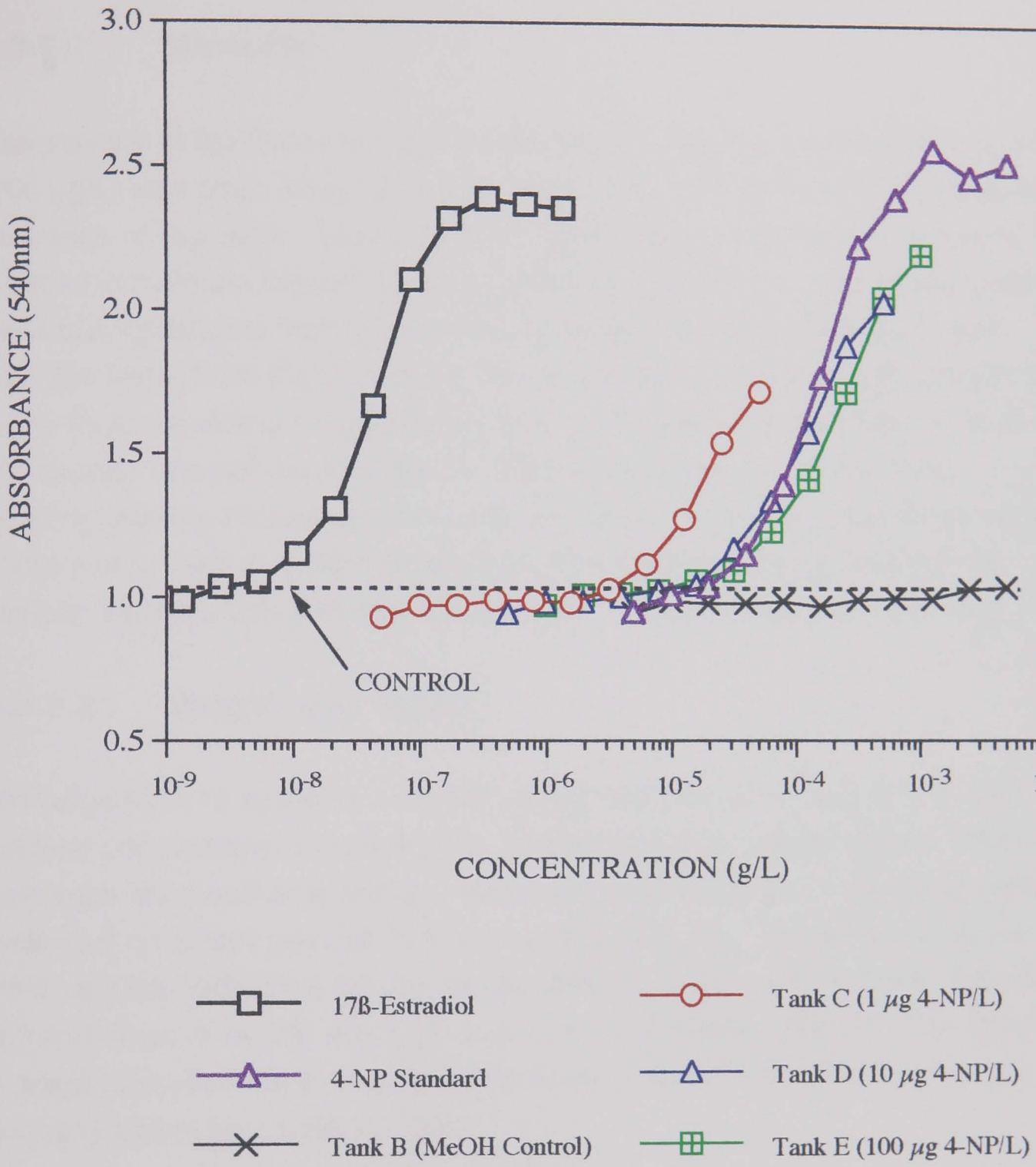


Figure 4.9
The estrogenic activity of extracted tank water samples (collected from the tanks at t=0, prior to the introduction of the fish) in the recombinant yeast assay. Results have been plotted based on the expected (nominal) concentrations of 4-NP, after taking into account the factor by which they were concentrated via solid phase extraction.
Full details are provided in the text (section 4.3.1).

4.3.2 The effect of 4-NP on general growth and reproductive endpoints of maturing female rainbow trout

4.3.2.1 Mortality

The survival of the fish in the tank containing the highest concentration of 4-NP (100 μg/L) was poor. Only 12 fish remained out of an initial 30 in this tank after 18 weeks of exposure. Each time a fish died, it was replaced with a 'spare' fish, in order to maintain similar stocking densities in each tank. These spare fish were distinguishable from the test fish because they were untagged, and samples taken from them were not included in the analyses. Only one fish died in the tank containing 10 μg 4-NP/L, and 100% survival was observed in all other treatments. A small number (two in total) of male fish, which had been indistinguishable (based on their external features) from the females at the start of the study, were identified amongst the experimental fish at the terminal sample; samples taken from these fish were also excluded from analyses.

4.3.2.2 Weight and length

From figures 4.10 and 4.11, it would appear that the fish in tank E (100 μg/L nominal concentration) were slightly smaller than the remaining fish. When the data were analysed as a whole, it was found that there was a significant effect of treatment on length ($p<0.001$) and on weight ($p<0.05$). However, when the data were split into individual timepoints, the difference in length between the control fish and those in tank E was significant only at 6 weeks ($p<0.05$). The difference in weight between the control fish and those in tank E was significant at t=0 ($p<0.01$) and at six weeks ($p<0.05$).

The coefficient of condition was calculated using the formula:

$$\text{coefficient of condition} = [(\text{weight} / \text{length}^3) \times 100]$$

The data are depicted in figure 4.12. This figure indicates that the fish exposed to the highest concentration of 4-NP were nonetheless apparently quite healthy, and that there was no significant difference in the coefficient of condition of the fish in the control vs. the treated fish at individual timepoints.

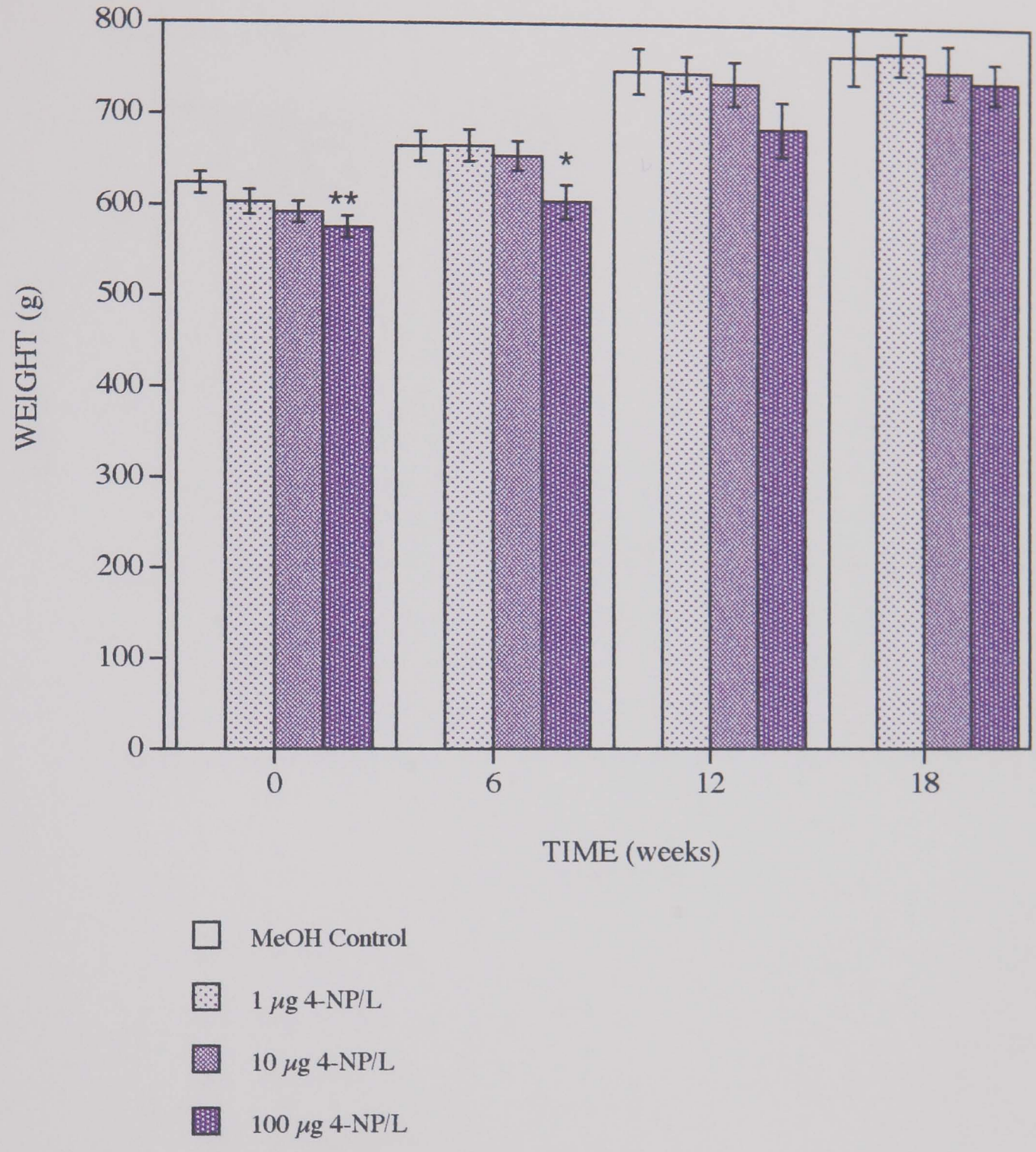


Figure 4.10
Weight of the fish over the duration of the experiment.
Significance from the control is denoted by * $p<0.05$; ** $p<0.01$.

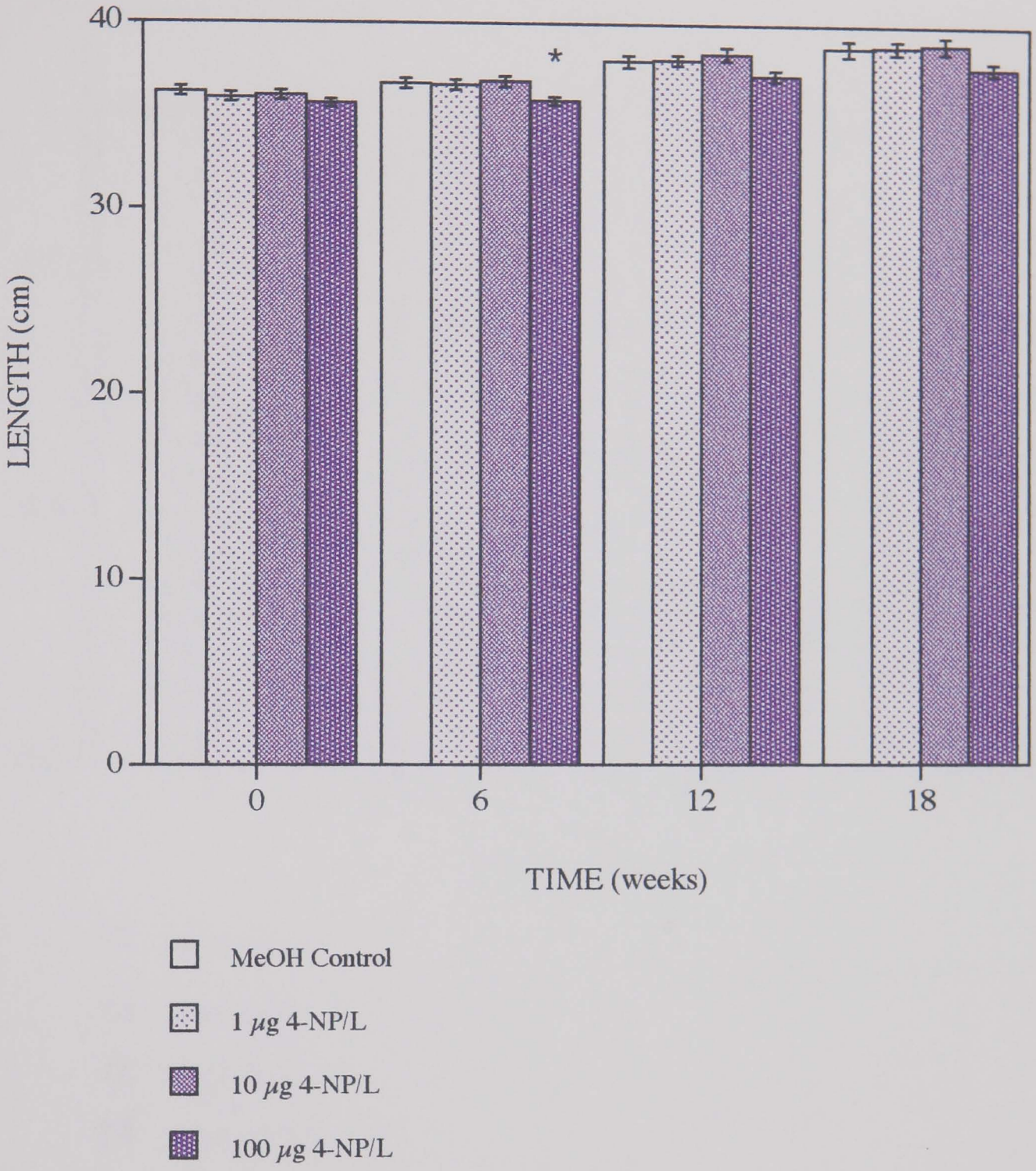


Figure 4.11
Fork length of the fish measured throughout the experiment. Significance from the control is denoted by * $p<0.05$.

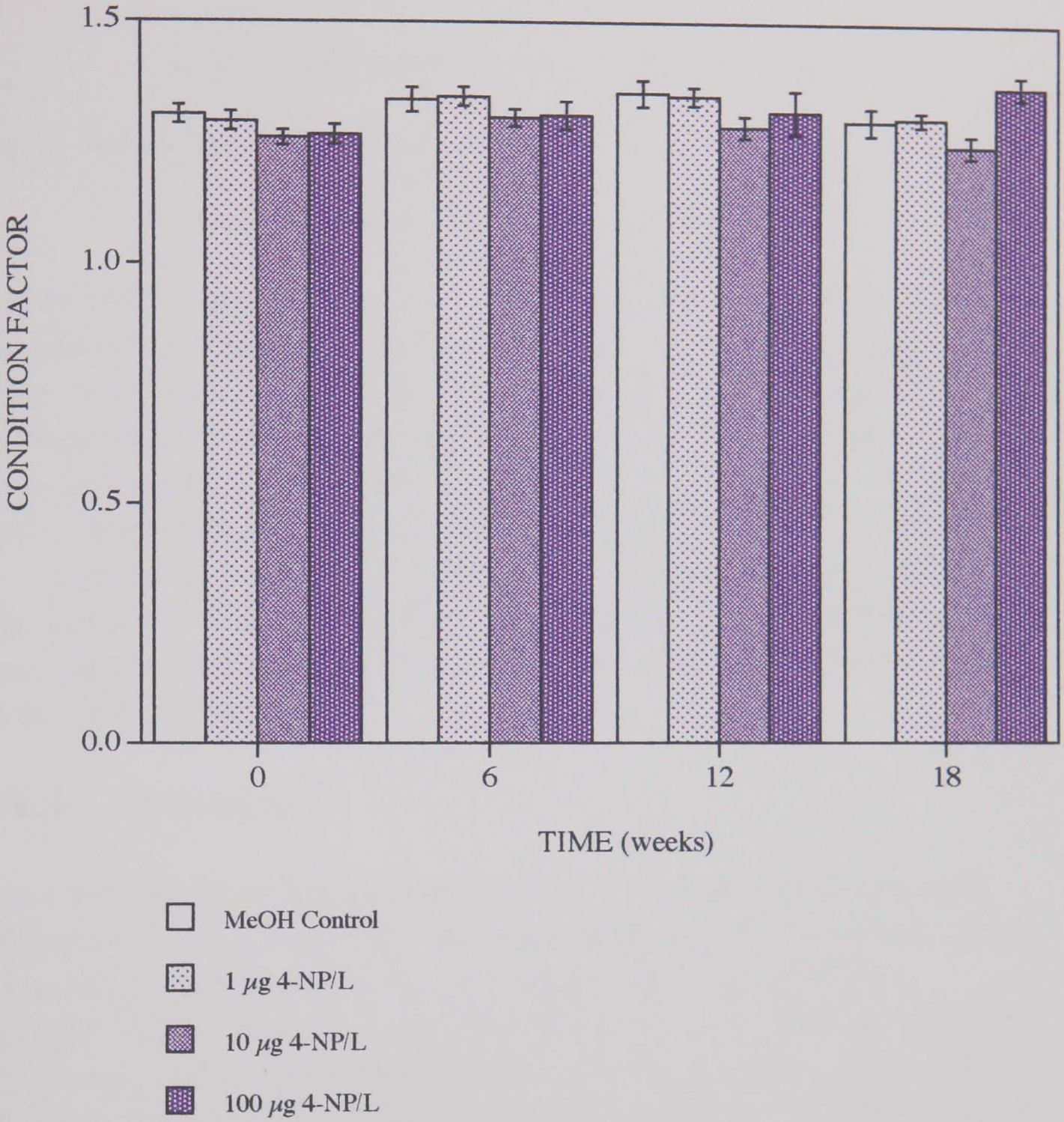


Figure 4.12
Coefficient of Condition of the fish throughout the experiment.

4.3.2.3 GSI and HSI

The gonadosomatic index (GSI) was calculated using the formula:

$$GSI = [(\text{gonad weight} / \text{total body weight}) \times 100]$$

Likewise, hepatosomatic index (HSI) was derived from the formula:

$$HSI = [(\text{liver weight} / \text{total body weight}) \times 100]$$

Both GSI and HSI (results shown in figures 4.13 and 4.14, respectively) in the fish exposed to the highest concentration of 4-NP were highly significantly different than those of the fish in the other tanks at the end of the experiment. In fact, the ovaries of the fish exposed to 100 μg/L for 18 weeks had not developed at all since the start of the experiment (see figure 4.15); the GSI at t=0 was 0.160 ± 0.008, and the GSI in the fish in tank E at 18 weeks was 0.163 ± 0.02). The HSI, in contrast, was significantly higher in the fish exposed to the highest concentration of 4-NP than it was in the control fish at the end of the study. This is most likely a result of the production of large amounts of vitellogenin, induced by 4-NP (see below).

4.3.2.4 Vitellogenin

There was a significant, dose-related increase in the induction of vitellogenin (VTG) by 4-NP (see figure 4.16). The concentration of VTG in the plasma of the control fish rose approximately 30-fold, as these fish underwent sexual maturation. A concentration of 1 μg 4-NP/L did not induce further synthesis of VTG. However, a concentration of 10 μg 4-NP/L did, especially initially, when VTG concentrations were approximately 20-fold higher than the concentration in the controls. The rise in concentration of VTG in plasma of fish exposed to the highest concentration of 4-NP was very pronounced, and was approximately 500-fold higher than that in the control fish after six weeks exposure. This was essentially a maximal concentration; VTG concentrations in mature female salmonids have been reported to reach 65 mg/ml (Fremont et al, 1984); around 50 mg/ml (Scott and Sumpter, 1983); and 13 mg/ml (van Bohemen and Lambert, 1981). The production of this protein is an estrogen-inducible response (Specker and Sullivan, 1993; Copeland et al, 1986) and this was the reason for

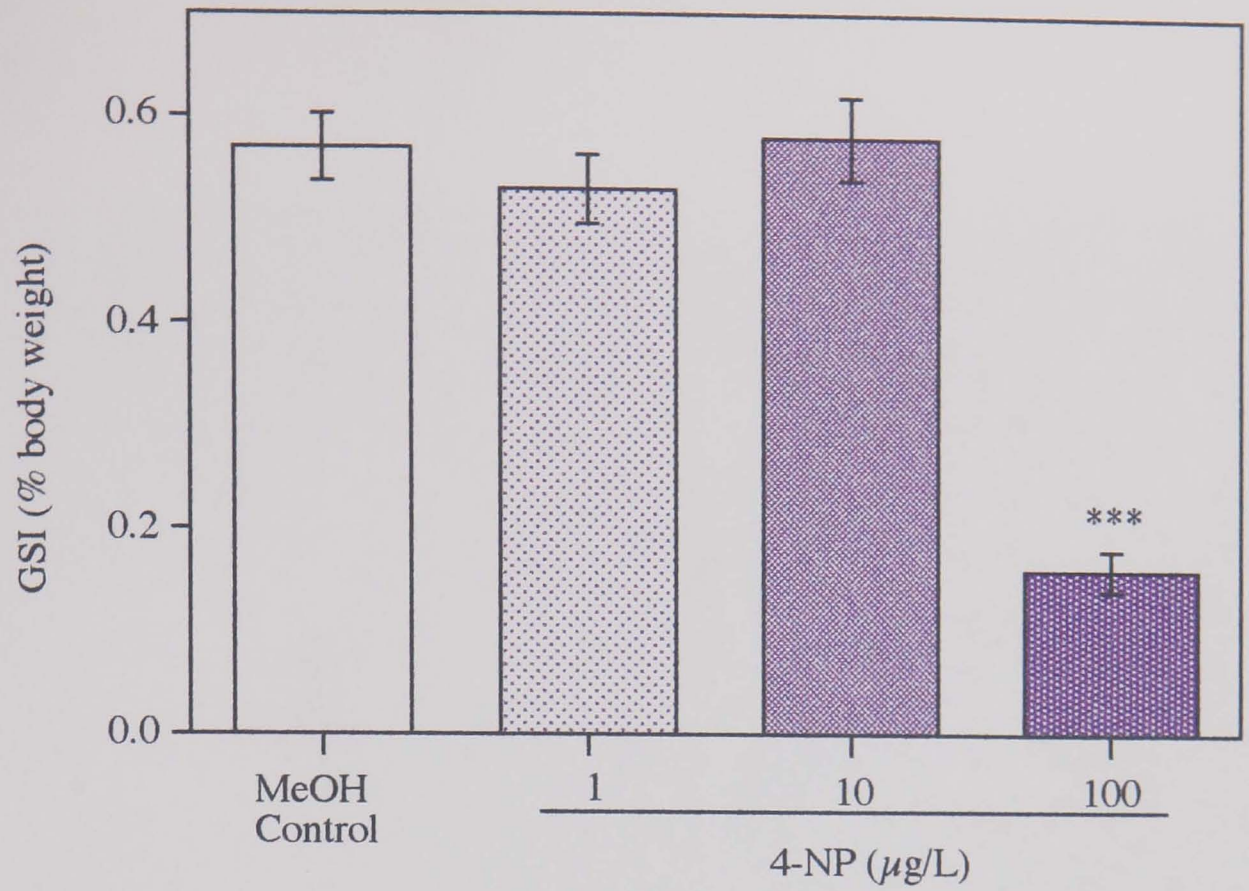


Figure 4.13
Gonadosomatic index of the fish at the end of the experiment. Significance from the control is denoted by *** $p<0.001$.

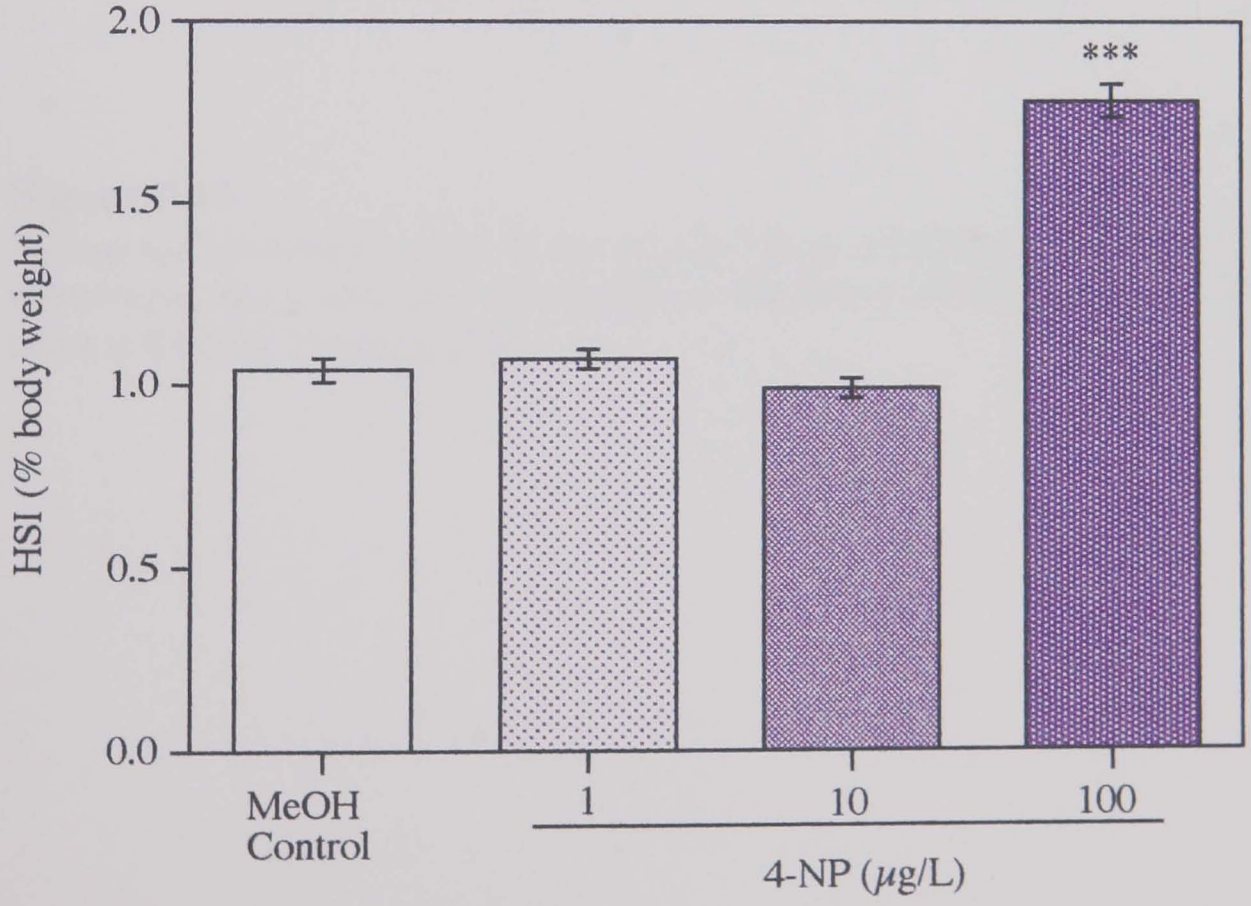


Figure 4.14
Hepatosomatic index of the fish at the end of the experiment. Significance from the control is denoted by *** $p<0.001$.

Figure 4.15
Photograph showing a typical ovary collected from the fish prior to the start of the experiment, along with one collected from each of the control and 100 μg 4-NP/L tanks at the final sampling point.

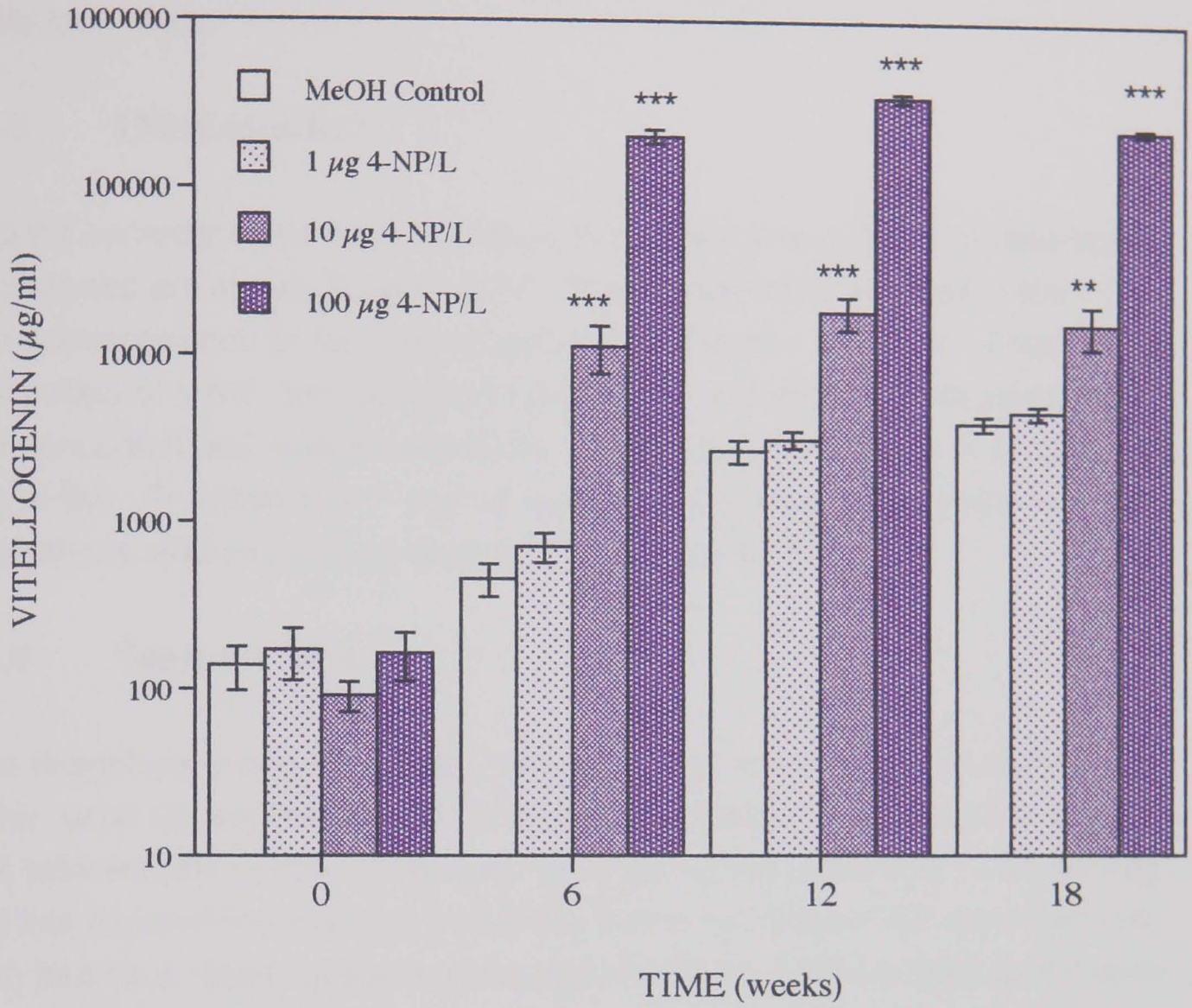


Figure 4.16
Plasma vitellogenin concentrations throughout the experiment.
Significance from the control is denoted by ** $p<0.01$; *** $p<0.001$.

measuring plasma vitellogenin. This set of data indicates that the 4-NP in these tanks was behaving, at least in this respect, in an estrogenic manner. Essentially, all of the effect has occurred after 6 weeks; thereafter, little further increase took place.

4.3.2.5 17ß-Estradiol

Plasma E2 concentrations were assessed throughout the study. The data from these analyses are shown in figure 4.17. There were no differences in the plasma concentrations of E2 between groups of fish prior to dosing. Following administration of 4-NP, fish exposed to the highest concentration had extremely low E2 concentrations, which were highly significantly lower those in all other groups of fish. Treatments of 1 and 10 µg 4-NP/L did not suppress plasma E2 concentrations relative to those observed in the control fish.

4.3.2.6 Testosterone

Plasma testosterone was not initially intended as an endpoint of this study. However, once the suppression of E2 had been noted, it was decided to assess plasma testosterone concentration in at least one of the timepoints. The thinking behind this decision involved the possibility that a decrease in E2 concentration could in part be a result of reduced aromatase activity, in which case an increase in plasma testosterone concentration would be observed. Figure 4.18, which portrays the testosterone concentrations measured after 6 weeks, indicates that the aforementioned theory is not the explanation for the low E2 levels observed in the fish. Testosterone concentrations were actually highly significantly ($p<0.001$) reduced in tank E, and were also significantly reduced ($p<0.05$) in tanks C and D. I therefore concluded that the suppression of E2 concentration in the treated fish was not a result of the adverse influence of 4-NP on aromatase activity.

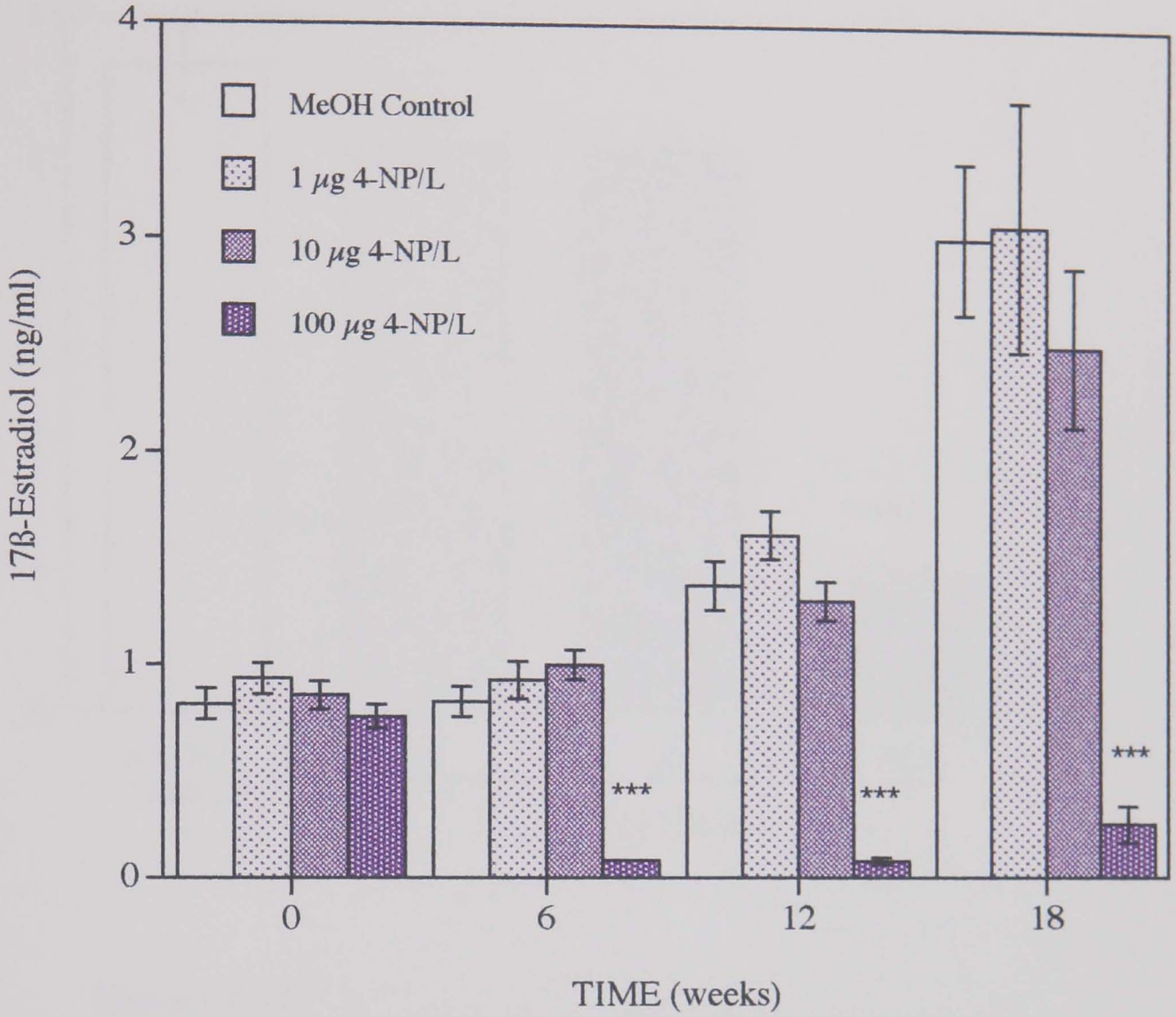


Figure 4.17
Plasma E2 concentrations throughout the experiment.
Significance from the control is denoted by *** $p<0.001$.

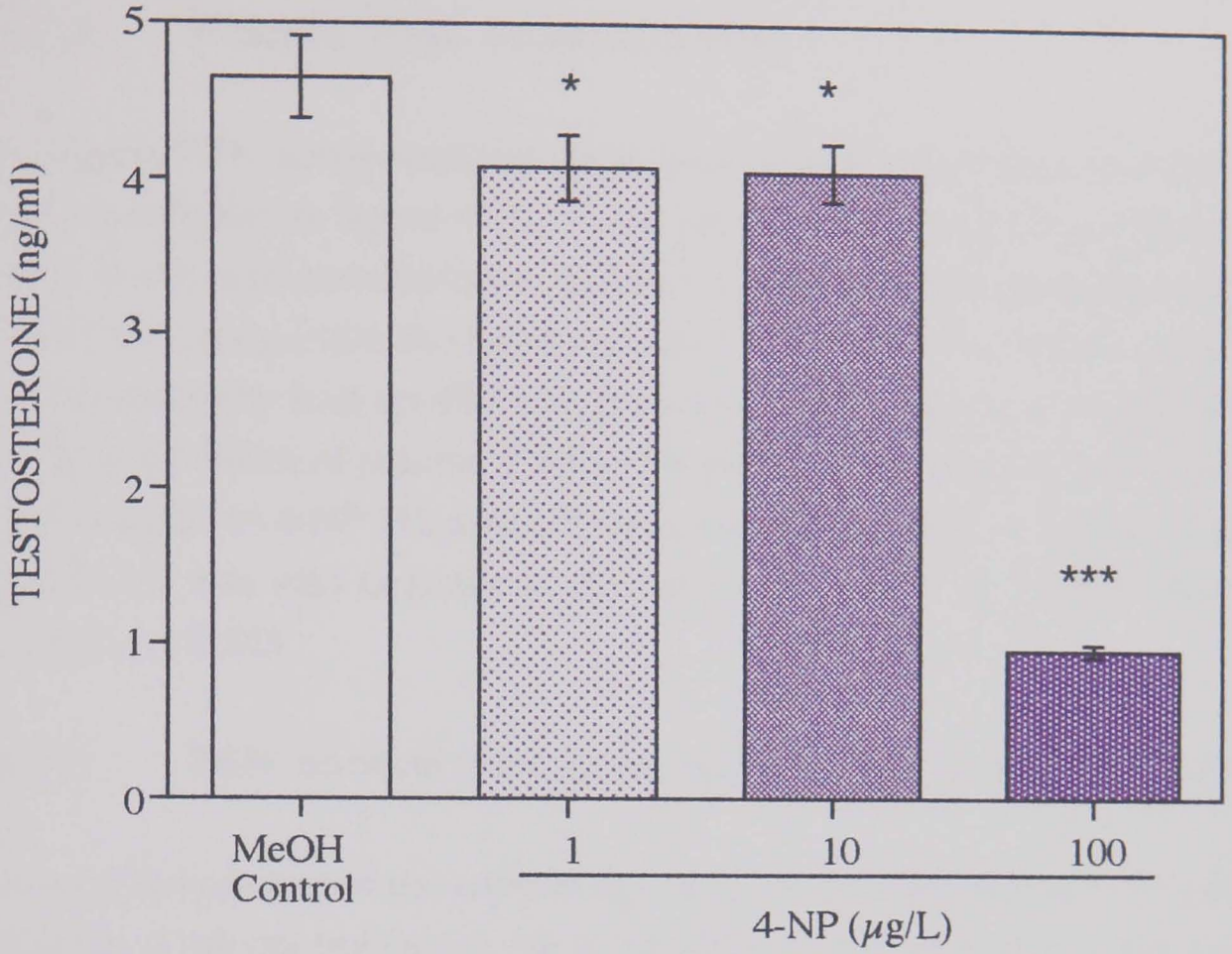


Figure 4.18
Plasma testosterone concentrations measured after 6 weeks of exposure. Significance from the control is denoted by * $p<0.05$; *** $p<0.001$.

4.3.3 The effect of 4-NP on gonadotropin concentrations in maturing female rainbow trout

4.3.3.1 Plasma FSH concentrations

The plasma FSH concentrations were measured at each timepoint in the study, and are presented in figure 4.19. In the last two sampling times (12 and 18 weeks), there was considerable variation in the FSH concentrations of individual fish within a group (note the large standard errors at these times). Nonetheless, 4-NP undoubtedly had an effect on plasma concentrations; a significant ($p<0.001$) inhibition of plasma FSH in the fish treated with the two highest concentrations of 4-NP (10 and 100 µg/L) was observed. A suppression of plasma FSH was also detected in the fish exposed to 1 µg 4-NP/L after 18 weeks exposure ($p<0.05$).

4.3.3.2 FSH content and gene expression in the pituitary

Pituitary FSH content is depicted in figure 4.20. The amount of FSH in the pituitaries of the control fish at the termination of the experiment was 14.8 ± 1.05 µg/pituitary. This figure corresponds well with the values reported by Gomez et al (1999), who found female rainbow trout pituitaries at the beginning of exogenous vitellogenesis to contain around 24 µg FSH/pituitary. This is the only study, as far as I know, to report these type of data.

The study revealed a clear and significant ($p<0.001$) inhibition of FSH synthesis in the fish maintained in the highest concentration of 4-NP (tank E). A nominal concentration of 10 µg 4-NP/L also led to reduced FSH content in the pituitaries of exposed fish ($p<0.01$).

The results of the dot blot analysis of FSH expression are expressed as arbitrary units of FSH mRNA / ß-actin mRNA ratios, and can be seen in figure 4.21. The pattern of gene expression of FSH is similar to that of the FSH content in the pituitary. FSH gene expression was highly significantly depressed ($p<0.001$) in the fish exposed to the highest concentration (100 µg/L) of 4-NP, and was also significantly lower in the fish exposed to 1 and 10 µg 4-NP/L.

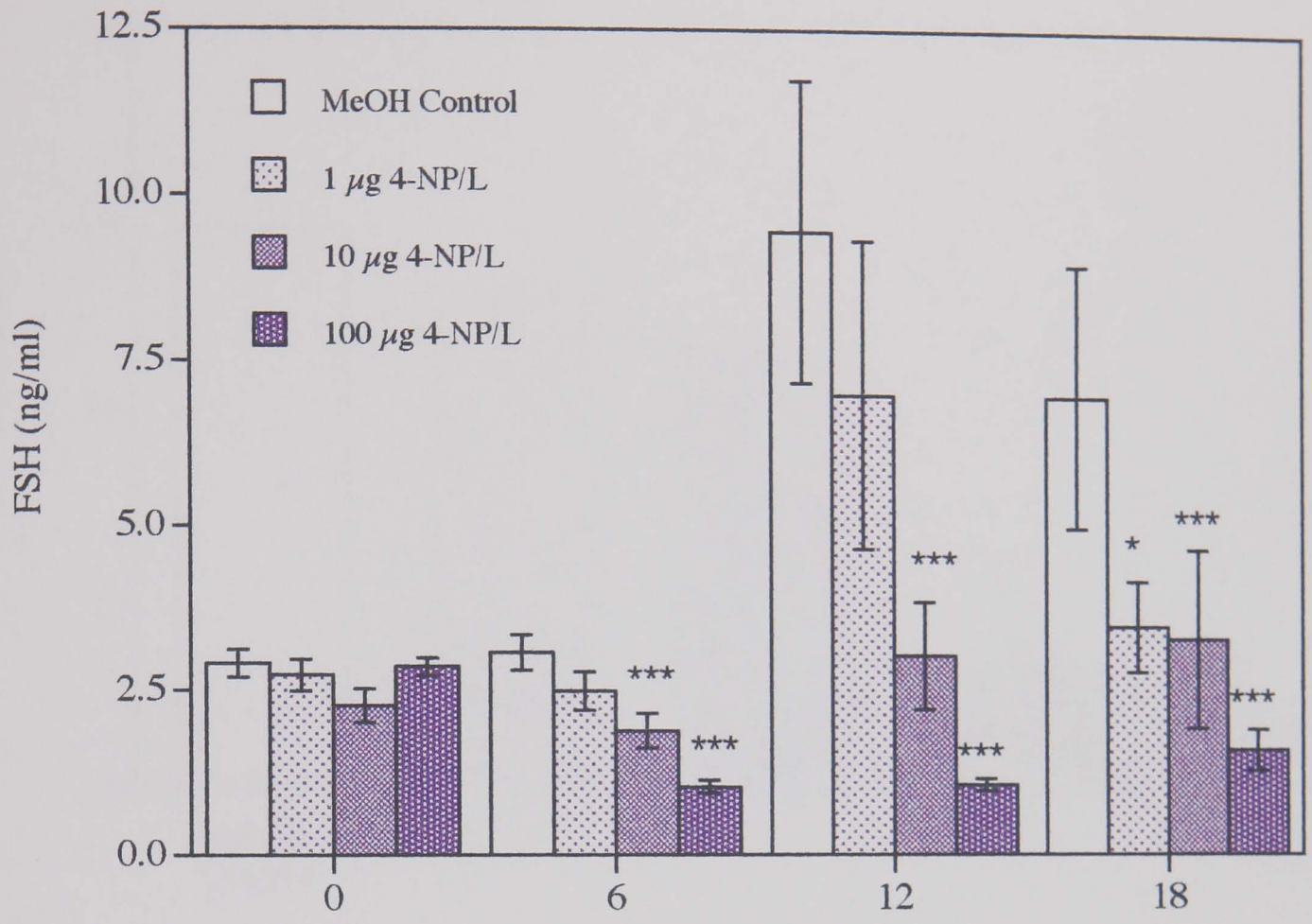


Figure 4.19
Plasma FSH concentrations throughout the experiment.
Significance from the control is denoted by * $p<0.05$; *** $p<0.001$.

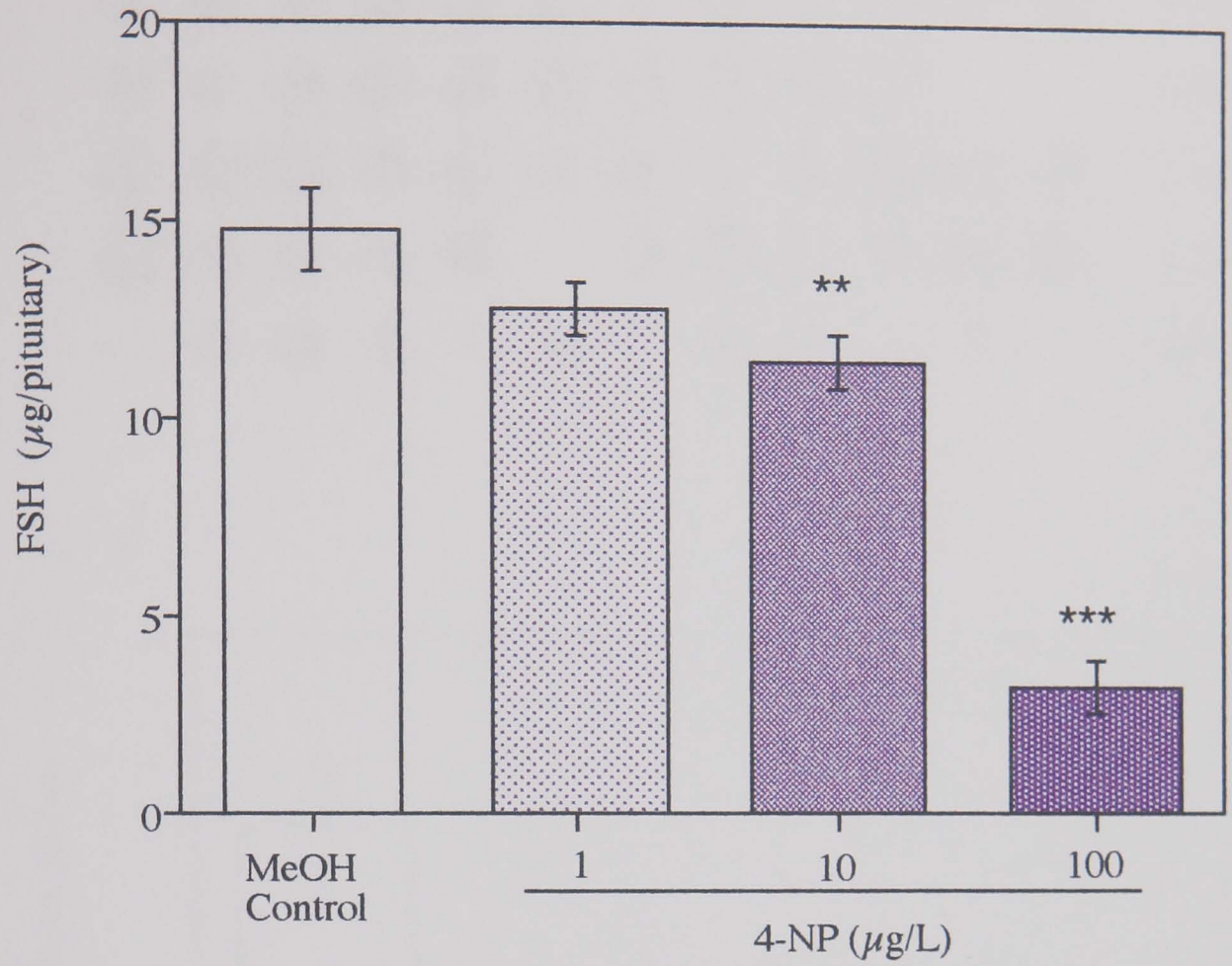
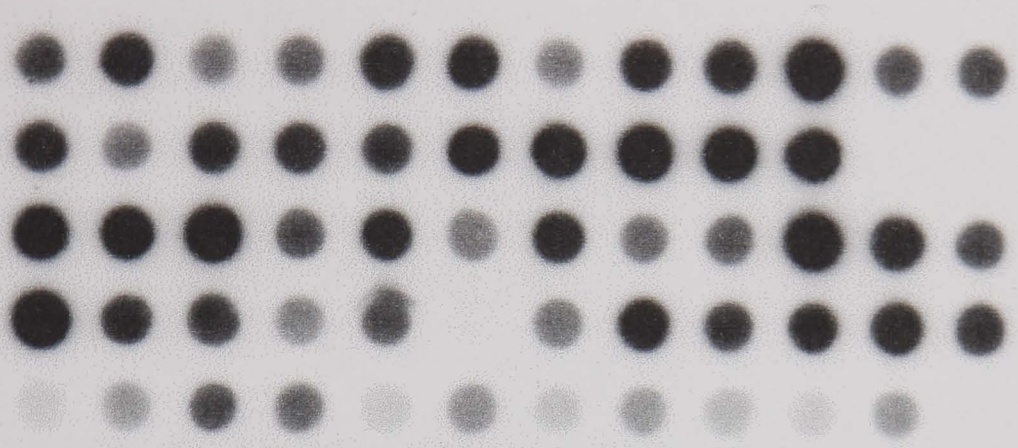


Figure 4.20
Pituitary FSH content at the end of the experiment.
Significance from the control is denoted by ** $p<0.01$; *** $p<0.001$.

A



Treatment

Absolute Control

MeOH Control

1 μg 4-NP/L

10 μg 4-NP/L

100 μg 4-NP/L

B

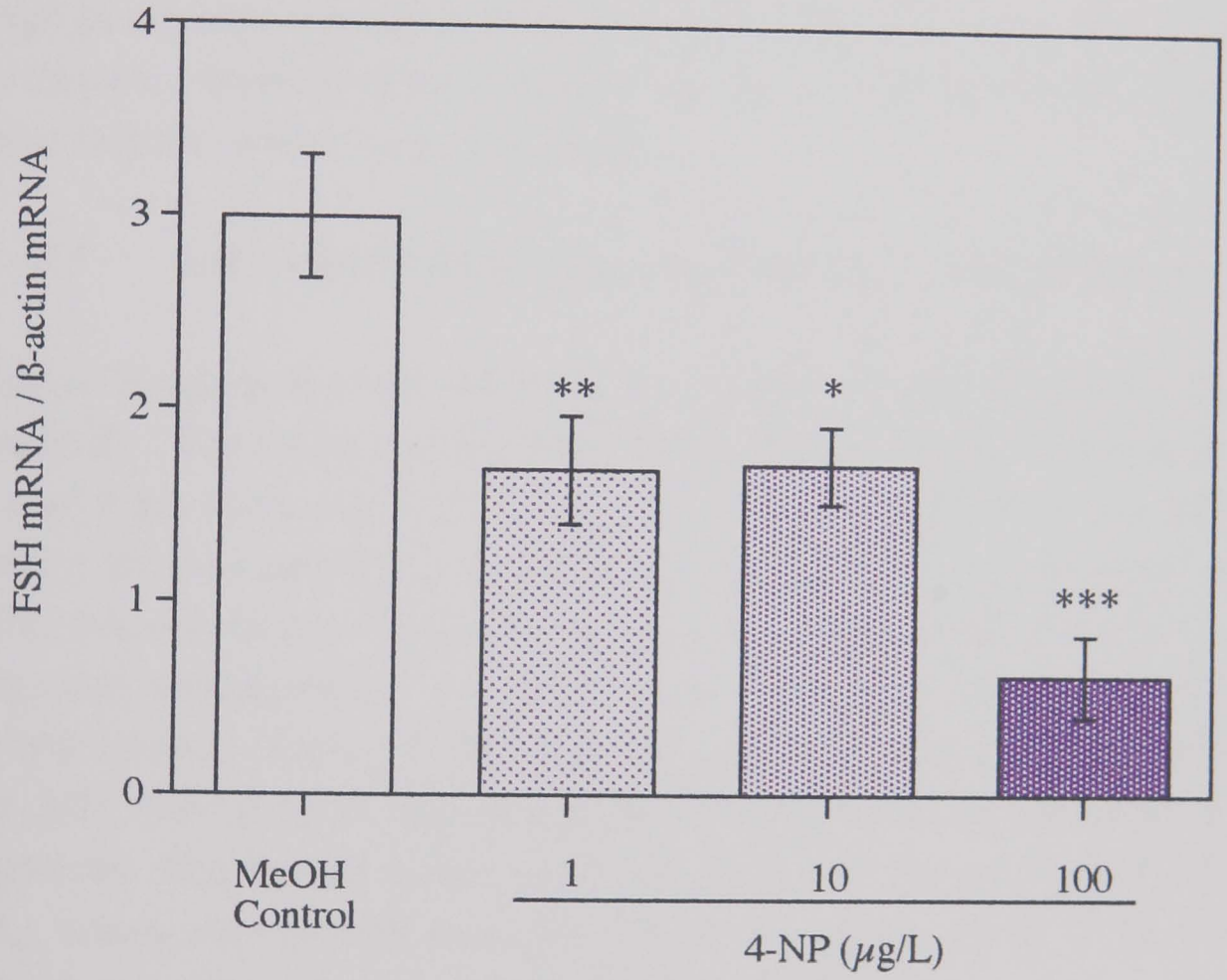


Figure 4.21

Figure 4.21A shows the results from the dot blot membrane, hybridised with FSH cDNA, in the form of a radiogram. Each row represents a different treatment tank, and each dot represents the expression of FSHß mRNA in an individual fish. Figure 4.21B shows these data (arbitrary units) corrected for the level of ß-actin expression. Significance from the control is denoted by * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

4.3.3.3 Plasma LH concentrations

Plasma LH concentrations in the fish, measured over the duration of the trial, are shown in figure 4.22. Mean plasma LH concentrations in the control fish remained low (around 0.15 - 0.25 ng/ml) for the duration of the experiment. These values are close to the detection limit of the assay (Prat et al, 1996). In contrast, the LH peak at ovulation can reach a concentration of around 70 ng/ml in rainbow trout (Prat et al, 1996).

In this study, the apparent effects of 4-NP on plasma LH concentrations were variable. For example, exposure to 4-NP appeared to increase LH concentrations at 6 weeks, but decrease it (in some, but not all groups) after 12 weeks, and have no effect after 18 weeks. In all cases these apparent changes, though statistically significant in at least some cases, were of small magnitude and degree, especially when viewed in the light of the very much higher LH concentrations which occur at ovulation.

4.3.3.4 LH content and gene expression in the pituitary

Figure 4.23 presents mean pituitary LH content in the fish at the termination of the experiment. The mean concentration of LH in the pituitaries of the control fish at the end of the study was 1.79 $\pm$ 0.42 μg/pituitary. As with the levels of FSH in the pituitary, the LH content also corresponds well with that observed by Gomez et al (1999), who reported LH levels to remain at $\leq$2 μg/pituitary until the end of exogenous vitellogenesis. A suppression of LH content was observed in treatment tank E (100 μg 4-NP/L), where the inhibition was highly significant ($p<0.001$), and where levels were suppressed by more than an order of magnitude. Pituitary LH content was also lower in the fish in tank D (10 μg 4-NP/L), where where levels were found to be less than half that of the control, although this difference was not statistically significant.

Results of dot blot analyses following hybridisation with LH cDNA are expressed as LH mRNA / ß-actin mRNA ratios, and are shown in figure 4.24. As with FSH, the pattern of LH gene expression is similar to the pattern of LH content in the pituitary. LH gene expression was highly significantly reduced in the fish exposed to 100 μg 4-NP/L, and was also significantly reduced in the fish exposed to 10 μg 4-NP/L.

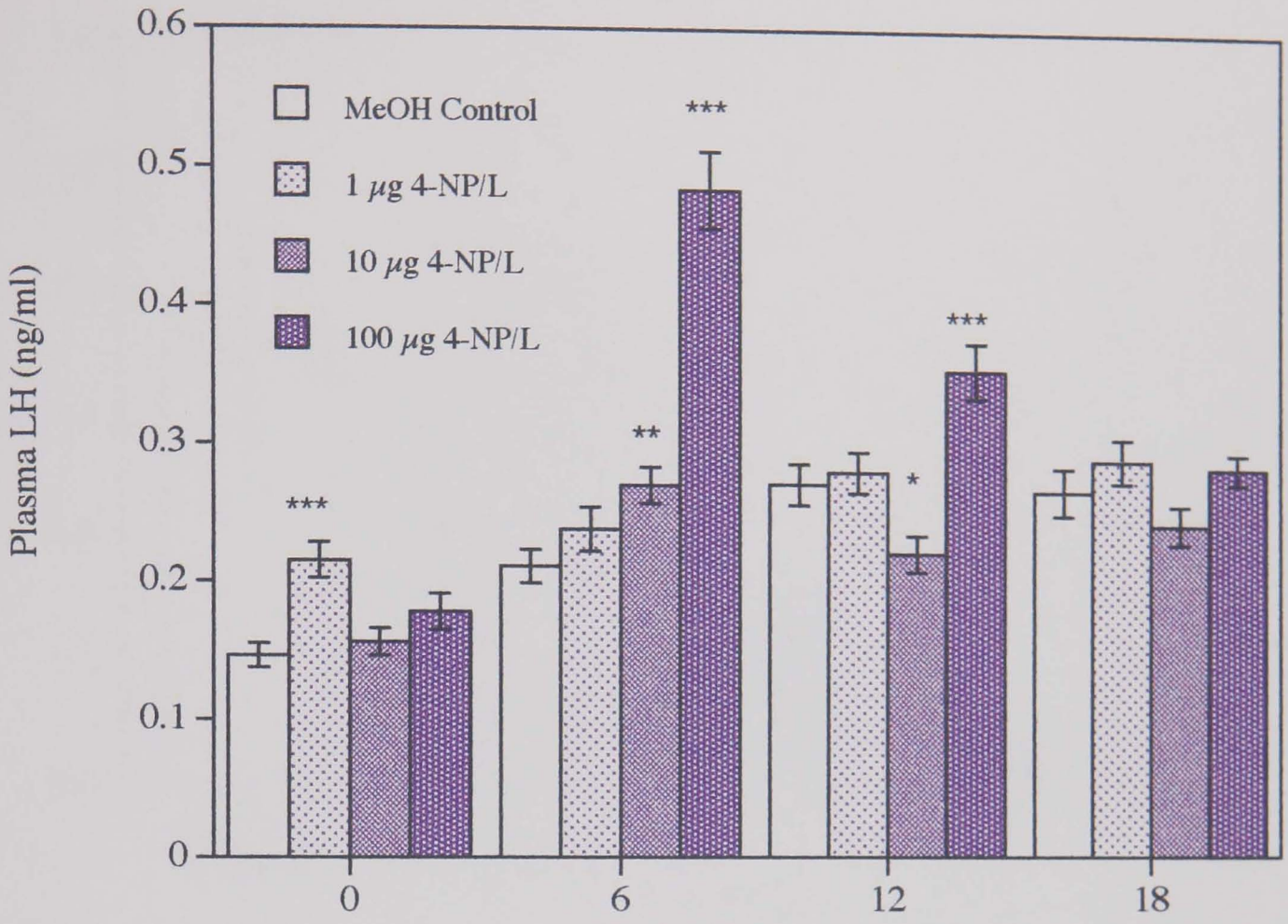


Figure 4.22
Plasma LH concentrations during the experiment.
Significance from the control is denoted by * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

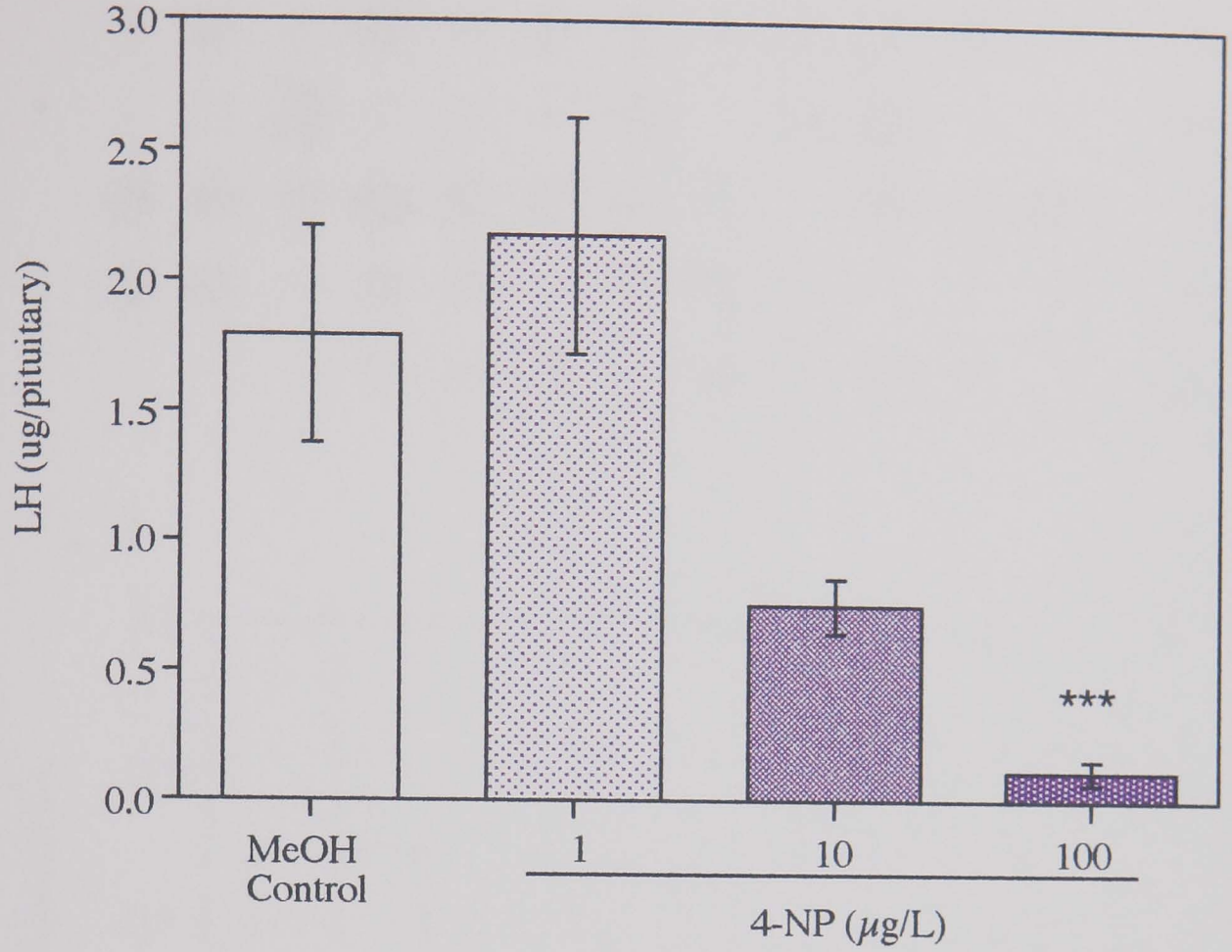


Figure 4.23
Pituitary LH content at the end of the experiment.
Significance from the control is denoted by *** $p<0.001$.

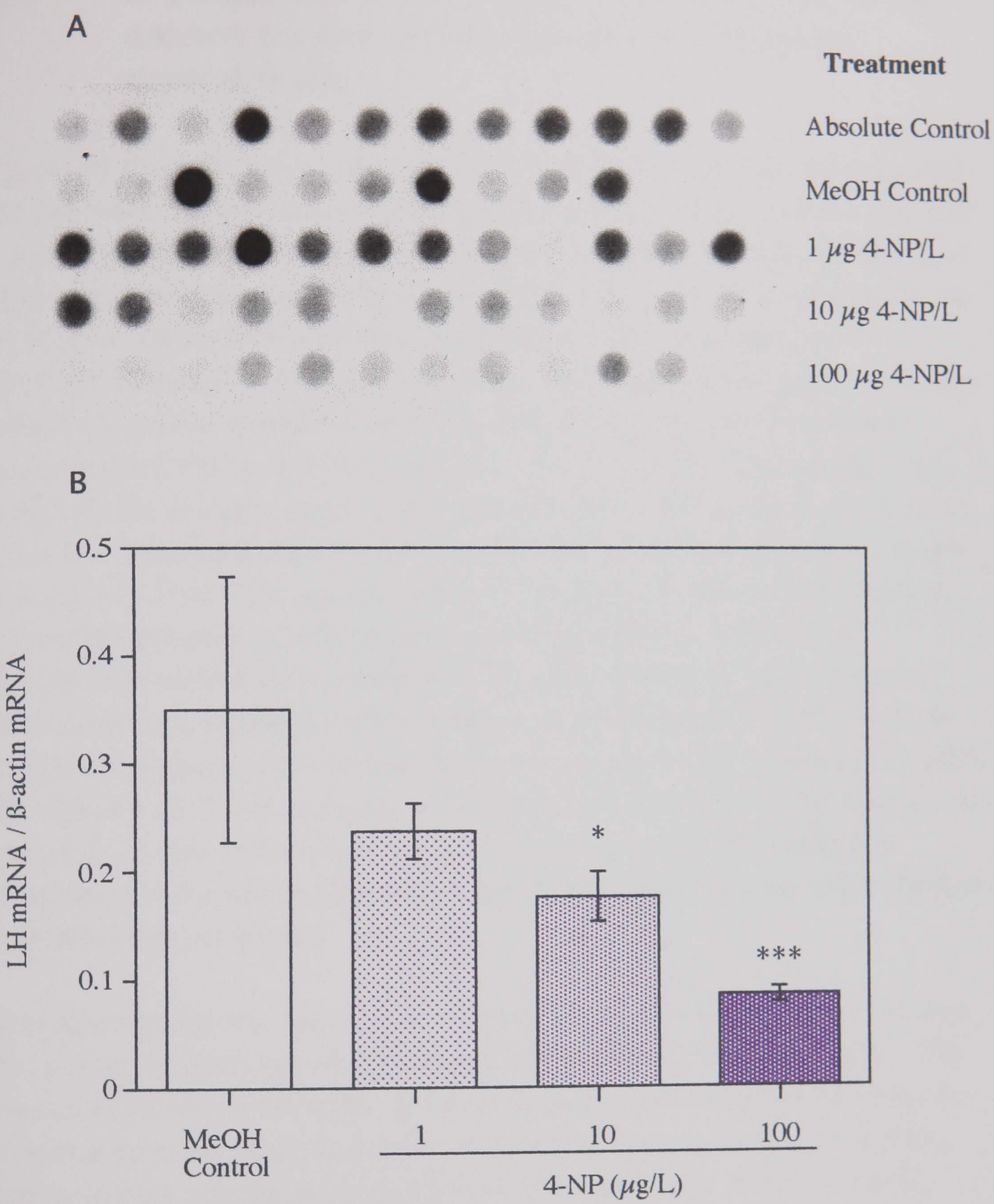


Figure 4.24
Figure 4.24A shows the results from the dot blot membrane, hybridised with LH cDNA, in the form of a radiogram. Each row represents a different treatment tank, and each dot represents the expression of LHß mRNA in an individual fish. Figure 4.24B shows these data (arbitrary units) corrected for the level of ß-actin expression. Significance from the control is denoted by * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

4.3.4 A comparison between the fish maintained in the solvent control versus those in the absolute control tanks

Although an absolute control tank was included in this study, data obtained from these fish have not been shown in the graphs presented so far. It was found that the solvent used (methanol), although commonly employed in such studies, and despite being used at a concentration below the maximum recommended by the EPA (Zucker, 1985), did in fact have a significant effect on some of the parameters assessed in this experiment. For this reason, and to avoid confusion, all data from the fish exposed to 4-NP have been compared with the correct scientific control, that is, with the solvent control. Some of the data comparing results from the absolute control with those from the solvent control are shown in figure 4.25. Essentially, MeOH had no impact on somatic parameters, such as K-factor (figure 4.25A), GSI, and HSI (data not shown), and also did not influence the gonadotropin content in the pituitary (data not shown). However, a suppression of plasma E2 concentration was observed in the fish exposed to MeOH alone compared to those in the absolute control (figure 4.25B). Also, an increase in the plasma FSH concentration occurred in the fish exposed to MeOH alone (figure 4.25C); this increase in FSH may well be related to the decrease in plasma E2, as may be the slight but significant drop in plasma vitellogenin concentration in the MeOH control fish compared to that observed in the absolute control fish (data not shown).

These data highlight the necessity for the inclusion of solvent controls in studies such as ours, in which sensitive hormonal endpoints are being monitored. The presence of solvent in the water, although apparently artificially challenging the fish compared to the absolute control, is not altogether unrealistic, since many effluents contain detectable concentrations of solvents (e.g. Kirchmann et al, 1991; Clark et al, 1991).

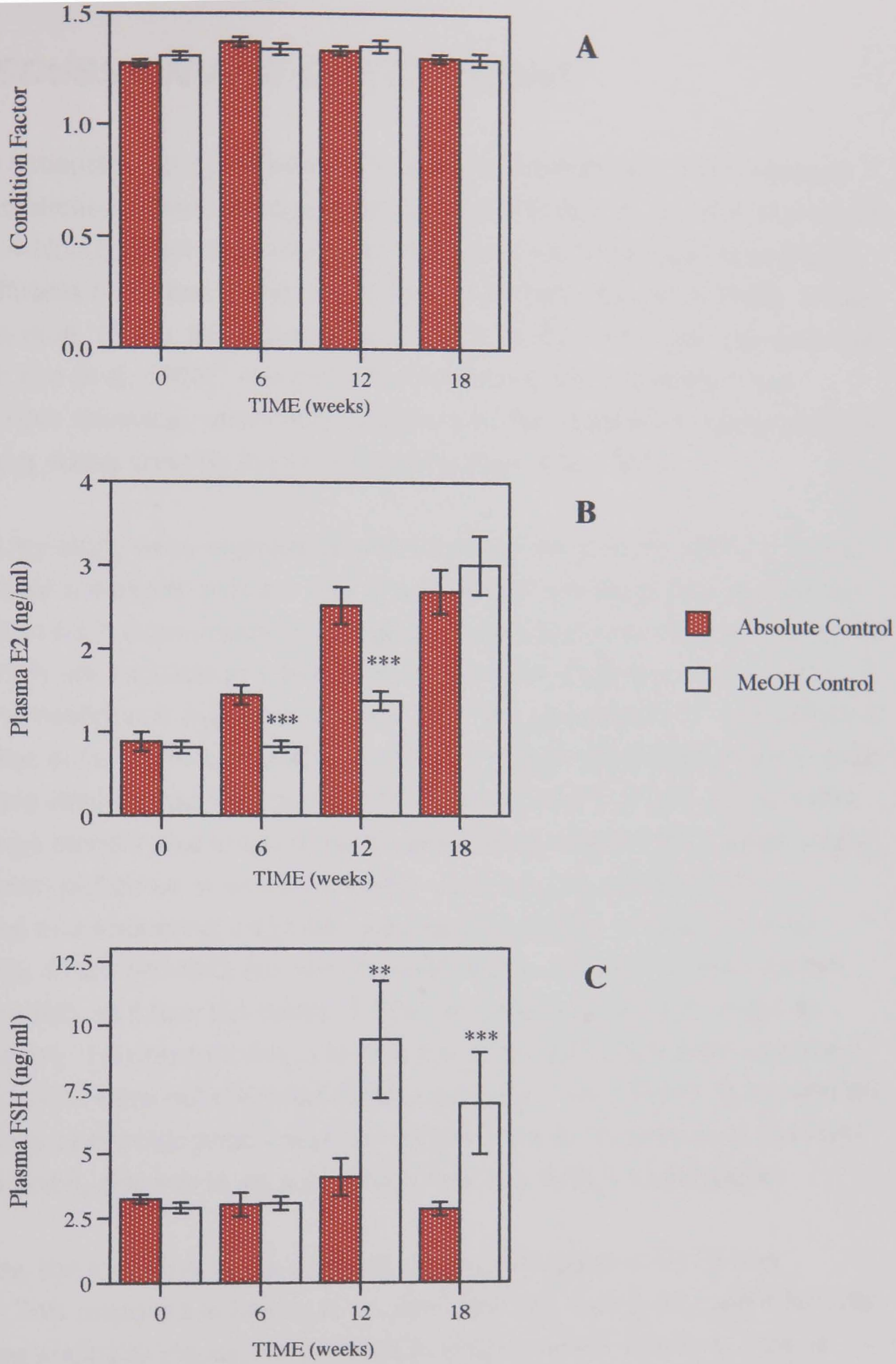


Figure 4.25
Comparison between some of the parameters measured in the fish in the absolute control and solvent (MeOH) control tanks. Significance between the two treatments is denoted by ** $p<0.01$; *** $p<0.001$.

4.6 *DISCUSSION AND CONCLUSIONS*

It is widely accepted that some sewage treatment effluents which are released into water courses possess estrogenic properties (Purdom et al, 1994; Harries et al, 1996). 4-Nonylphenol is an industrial chemical which has been detected in sewage effluents (Ahel and Giger, 1985; Pirie et al; 1996; Lye et al 1999), in river water (Ahel et al, 1994b; Blackburn and Waldock, 1995) and in fish tissues (Ahel et al, 1993; Lye et al, 1999). It is clear that some wild fish populations are exposed to this chemical, which may contribute to the estrogenic activity of some effluents (e.g. those entering the River Aire; Harries et al, 1997).

The fish in my study were exposed to various concentrations of 4-NP (1, 10 and 100 µg/L) over a 4 month period. This is a far longer exposure than is normally undertaken in such experiments, although to understand what happens to fish in the wild which are exposed to 4-NP, even longer term studies are required to simulate the continuous exposure to which wild fish are subjected. In addition, a large number of fish were sampled at each timepoint (n=30 per treatment in most cases); many similar experiments use a sample size of 4 to 8 fish. It is possible that this large sample size allowed me to detect more subtle effects which might not have been picked up in smaller studies. Furthermore, a wider suite of reproductive and endocrine endpoints was assessed than is typical in such experiments, which provided me with an overview of the general reproductive status of the fish, and how the effect of 4-NP on certain parameters might be linked to others. Having said this, it is also true that 4-NP might have effects on reproduction that were not detected in this study, such as influencing expression and/or activity of steroidogenic enzymes. Effects of such chemicals at this level remain unknown, and this is an area which requires further investigation.

In this study, the induction of the egg yolk protein vitellogenin (VTG) was observed. This response is known to be stimulated by estrogens, and 4-NP has been demonstrated to induce the production of this protein in vivo in male or immature female rainbow trout (Lech et al, 1996; Jobling et al, 1996). The highest nominal concentration of 4-NP employed by Jobling et al was 65 µg/L (mean measured concentration 54.3 µg/L). Although only a three week exposure was undertaken in that study, a 10,000-fold increase in plasma VTG concentration was observed. In the present study, the increase was approximately 1000-fold in response to the highest concentration, although my

study used female fish which had a higher initial concentration of VTG compared to that of the male fish used in the study by Jobling et al (1996). To date, the induction of vitellogenin by 4-NP in maturing female rainbow trout has not been fully characterised, but the data from my study indicate that the fish had been exposed to an estrogenic influence. The level of VTG reached in the tank with the highest concentration of 4-NP (in 100's of mg/ml) was far higher than maximal concentrations found in female rainbow trout during ovarian development (which is between 50 and 100 mg/ml, see section 4.3.2.4). This may be a reason for the high mortality I observed in these fish, as has also been observed in juvenile females (Herman and Kincaid, 1988). In that study, excess vitellogenin (induced by exposure to exogenous E2) was found to accumulate in the liver and kidney, thus impairing the function of these organs. It is unknown how the apparent toxicity observed in my experiment may have affected other parameters measured over the course of the experiment. In addition to monitoring gonadotropin concentrations, several other endpoints were assessed, which may help to explain whether any observed adverse effects were due to the endocrine disrupting properties of 4-NP, or simply due to a toxic effect of unknown mechanism. Nevertheless, the size of the remaining fish, although apparently smaller overall, was not much different to that of the control fish, and the weight of the fish was only significantly different at the earlier timepoints in the study. Thus, even the highest concentration of 4-NP I employed had little effect on growth. An effect of 4-NP on growth of juvenile female rainbow trout was demonstrated by Ashfield et al (1998). They reported that 4-NP at 10 and 30 μg/L suppressed growth of these fish, with the effect at 30 μg/L appearing to be permanent, and persisting for over a year. However, the fish were exposed from an extremely young age (from hatch), and it is therefore likely that they were more sensitive to factors which may influence growth than the adults which were used in our experiment.

The coefficient of condition might be expected to decrease in stressed fish (Fagerlund et al, 1981), but the coefficient of condition of the fish exposed to the highest concentration of 4-NP was, in fact, slightly higher (although not significantly so) than that of the control fish at the end of the experiment. A lower coefficient of condition can be interpreted as representing depleted energy reserves of the fish. As well as being influenced by stressors, this can also change seasonally; for example, as a fish approaches sexual maturity, the focus of energy storage alters, as resources are diverted into gonadal growth. This

may be one explanation for the higher coefficient of condition of the fish in tank E; since there was no gonadal development, the stored energy of these fish was not being consumed by ovarian maturation, but instead continued to be used for somatic growth. In any case, the lack of any decrease in coefficient of condition of the fish exposed to 4-NP strongly suggests that these fish were normal and healthy; that is, that they clearly were not moribund.

The HSI can increase in response to stress, particularly pollutant stress, as the liver's p450 system becomes activated to deal with such foreign influences. In contrast, a drop in HSI can sometimes be seen in cases of chronic stress, due to starvation, or increased metabolic rate. However, during the reproductive cycle of females, the HSI normally increases as a result of the increased synthesis of vitellogenin, and consequently increased tissue mass to manufacture this protein (van Bohemen et al, 1981, 1982). There was evidence of increased vitellogenin production in the fish maintained in 10 μg 4-NP/L, and I might also have expected to see a concurrent increase in HSI of these fish. However, the only treatment which led to a higher HSI was 100 μg 4-NP/L.

The experiment was started when the fish were at the pre-vitellogenic stage of reproduction (see figure 4.26 for a schematic representation of the stages of oocyte development and approximate relationship with plasma GTH concentration and GSI). Subsequently, the ovaries in all fish, except those in tank E, increased in size as the oocytes sequestered vitellogenin during the early phase of vitellogenesis. The GSI of the fish in tank E, however, did not increase in size over the period of the experiment. It is thought that FSH plays a role in mediating oocyte recruitment into the maturing pool, either before or during early vitellogenesis (Tyler and Sumpter, 1996; Tyler et al, 1997). In addition, it has been demonstrated that FSH stimulates vitellogenic uptake into rainbow trout oocytes at the mid-vitellogenic stage (Tyler et al, 1991). It is therefore possible that the lack of growth of the ovaries in the fish exposed to a nominal concentration of 100 μg 4-NP/L can be attributed to the suppression of the plasma FSH concentration by this chemical. The absolute weight of the ovaries increased slightly during the experiment, from 1.0 ± 0.05 g at the beginning of the experiment, to 1.2 ± 0.162 g at the terminal sample, in the fish maintained in tank E. However, the weight of the ovaries relative to body weight (GSI) did not change over this period, and the slight increase in absolute mass can

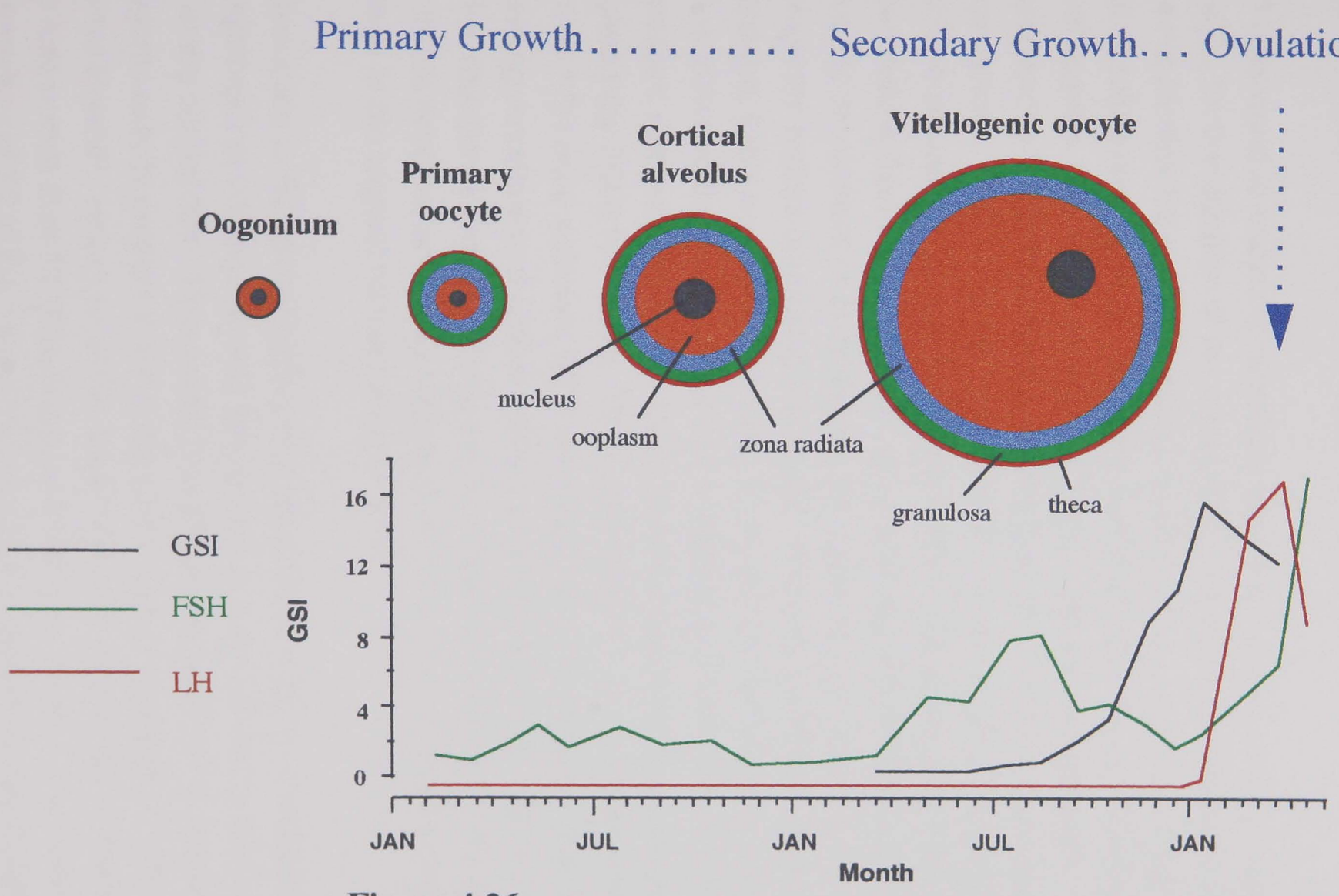


Figure 4.26
Schematic diagram illustrating the seasonal profiles of gonadotropins and GSI in female rainbow trout in relation to oocyte development.

presumably be attributed to normal somatic growth. Van den Hurk and Slof (1981) demonstrated that ovarian weight of female rainbow trout increases in proportion to the size of the fish, and therefore a small increase in ovarian mass of non-maturing fish might be expected. Overall, I conclude that, despite the very high VTG concentrations in the fish exposed to the highest concentration of 4-NP, no vitellogenin was sequestered by the oocytes, and hence the ovary did not grow, as it would normally have done at this time.

4-NP was found to markedly suppress plasma E2 levels in the fish exposed to 100 μg/L, for the duration of the experiment. However, MeOH alone was found to suppress plasma E2 concentrations relative to those observed in fish maintained in the absolute control tank. This pattern of effect is similar to that observed with the vitellogenin data (data not shown); in fact, the lower vitellogenin concentrations in the fish exposed to MeOH probably reflect the suppressed E2 concentrations. This is not the case with the fish exposed to 100 μg 4-NP/L, which had elevated vitellogenin concentrations, despite having lower E2 concentrations than the control fish. As discussed above, FSH is thought to induce the recruitment of oocytes into the maturing pool (Tyler et al, 1997). Although the factors controlling this aspect of oocyte development are not yet understood, if this were to be the case, it is possible that very few, if any, oocytes were recruited in the fish exposed to the highest concentration of 4-NP, due to suppressed plasma FSH levels. Once recruited, the maturing follicles are stimulated (by FSH) to produce E2 (in mammals, E2 has a negative feedback effect on FSH once sufficient E2 is produced from a maturing follicle, thus preventing recruitment of further follicles, e.g. Zeleznik et al, 1985; it seems likely that the situation is similar in fish), but non-maturing oocytes will not produce E2. This is one explanation for the suppression of E2 levels demonstrated in the fish exposed to the highest concentration of 4-NP.

The reduction in E2 concentration also provides us with an interesting anomaly. The synthesis of vitellogenin was greater in the fish exposed to 100 μg 4-NP/L than in the control fish. This would, theoretically, mean that the amount of estrogenic activity present in the fish's blood system was greater in the fish exposed to 4-NP. However, 4-NP, when tested in vitro, is approximately 10,000 times less potent than E2 (Routledge and Sumpter, 1996). The mean concentration of E2 in the control fish reached a value of around 3 ng/ml (≡ 3 μg/L) and, therefore, were the mechanism of action of 4-NP to be purely an

'estrogenic' one, the concentration of 4-NP in the plasma would be expected to attain a value of around 30,000 µg/L (= 30 mg/L) before the estrogenic response was equal to that in the control fish. No analyses were undertaken on the chemical (4-NP) content of the blood, and hence the actual 4-NP concentration in the fish remains unknown. There are several possible explanations to account for why a weak estrogen such as 4-NP is considerably more potent as an estrogen in vivo than one might initially think; these are:-

1. That the BCF of 4-NP over the duration of the experiment was >300, and therefore the concentration of 4-NP in the blood did in fact reach around 30,000 µg/L.
2. That, in the control fish, a high proportion of E2 in the blood was unavailable, due to it being bound to sex hormone binding globulin (SHBG), whereas the fish exposed to 4-NP had higher 'free' E2 levels, because 4-NP has been found to displace E2 from SHBG in vitro (Danzo, 1997), therefore theoretically increasing the available E2 in the blood of exposed fish.
3. That the 4-NP in the fish became more concentrated in the livers of the fish than elsewhere (as has been reported: Ahel et al, 1993; Lewis and Lech, 1996), which is where vitellogenin synthesis takes place.
4. That 4-NP was working in conjunction with the residual E2 in the system to induce a greater response than would be expected if it was stimulating VTG synthesis on its own.
5. That 4-NP, relative to E2, is more potent (for an unknown reason/s) in vivo than in vitro.

Plasma LH concentrations in the fish in my study essentially remained at baseline concentrations throughout the experiment (compared with the data presented by Prat et al, 1996). Other studies have found similar patterns of LH (GTH II) in maturing female rainbow trout to that reported by Prat et al. Plasma LH concentrations remain at levels close to or less than the detection limit for most of the cycle, rising to around 15 ng/ml (Gomez et al, 1999), or over 40 ng/ml (Sumpter & Scott, 1989) during final maturation. Although it appears from several studies that E2 has a positive feedback effect on LH synthesis in immature / early maturing fish, but a negative effect in mature / spawning fish, it is not entirely clear at what precise stage the transition occurs. In the majority of studies with immature fish, induction of LH mRNA, or of pituitary LH content, has been shown to be enhanced by E2, whereas the plasma LH concentration remains undetectable. This contrasts with my data, where a clear dose-response

indicates inhibition of pituitary LH synthesis together with inhibited LH gene expression (after 18 weeks exposure), although a small increase in plasma LH was observed after 6 weeks exposure (but not subsequently). Since pituitary samples were not collected at each timepoint, particularly at the time of increased plasma LH concentration, this scenario is difficult to interpret. It is, therefore, possible that at the time of the increased plasma LH concentration, pituitary LH synthesis was also stimulated, and that subsequently the negative feedback pathway was activated (as described by Saligaut et al, 1999), thus depleting LH reserves in the pituitary. This theory, however, is based on the inhibitory influence of dopamine, which has been found not to alter the steady state pituitary LH levels in mature tilapia pituitary cells, and therefore was thought to act only on the release of this hormone (Melamed et al, 1998). The differential effect between the (increased) plasma levels compared with the (decreased) pituitary levels might suggest that the controlling factor is acting on release and, therefore, the theory of activation of the DA inhibitory tone is a possible explanation, but it is also evident that the NP treatment is, in some (unknown) way, interfering with the synthesis of LH in the pituitary. In addition, if DA acts only on the release of LH, and not on its synthesis, then the suggestion proposed by Saligaut et al (1999) does not fit with the data presented by Breton et al (1997), Dickey and Swanson (1998), and Amano et al (1997), who all showed that it was the pituitary LH concentration which was stimulated by E2 in immature fish, and not the plasma concentration. Another possibility is that the decreased endogenous plasma E2 levels were not sufficient to induce LH synthesis in the fish exposed to 100 μg 4-NP/L, and/or that 4-NP was acting as an anti-estrogen in this respect, although there is no additional evidence to suggest this is the case. In any case, LH concentrations in the fish in my experiment were increased by such a minor absolute amount that it is most unlikely that this increase would have had a physiological impact on the fish concerned.

Very few studies have investigated the influence of environmental estrogens on gonadotropin levels in fish. Khan and Thomas (1998) found that o,p'-DDT (a relatively weak environmental estrogen; Routledge and Sumpter, 1996) stimulated the release of LH in early recrudescing female atlantic croaker. E2 (in a separate experiment) also enhanced plasma LH concentrations. These data indicate a similar effect to that seen in our study, in which LH release appears to be affected by an environmental estrogen. Pituitary LH content was not assessed in the study by Khan and Thomas (1998), nor were pituitary or plasma

FSH concentrations measured, because no assay is available presently for FSH in this species. In addition, van Baal et al (2000) measured pituitary LH content in juvenile catfish following exposure to a single dose of 4-NP (10 µg/L). No changes in plasma LH were observed, and no significant increase in pituitary LH occurred in female fish (although pituitary LH content was increased in the male fish in that experiment). Zilberstein et al (2000) also exposed maturing fish to 10 µg 4-NP/L (in this case, male tilapia). They found that FSHß mRNA levels were suppressed by 4-NP. In contrast, LHß mRNA levels were not affected, although LH release was stimulated in vitro in pituitary cell cultures by 4-NP (Zilberstein et al, 2000). With respect to mammals, alkylphenols (specifically octylphenol) have been shown to suppress FSH concentrations in prenatally exposed sheep (Brooks et al, 1996) and in rats (Blake and Boockfor, 1997). The latter study also demonstrated a decrease in LH levels, in both plasma and the pituitary gland. Thus, my results agree with those reported for mammals exposed to an alkylphenol.

In the control fish, mean plasma FSH concentration reached a high of 9.46 ng/ml. This lies in the same range as, although is slightly lower than, that reported by Prat et al (1996). They found maximal FSH concentrations, during early gonadal development, of 17 ng/ml. Gomez et al (1999) have also studied plasma and pituitary levels in rainbow trout, and found the plasma FSH concentration in maturing females to peak (during the early vitellogenic stage) at around 14 ng/ml, which is similar to the value reported by Prat et al (1996). In my study, the variation of FSH concentration between individual fish in the control tank was large, and after 12 weeks exposure, there were a number of individual fish with a plasma FSH concentration exceeding 10 ng/ml (with a maximum in one fish of 55 ng/ml), although the majority had lower levels. It is possible that, despite the GSI of the fish at termination being very similar to the GSI of fish having the highest FSH concentrations in the Prat et al study, the fish were sampled after the time when their FSH concentrations had reached their maxima. This idea is supported by the fact that two thirds of the fish in the control tank had lower FSH concentrations at 18 weeks compared to 12 weeks, but it is also possible that the peak FSH concentration in the fish used in this experiment was less than that demonstrated in the fish used by Prat et al (1996). Nonetheless, the level of FSH in the plasma of the fish exposed to 100 µg 4-NP/L was suppressed compared to that of the control fish. Pituitary FSH content was also depleted, although the quantity of FSH in the pituitaries of the fish in tank E (mean = 3.2 ± 0.68

μg/pituitary) still appears to be, in effect, a huge reservoir of this hormone, as the amounts found are 1000-fold greater than that in 1 ml of plasma, however, the rates of release/degradation to/in the blood are not known. The pituitary FSH levels appear to have been affected directly at the level of gene expression. as observed by the similar pattern of response of these two parameters in the exposed fish compared to the control fish. These data indicate a negative feedback response of FSH to the estrogen-mimic, 4-NP. This fits well with the data from the small number of studies which have demonstrated E2 to inhibit either FSH synthesis (Breton et al, 1997) or secretion (Saligaut et al, 1998) in rainbow trout, or FSH secretion in coho salmon (Dickey and Swanson, 1998). The mechanisms behind this regulatory control have not been fully investigated, primarily because the measurement of FSH itself has only become possible in the last decade, and then in only a few species. Saligaut et al (1998) suggested that, although LH is influenced by the inhibitory actions of DA, FSH is not. It is possible that the production of FSH is directly regulated by E2, but to date this has not been determined. In mammals, the secretion of FSH is controlled primarily by GnRH, but this has not yet been clearly established in teleost species, although sGnRH has been found to stimulate secretion of FSH and also to enhance FSH subunit expression in coho salmon pituitary cell cultures (Dickey and Swanson, 2000), and Weil et al (1999) found both FSH and LH cells to be responsive to GnRH. These data are in contrast with those presented by Breton et al (1998), who found that GnRH could not stimulate FSH secretion in rainbow trout, and Kitahashi et al (1998), who demonstrated the lack of change in levels of FSHß mRNA in male and female sockeye salmon in response to GnRH-analog stimulation. It is probable that FSH and LH are regulated by different factors, the nature and extent of which have not yet been elucidated.

CONCLUSIONS

In this study, I exposed maturing female rainbow trout to a widely distributed aquatic pollutant, 4-NP, at a critical stage of ovarian development. The highest concentration of this chemical employed in the study essentially shut off reproduction altogether; vitellogenin was produced in large quantities, but was not sequestered by the oocytes, which consequently did not develop further. This would obviously depress fecundity of these fish. The primary reason for this

may have been the suppression of FSH synthesis (in the pituitary) and/or release (to the plasma) by 4-NP at this concentration, since FSH is thought to mediate oocyte recruitment (Tyler et al,1997), and also stimulates uptake of vitellogenin into developing oocytes (Tyler et al, 1991). Lower concentrations of 4-NP, although capable of suppressing the concentration of FSH in the plasma, at least temporarily, did not affect the overall development of the ovary, in so far as the GSI did not differ from that of the control fish. Although the mechanisms underlying these responses are unknown, this study undoubtedly demonstrates a feedback response of gonadotropic hormones to an environmental hormone mimic. Thus, environmental estrogens probably have multiple sites of action in fish.

CHAPTER 5

GENERAL DISCUSSION

Prior to this work, phthalates as a class of chemicals were regarded by many in the media, public, and even in some cases, scientific arenas, as estrogen mimics. The data described in chapter 3 indicate that:-

1. Not all of the phthalates exhibit estrogenic activity in vitro.
2. Those that do are among the weakest xenoestrogens (relative to E2) assessed in such assays to date.
3. None of the metabolites of the phthalates tested were estrogenic.
4. The most potent of the phthalates (BBP) did not induce an estrogenic response in vivo in a relatively sensitive species of fish, the fathead minnow.

These data, had they been taken as read, might have indicated that the phthalates did not pose a substantial risk as endocrine disrupters. When the study was initiated, the equivalent yeast assay to test for androgen mimics had not been properly validated, and these chemicals were not assessed for their androgenic or anti-androgenic activity. At a later date, I was able to test some of the phthalates in this assay, and the data obtained from these experiments is potentially the most significant of all the data presented in chapter 3. This is partly because of the relative potency of these chemicals compared to flutamide (they were reasonably potent anti-androgens), but also partly due to the observation that some of the metabolites of the phthalates were androgen antagonists. Even more significant is the fact that MEHP, the metabolite of the most prolific phthalate, DEHP, was strongly anti-androgenic. Although it is generally accepted that DEHP acts as a reproductive toxicant in certain mammalian species, it had until very recently not been considered an endocrine disrupter, since it does not display 'estrogenic' properties. These data, taken together, highlight the necessity for in vivo studies which account for both the metabolites of pollutants, as well as the parent chemicals, and for a range of modes of action of such chemicals. The reproductive performance test employing fathead minnows may be such a test, since this determines overall reproductive fitness in successfully breeding pairs of minnows which are exposed to the test chemical via the water. Furthermore, spawning events may be affected by both physiological and behavioural aspects of both sexes, albeit that the mechanisms behind any effects observed in this test are not always clear. It is for this latter reason that a combination of in vivo (holistic) and in vitro (mechanistic) assays should be employed. It would clearly be desirable to conduct in vivo experiments on a multigenerational basis, therefore accounting for effects at the developmental stage, as well as transgenerational influences,

rather than solely considering responses in adults, which may be less sensitive than earlier stages. However, such studies are extremely costly and time consuming, it can take a matter of years to test a single compound (at several concentrations), and it is therefore advisable to conduct initial short-term trials as preliminary studies.

One aspect which must be given some thought in these types of studies is the endpoint to be assessed. Clearly, any response in vivo bears more relevance to a wildlife situation than an in vitro test, since factors such as bioaccumulation and metabolism are accounted for. Nonetheless, the relevance of certain parameters frequently used as markers for estrogen exposure to the fundamental question of populational effects is unknown. For example, vitellogenin is an extremely sensitive biomarker for estrogen exposure, and a vitellogenic response in vivo has been correlated with a reduced GSI in male rainbow trout (Jobling et al, 1996). However, where induction of vitellogenin is observed, but other reproductive parameters are either not monitored, or are not adversely affected, it is unclear as to the significance of an elevated plasma vitellogenin concentration. For example, in this thesis an increase in vitellogenin concentration was observed in fish exposed to 10 μg 4-NP/L, but the GSI of these fish was unaffected. Similarly, in the studies undertaken by Harries et al (2000), an elevation of the vitellogenin concentration was reported in fathead minnows exposed to 10 μg 4-NP/L, but there was no significant adverse effect on fecundity (the mean number of total eggs spawned by these fish was reduced, but this was not statistically significant). It is possible that the increased energy reserves required to synthesise vitellogenin would detract from energy invested in general growth and health of the fish, and also that pronounced increases in the vitellogenin concentration may result in pathological effects on the liver (as was hypothesised in this thesis), but any long term consequences of lesser rises in plasma vitellogenin concentration are as yet unknown. Length and timing of exposure is important in this regard, as a short-term vitellogenic response may be reflected in the long term, or during more sensitive periods of development, by more profound reproductive effects, but this relationship (if it exists) has not yet been demonstrated. What the vitellogenin response undoubtedly *does* show is that the exposure medium is, at least in part, estrogenic, and, in a similar way to trout being considered a sentinel species due to their sensitivity (see below), this parameter may be considered a 'sentinel biomarker'. Although it might be said that any physiological change induced in an organism by a foreign chemical is

unnatural, and therefore undesirable, regulatory bodies might have difficulty in making decisions where only data such as those described above are presented.

When conducting in vivo trials with fish, it is also worth giving some thought to the method by which the chosen chemical is administered. There are essentially two realistic possibilities, via food and via water, and one method which bears no relevance to a wildlife situation, that of subcutaneous injection. Organic, hydrophobic chemicals (as the majority of the EDCs are) might accumulate in food eaten by fish, and therefore this may be the principal route of exposure of wild fish to such pollutants. However, exposure via the water would result in chemical uptake via the gills, therefore directly entering the bloodstream and reaching many vital organs before being metabolised in the liver. This latter route of exposure might therefore endow the chemical in question with increased potency, despite it being present at lower concentrations in water than in food. It is currently unclear as to the difference in potency and/or effects elicited by an individual chemical administered via these two different exposure routes.

It is also true that the experiments described in this thesis were conducted somewhat unrealistically (in an environmental sense), since only a single compound was assessed at one time. In reality, wildlife are simultaneously exposed to multiple contaminants, probably with multiple mechanisms of action. Only when environmentally relevant combinations of pollutants are assessed together will we be able to envisage the wider picture of how endocrine disrupters in real situations may influence wildlife populations. This is not an easy topic to address, however, since complications arise with respect to what the additive effect of two chemicals, let alone several chemicals, may be. This issue has already seen some controversy, when an extreme case of synergism was reported by Arnold et al (1996), whereby a 1000-fold increase in estrogenic activity was observed when two chemicals were combined, compared to the response of those chemicals alone. These results were the stimulus for a number of other research groups to begin investigating the interactive effects of chemicals. However, no synergism was observed (using the same chemicals as employed by Arnold et al, 1996) by any of these scientists (e.g. Ashby et al, 1997b; Ramamoorthy et al, 1997). The original paper was subsequently withdrawn (McLachlan, 1997) and, to date, it is unclear whether synergistic processes are a reality. Ultimately, it would be wise to assess realistic mixtures of compounds in vivo, and determine the overall response of organisms to such

combinations. The essence of ecotoxicology lies in answering the question "are contaminants, as encountered in the 'real' environment, capable of inducing physiological responses in wildlife, and will these responses lead to harmful effects upon the population/community/ecosystem ?" This is the question to which we are currently trying to obtain more comprehensive answers.

Chapter 4 of this thesis presents the first evidence to date that a xenoestrogenic compound can affect higher levels of the BPG axis. This study was undertaken using maturing female rainbow trout, and those exposed to the highest concentration of 4-NP, together with exhibiting suppressed plasma FSH levels compared to the control fish, also did not undertake normal ovarian development. In vivo studies are specific to life stage and duration of exposure, as well as to the particular species employed. Rainbow trout may be considered a particularly sensitive species, as they have been reported as being responsive to lower concentrations of environmental estrogens than, for example, the roach (Harries et al, 1996; Routledge et al, 1998). Nonetheless, they can be an important sentinel species in certain circumstances, and short-term effects observed in the rainbow trout may occur as a result of prolonged exposure, or exposure at a critical life-stage, in other species where the adults are less sensitive. It would be interesting to establish whether there is a link between the data concerning the effect of 4-NP on gonadotropins, and those reported by Harries et al (2000), whereby 100 μg 4-NP/L inhibited spawning in paired adult fathead minnows. Although in the latter experiments the GSI of the females was not reduced to the extent observed in my experiment with rainbow trout, it is possible that 4-NP had a similar suppressive effect on LH synthesis in the two experiments, and therefore that the fathead minnows did not experience the appropriate levels of LH to induce gonadal maturation/ovulation. Unfortunately, there are as yet no assays available to measure gonadotropin concentrations in cyprinid species. Such assays would be of great use in investigating intersexuality in wild populations of roach (Jobling et al, 1998). These authors reported a high incidence of intersexuality, up to 100%, in some populations of roach. It is not known at what stage these fish were subject to exposure to endocrine disrupters, or more specifically, at what stage their phenotype has been altered in this way. Furthermore, the role of gonadotropins in gonadal differentiation is unknown. However, FSH producing cells have been found to appear early in development of rainbow trout (Saga et al, 1993), prior to the first signs of gonadal sex differentiation in these fish. It is therefore possible that FSH plays a part in early

gonadal development in rainbow trout. LH cells, in contrast, do not make an appearance until the onset of gametogenesis in rainbow trout (Nozaki et al, 1990b). In juvenile fish, the role of gonadotropins in puberty has likewise not yet been defined, since in most cases they have not been detected in immature fish (due to the limitations of the available assays). In humans, however, gonadotropins play a significant role at puberty, when they induce the secretion of hormones from the gonads, which in turn initiate the maturation of the reproductive system and development of the secondary sex characteristics. In addition, FSH concentrations in mammals are very high in utero, and shortly after birth. These high FSH concentrations stimulate Sertoli cell proliferation. In turn, Sertoli cell number regulates sperm production. So, if FSH levels are low early in life, sperm production will be low in adult life. If similar scenarios were to ensue in teleosts, the implications of inhibition of gonadotropin secretion following exposure to xenoestrogens for reproductive development would be profound.

It is, in fact, not only 'xenoestrogens' which may result in such harmful effects. Natural estrogens too are present in sewage effluents, accounting for the majority of the estrogenic activity in the case of many domestic effluents (Desbrow et al, 1998). It has been established that these chemicals can elicit estrogenic responses at the concentrations at which they were found in the effluents. Presumably these natural estrogens will have similar effects on gonadotropin levels as did 4-NP, although their potency may differ. Bearing this in mind, a significant factor in the effects that such chemicals have on wild fish is the timing of the exposure, and also the estrogenic 'strength' of the effluent. Sex differentiation in roach, for example, takes place at around 60 days, and since this species spawns in early summer, it seems likely that the juveniles will be exposed to relatively concentrated effluents during the period of sexual differentiation. With respect to 4-NP in particular, this would appear to be a short-term problem (in Europe at least), since the use of this chemical in agricultural and industrial cleaning applications is in the process of being phased out (ENDS, 1999a), but certainly the presence of natural estrogens will need to be tackled by water companies. Although an expensive process, the more efficient degradation of these chemicals, via biological or chemical processes during treatment, is required to reduce their concentration in effluents discharging into aquatic systems.

LIST OF MY PUBLICATIONS TO DATE

Alcock RE, Halsall CJ, Harris CA, Johnston AE, Lead WA, Sanders G, Jones KC (1994) Contamination of environmental samples prepared for PCB analysis. *Environ Sci Technol* 28: 1838-1842

Ashby J, Harris CA, Lefevre PA, Odum J, Routledge EJ, Sumpter JP (1997) Failure to confirm oestrogenic synergism between dieldrin and endosulfan. *Nature* 385: 494

Harris CA, Henttu P, Parker MG, Sumpter JP (1997) The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 105: 802-811

Beresford N, Routledge EJ, Harris CA, Sumpter JP (2000) Issues arising when interpreting results from an in vitro assay for estrogenic activity. *Toxicol Appl Pharmacol* 162: 22-33

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

In Press:

Harris CA and Sumpter JP. The endocrine disrupting potential of phthalates. In: Metzler, M. (Ed) *Endocrine Disruptors in the Environment* , The Handbook of Environmental Chemistry, Springer-Verlag, Heidelberg, Germany

Submitted:

Harris CA, Santos EM, Janbakhsh A, Pottinger TG, Tyler CR, Sumpter JP. Some of the adverse reproductive effects of 4-nonylphenol in female rainbow trout occur at the level of the pituitary gland.

REFERENCES

Adams NR (1998) Clover phyto-oestrogens in sheep in Western Australia. *Pure Appl Chem* 70: 1855-1862

Adams NR, Sanders MR, Ritar AJ (1988) Oestrogenic damage and reduced fertility in ewe flocks in South Western Australia. *Aust J Agric Res* 39: 71-77

Adlercreutz H (1990) Diet, breast cancer, and sex hormone metabolism. *Annals NY Acad Sci* 595: 281-290

Adlercreutz H (1995) Phytoestrogens: Epidemiology and a possible role in cancer protection. *Environ Health Perspect* 103(Suppl 7): 103-112

Adlercreutz H, Markkanen H, Watanabe S (1993) Plasma concentrations of phyto-oestrogens in Japanese men. *The Lancet* 342: 1209-1210

Ahel M and Giger W (1985) Determination of alkylphenols and alkylphenol mono- and diethoxylates in environmental samples by high-performance liquid chromatography. *Anal Chem* 57: 1577-1583

Ahel M and Giger W (1993a) Aqueous solubility of alkylphenols and alkylphenol polyethoxylates. *Chemosphere* 26: 1461-1470

Ahel M and Giger W (1993b) Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. *Chemosphere* 26: 1471-1478

Ahel M, McEvoy J, Giger W (1993) Bioaccumulation of the lipophilic metabolites of nonionic surfactants in freshwater organisms. *Environ Pollution* 79: 243-248

Ahel M, Giger W, Koch M (1994a) Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment - I. Occurrence and transformation in sewage treatment. *Water Res* 28: 1131-1142

Ahel M, Giger W, Schaffner C (1994b) Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment - II. Occurrence and transformation in rivers. *Water Res* 28: 1143-1152

Aherne GW and Briggs R (1989) The relevance of the presence of certain synthetic steroids in the aquatic environment. *J Pharm Pharmacol* 41: 735-736

Albro PW and Lavenhar SR (1989) Metabolism of di(2-ethylhexyl)phthalate. *Drug Metab Reviews* 21: 13-34

Alcock RE, Halsall CJ, Harris CA, Johnston AE, Lead WA, Sanders G, Jones KC (1994) Contamination of environmental samples prepared for PCB analysis. *Environ Sci Technol* 28: 1838-1842

Amano M, Ikuta K, Kitamura S, Aida K (1997) The maturation of the salmon GnRH system and its regulation by gonadal steroids in masu salmon. *Fish Physiol Biochem* 17: 63-70

Anderson DW, Jehl JR Jr, Riseborough RW, Woods LA Jr, Deweese LR, Edgecombe WG (1975) Brown pelicans: Improved reproduction off the southern California coast. *Science* 190: 806-808

Antonopoulou E, Bornestaf C, Swanson P, Borg B (1999) Feedback control of gonadotropins in atlantic salmon, *Salmo salar*, male parr I. Castration effects in rematuring and non-rematuring fish. *Gen Comp Endocrinol* 114: 132-141

Arme C (1965) A hermaphrodite specimen of roach, *Rutilus rutilus* (L). Proceedings of the Leeds Philosophical and Literary Society, Vol IX, Part XI: 277-281

Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette LJ Jr, McLachlan JA (1996) Synergistic activation of estrogen-receptor with combinations of environmental chemicals. *Science* 272: 1489-1491

Arukwe A, Knudsen FR, Goksoyr A (1997) Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. *Environ Health Perspect* 105: 418-422

Ashby J, Tinwell H, Lefevre PA, Odum J, Paton D, Millward SW, Tittensor S, Brooks AN (1997a) Normal sexual development of rats exposed to butyl benzyl phthalate from conception to weaning. *Regulatory Toxicol Pharmacol* 26: 102-118

Ashby J, Harris CA, Lefevre PA, Odum J, Routledge EJ, Sumpter JP (1997b) Failure to confirm oestrogenic synergism between dieldrin and endosulfan. *Nature* 385 :494

Ashfield LA, Pottinger TG, Sumpter JP (1998) Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environ Toxicol Chem* 17: 679-686

Auger J, Kunstmann JM, Czyglik F, Jouannet P (1995) Decline in semen quality among fertile men in Paris during the past 20 years. *New England Journal of Medicine* 332: 281-285

Baker VA, Hepburn PA, Kennedy SJ, Jones PA, Lea LJ, Sumpter JP, Ashby, J (1999) Safety evaluation of phytosterol esters. Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays. *Food Chem Toxicol* 37: 13-22

Beard AP, McRae AC, Rawlings NC (1997) Reproductive efficiency in mink (Mustela vison) treated with the pesticides lindane, carbofuran and pentachlorophenol *J Reprod Fertil* 111: 21-28

Bennetts HW, Underwood EJ, Shier FL (1946) A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust Vet J* 22: 2-12

Beresford N, Routledge EJ, Harris CA, Sumpter JP (2000) Issues arising when interpreting results from an in vitro assay for estrogenic activity. *Toxicol Appl Pharmacol* 162: 22-33

Bergeron JM, Crews D, McLachlan JA (1994) PCBs as environmental estrogens: Turtle sex determination as a biomarker of environmental contamination. *Environ Health Perspect* 102: 780-781

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

Billard R (1978) Testicular feedback on the hypothalamic-pituitary axis in rainbow trout (*Salmo gairdneri* R.). *Annales de Biologie Animale, Biochimie, Biophysique* 18: 813-818

Billard R, Richard M, Breton B (1976) Stimulation de la secretion gonadotrope hypophysaire apres castration chez la truite arc-en-ciel. Variation de la response au cours du cycle reproducteur. *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences. D: Sciences Naturelles* 283:171-174

Billard R, Richard M, Breton B (1977) Stimulation of gonadotropin secretion after castration in rainbow trout. *Gen Comp Endocrinol* 33: 163-165

Birnbaum LS (1994) Endocrine effects of prenatal exposure to PCBs, dioxins, and other xenobiotics: Implications for policy and future research. *Environ Health Perspect* 102: 676-679

Bitman J and Cecil HC (1970) Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J Agric Food Chem* 18: 1108-1112

Bitman J, Cecil HC, Harris SJ, Fries GF (1969) Estrogenic activity of o,p' DDT in the mammalian uterus and the avian oviduct. *Science* 162: 371-372

Blackburn MA and Waldock MJ (1995) Concentrations of alkylphenols in rivers and estuaries in England and Wales. *Water Res* 29: 1623-1629

Blackburn MA, Kirby SJ, Waldock MJ (1999) Concentrations of alkylphenol polyethoxylates entering UK estuaries. *Mar Poll Bull* 38: 109-118

Blake CA and Boockfor FR (1997) Chronic administration of the environmental pollutant 4-tert-octylphenol to adult male rats interferes with the secretion of luteinising hormone, follicle-stimulating hormone, prolactin, and testosterone. *Biol Reprod* 57: 255-266

Blom A, Ekman E, Johannisson A, Norrgen L, Pesonen M (1998) Effects of xenoestrogenic environmental pollutants on the proliferation of a human breast cancer cell line (MCF-7). *Arch Environ Contam Toxicol* 34: 306-310

Bolger R, Wiese TE, Ervin K, Nestich S, Checovich W (1998) Rapid screening of environmental chemicals for receptor binding capacity. *Environ Health Perspect* 106: 551-557

Bommelaer MC, Billard R, Breton B (1981) Changes in plasma gonadotropin after ovariectomy and estradiol supplementation at different stages at the end of the reproductive cycle in the rainbow trout (*Salmo gairdneri* R.). *Reproduction, Nutrition, Development* 21: 989-997

Braunbeck T, Görge G, Storch V, Nagel R (1990) Hepatic steatosis in zebra fish (*Brachydanio rerio*) induced by long-term exposure to γ-hexachlorocyclohexane. *Ecotox Environ Safety* 19: 355-374

Breton B, Fostier A, Zohar Y, Le Bail PY, Billard R (1983) Gonadotropine glycoproteique maturante et oestradiol-17ß pendant le cycle reproducteur chez la truite fario (*Salmo trutta*) femelle. *Gen Comp Endocrinol* 49: 220-231

Breton B, Sambroni E, Govoroun M, Weil C (1997) Effects of steroids on GTH I and GTH II secretion and pituitary concentration in the immature rainbow trout *Oncorhynchus mykiss*. *CR Acad Sci Paris, Life Sciences* 320: 783-789

Breton B, Govoroun M, Mikolajczyk T (1998) GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: Relationship with pituitary responsiveness to GnRH-A stimulation. *Gen Comp Endocrinol* 111: 38-50

Brooks AN, Hagan DM, Sheng C, McNeilly AS, Sweeney T (1996) Prenatal gonadotropins in the sheep. *Animal Reprod Sci* 42: 471-481

Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N (1995) Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect* 103: 608-612

Bulger WH, Muccitelli RM, Kupfer D (1978a) Studies on the in vivo and in vitro estrogenic activities of methoxychlor and its metabolites. Role of hepatic mono-oxygenase in methoxychlor activation. *Biochem Pharmacol* 27: 2417-2423

Bulger WH, Muccitelli RM, Kupfer D (1978b) Interactions of methoxychlor, methoxychlor base contaminant, and 2,2-bis(p-hydroxy-phenyl)-1,1,1-trichloroethane with rat uterine receptor. *J Toxicol Environ Health* 4: 881-893

Carlsen E, Giwercman A, Keiding N, Skakkebaek N (1992) Evidence for decreasing quality of semen during past 50 years. *British Med J* 305: 609-613

Carr KH, Coyle GT, Kimerle RA (1997) Bioconcentration of [^{14}C]butyl benzyl phthalate in bluegill sunfish (*Lepomis macrochirus*). *Environ Toxicol Chem* 16: 2200-2203

Carragher JF (1988) The effects of stress on reproductive function in trout. PhD Thesis, Brunel University, Uxbridge, UK

Celius T, Haugen TB, Grotmol T, Walther BT (1999) A sensitive zonagenetic assay for rapid in vitro assessment of estrogenic potency of xenobiotics and mycotoxins. *Environ Health Perspect* 107: 63-68

Cheesman RV and Wilson AL (1989) Manual on analytical quality control for the water industry. WRc (UK)

Christiansen LB, Pedersen KL, Korsgaard B, Bjerregaard P (1998) Estrogencity of xenobiotics in rainbow trout (*Oncorhynchus mykiss*) using in vivo synthesis of vitellogenin as a biomarker. *Marine Environ Res* 46: 137-140

Christiansen LJ, Korsgaard B, Bjerregaard P (1999) The effect of 4-nonylphenol on the synthesis of vitellogenin in the flounder Platichthys flesus. *Aquatic Toxicol* 46: 211-219

Clark LB, Rosen RT, Hartman TG, Alaimo LH, Louis JB, Hertz C, Ho C-T, Rosen JD (1991) Determination of nonregulated pollutants in three New Jersey publicly owned treatment works (POTWs). *Res J Water Pollut Control Fed* 63: 104-113

Coldham NG, Dave M, Sivapathasundaram S, McDonnell DP, Connor C, Sauer MJ (1997) Evaluation of a recombinant yeast cell estrogen screening assay. *Environ Health Perspect* 105: 734-742

Coldham NG, Sivapathasundaram S, Dave M, Ashfield LA, Pottinger TG, Goodall C, Sauer MJ (1998) Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout. *Drug Metab & Disposition* 26: 347-354

Cook DL, LaFleur L, Parrish A, Jones J, Hoy D (1996) Characterization of plant sterols in a select group of US pulp and paper mills. *Proceedings*, 5th International Association on Water Quality, Vancouver, Canada, June 10-13; pp1-8

Copeland PA, Sumpter JP, Walker TK, Croft M (1986) Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri*, Richardson) at various stages of the reproductive cycle. *Comp Biochem Physiol* 83B; 487-493

Cox RI and Braden AW (1974) The metabolism and physiological effects of phytoestrogens in livestock. *Proc Aust Soc Anim Prod* 10: 122-129

Crim LW and Evans DM (1979) Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (*Salmo gairdneri*). *Gen Comp Endocrinol* 37: 192-196

Crim LW and Peter RE (1978) The influence of testosterone implantation in the brain and pituitary on pituitary gonadotropin levels in Atlantic salmon parr. *Annales de Biologie Animale, Biochemie et Biophysique* 18: 689-694

Danzo BJ (1997) Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. *Environ Health Perspect* 105: 294-301

Darnerud PO, Sinjari T, Jönsson C-J (1996) Foetal uptake of coplanar polychlorinated biphenyl (PCB) congeners in mice. *Pharmacol Toxicol* 78: 187-192

Davis WP and Bortone SA (1992) Effects of kraft mill effluent on the sexuality of fishes: An environmental early warning? In: Colborn T and Clement C (Eds); Advances in Modern Environmental Toxicology. Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton Scientific, Princeton, Vol. 21: 113-128

Davis BJ, Maronpot RR, Heindel JJ (1994) Di(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol Appl Pharmacol* 128: 216-223

Denhardt DT (1966) A membrane-filter technique for the detection of complementary DNA. *Biochem Biophys Res Commun* 23: 641-644

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: 1549-1558

Di Corcia A, Costantino A, Crescenzi C, Marinoni E, Samperi R (1998) Characterization of recalcitrant intermediates from biotransformation of the branched alkyl side chain of nonylphenol ethoxylate surfactants. *Environ Sci Technol* 32: 2401-2409

Dickey JT and Swanson P (1998) Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). *J Mol Endocrinol* 21: 291-306

Dickey JT and Swanson P (2000) Effects of salmon gonadotropin-releasing hormone on follicle stimulating hormone secretion and subunit gene expression in coho salmon (*Oncorhynchus kisutch*). *Gen Comp Endocrinol* 118: 436-449

Dirksen S, Boudewijn TJ, Slager LK, Mes RG, van Schaick MJM, de Voogt P (1995) Reduced breeding success of cormorants (*Phalacrocorax carbo sinensis*) in relation to persistent organochlorine pollution of aquatic habitats in the Netherlands. *Environ Pollution* 88: 119-132

Dirven HAAM, van den Broek PHH, Arends T, Noordkamp EM, de Lepper AJTM, Henderson PTh, Jongeneelen FJ (1993) Metabolites of the plasticizer di(2-ethylhexyl) phthalate in urine samples of workers in PVC processing industries. *Int Arch Occup Environ Health* 64:549-554

Dodds EC and Lawson W (1938) Molecular structure in relation to oestrogenic activity. Compounds without a phenanthrene nucleus. *Proc R Soc Lond Biol Sci* 125: 222-232

DoE (1992) First report of the technical committee on detergents and the environment. London: Department of the Environment

Donohoe RM and Curtis LR (1996) Estrogenic activity of chlordecone, o,p'-DDT, and o,p'-DDE in juvenile rainbow trout: Induction of vitellogenesis and interaction with hepatic estrogen binding sites. *Aquatic Toxicol* 36: 31-52

ECPI (1996) Phthalate esters used in PVC: Assessment of the release, occurrence and possible effects of plasticisers in the environment [Partial copy]. Brussels: European Chemical Industry Council (European Council for Plasticisers and Intermediates)

Ejlertsson J, Meyerson U, Svensson BH (1996) Anaerobic degradtion of phthalic acid esters during digestion of municipal solid waste under landfilling conditions. *Biodegradation* 7: 345-352

Ejlertsson J, Alnervik M, Jonsson S, Svensson BH (1997) Influence of water solubility, side-chain degradability, and side-chain structure on the degradation of phthalic acid esters under methanogenic conditions. *Environ Sci Technol* 31: 2761-2764

Ema M, Kurosaka R, Amano H, Ogawa Y (1994) Embryolethality of butyl benzyl phthalate during early pregnancy in rats. *Reprod Toxicol* 8: 231-236

Ema M, Harazono A, Miyawaki E, Ogawa Y (1997) Embryolethality following maternal exposure to dibutyl phthalate during early pregnancy in rats. *Bull Environ Contam Toxicol* 58: 636-643

Ema M, Miyawaki E, Kawashima K (1998) Further evaluation of developmental toxicity of di-n-butyl phthalate following administration during late pregnancy in rats. *Toxicol Letters* 98: 87-93

ENDS (1997) Firms begin APE phase-out. *ENDS Report* 267: 27

ENDS (1999a) Risk reduction strategy proposed for NP, NPEs. *ENDS Report* 295: 44-45

ENDS (1999b) APE manufacturers complain of risk assessment "witch hunt". *ENDS Report* 292: 11

Ensor DM and Tinsley D (1984) Intersex roach. Internal Report, Thames Water, Lea Division, Herts, UK

Eroschenko VP and Palmiter RD (1980) Estrogenicity of kepone in birds and mammals In: McLachlan JA (Ed); *Estrogens in the Environment* 305-324, New York: Elsevier

Evans MS, Noguchi GE, Rice CP (1991) The biomagnification of polychlorinated biphenyls, toxaphene, and DDT compounds in a Lake Michigan offshore food web. *Arch Environ Contam Toxicol* 20: 87-93

Facemire CF, Gross TS, Guillette LJ Jr (1995) Reproductive impairment in the florida panther: Nature or nurture? *Environ Health Perspect* 103(Suppl 4): 79-86

Fagerlund UHM, McBride JR, Stone ET (1981) Stress-related effects of hatchery rearing density of coho salmon. *Trans Amer Fish Soc* 110: 644-649

Folmar LC, Denslow ND, Rao V, Chow M, Crain A, Enblom J, Marcino J, Guillette LJ Jr (1996) Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ Health Perspect* 104: 1096-1101

Frakes RA, Zeeman CQT, Mower B (1993) Bioaccumulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) by fish downstream of pulp and paper mills in Maine. *Ecotox Envrion Safety* 25: 244-252

Fremont L, Leger C, Petridou B, Gozzelino MT (1984) Effects of a (n-3) polyunsaturated fatty acid-deficient diet on profiles of serum vitellogenin and lipoprotein in vitellogenic trout, *Salmo gairdneri. Lipids* 19: 522-529

Fry DM (1995) Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ Health Perspect* 103(Suppl 7): 165-171

Fry DM and Toone CK (1981) DDT-induced feminization of gull embryos. *Science* 213: 922-924

Fry DM, Toone CK, Speich SM, Peard RJ (1987) Sex ratio skew and breeding patterns of gulls: Demographic and toxicological considerations. *Studies in Avian Biol* 10: 26-43

Furtmann RNK (1996) Phthalates in the aquatic environment. CEFIC Report No. 6/93 (Brussels)

Gagnon MM, Bussieres D, Dodson JJ, Hodson PV (1995) White sucker (*Catostomus commersoni*) growth and sexual maturation in pulp mill-contaminated and reference rivers. *Environ Toxicol Chem* 14: 317-327

Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, McDonnell DP (1997) Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. *Toxicol Appl Pharmacol* 143: 205-212

Gaido K, Dohme L, Wang F, Chen I, Blankvoort B, Ramamoorthy K, Safe S (1998) Comparative estrogenic activity of wine extracts and organochlorine pesticide residues in food. *Environ Health Perspect* 106(Suppl 6): 1347-1352

Gangolli SD (1982) Testicular effects of phthalate esters. *Environ Health Perspect* 45: 77-84

Ge W, Chang JP, Peter RE, Vaughan J, Rivier J, Vale W (1992) Effects of porcine follicular fluid, inhibin-A and activin-A on goldfish gonadotropin release in vitro. *Endocrinology* 131: 1922-1929

Gellert RJ (1978) Uterotrophic activity of polychlorinated biphenyls (PCB) and induction of precocious reproductive aging in neonatally treated female rats. *Environ Res* 16: 123-130

Giam CS, Atlas E, Chan HS, Neff GS (1980) Phthalate esters, PCB and DDT residues in the gulf of Mexico atmosphere. *Atmos Environ* 14: 65-69

Giam CS, Atlas E, Powers MA, Leonard JE Jr. (1984) Phthalic acid esters. In: Hutzinger O (ed) Handbook of Environmental Chemistry. Springer, Berlin Heidelberg, pp 67-142

Gielen JTh and Goos HJTh (1983) The brain-pituitary-gonadal axis in the rainbow trout, *Salmo gairdneri*. II. Direct effect of gonadal steroids on the gonadotropic cells. *Cell and Tissue Research* 233: 377-388

Giesy JP, Ludwig JP, Tillit DE (1994) Deformities in birds of the Great Lakes region: Assigning causality. *Environ Sci Technol* 28: A128-A135

Giger W, Brunner PH, Schaffner C (1984) 4-Nonylphenol in sewage sludge: Accumulation of toxic metabolites from nonionic surfactants. Science 225: 623-625

Gilbertson M, Kubiak TJ, Ludwig JP, Fox G (1991) Great Lakes embryo mortality, edema and deformaties syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick edema disease. *J Toxicol Environ Health* 33: 455-520

Gill WB, Schumacher GFB, Bibbo M, Straus FH II, Schoenberg HW (1979) Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. *J Urol* 122: 36-39

Gill WB (1989) Effects on human males of in utero exposure to exogenous sex hormones. In: Mori T and Nagasawa H (Eds); *Toxicity of Hormones in Perinatal Life*. CRC Press; Boca Raton, Fla: 161-177

Gomez JM, Weil C, Ollitrault M, Le Bail P-Y, Breton B, Le Gac F (1999) Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 113: 413-428

Gray LE Jr (1982) Neonatal chlordecone exposure alters behavioural sex differentation in female hamsters. *Neuroendocrinol* 3: 67-80

Gray LE Jr (1998) Tiered screening and testing strategy for xenoestrogens and antiandrogens. *Toxicol Letters* 103: 677-680

Gray LE Jr and Kelce WR (1996) Latent effects of pesticides and toxic substances on sexual differentiation of rodents. *Toxicol Indust Health* 12: 515-531

Gray LE Jr and Ostby JS (1995) In utero 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. *Toxicol Appl Pharmacol* 133: 285-294

Gray LE Jr, Ostby JS, Ferrell JM, Sigmon ER, Goldman JM (1988) Methoxychlor induces estrogen-like alterations of behavior and the reproductive-tract in the female rat and hamster - effects on sex behavior, running wheel activity, and uterine morphology. *Toxicol Appl Pharmacol* 96: 525-540

Gray LE Jr, Ostby JM, Marshall R (1993) The fungicide vinclozolin inhibits morphological sex differentiation in the male rat. *Biol Reprod* 48(Suppl.1): 97

Gray LE Jr, Kelce WR, Monosson E, Ostby JS, Birnbaum LS (1995) Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: Reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. *Toxicol Appl Pharmacol* 131: 108-118

Gray LE Jr, Ostby JS, Mylchreest E, Foster PMD, Kelce WR (1998) Dibutyl phthalate (DBP) induces antiandrogenic but not estrogenic in vivo effects in long evans hooded rats. *Biol Reprod* 58(S1): 411

Gray MA and Metcalfe CD (1997) Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. *Environ Toxicol Chem* 16: 1082-1086

Gray TJB and Gangolli SD (1986) Aspects of the testicular toxicity of phthalate esters. *Environ Health Perspect* 65: 229-235

Guillette LJ Jr, Gross TS, Masson GR, Matter JM, Percival F, Woodward AR (1994) Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect* 102: 680-688

Guillette LJ Jr, Pickford DB, Crain DA, Rooney AA, Percival HF (1996) Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. *Gen Comp Endocrinol* 101: 32-42

Gulati DK, Hommel-Barnes L, Chapin RE, Heindel J (1991) The reproductive toxcity of di-n-butyl phthalate in Sprague -Dawley rats; Report No. T-0035C; National Institute of Environmental Health Services, New York, USA

Hagenmaier H, She J, Benz T, Dawidowsky N, Düsterhöft, Lindig C (1992) Analysis of sewage sludge for polyhalogenated dibenzo-p-dioxins, dibenzofurans, and diphenylethers. *Chemosphere* 25: 1457-1462

Hammond B, Katzenellenbogen B, Krauthammer N, McConnel J (1979) Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptors. *Proc Natl Acad Sci USA* 76: 6641-6645

Hansen LG and O'Keefe PW (1996) Polychlorinated dibenzofurans and dibenzo-p-dioxins in subsurface soil, superficial dust, and air extracts from a contaminated landfill. *Arch Environ Contam Toxicol* 31: 271-276

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

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N (1997) Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* 16: 534-542

Harries JE, Janbakhsh A, Jobling S, Matthiessen P, Sumpter JP, Tyler CR (1999) Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environ Toxicol Chem* 18: 932-937

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

Harris CA, Henttu P, Parker MG, Sumpter JP (1997) The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 105: 802-811

Harrison N (1998) Migration of plasticizers from cling film. *Food Additives & Contaminants* 5: 493-499

Hassin S, Gothilf Y, Blaise O, Zohar Y (1998) Gonadotropin-I and -II subunit gene expression of male striped bass (*Morone saxatilis*) after gonadotropin-releasing hormone analogue injection: Quantitation using an optimized ribonuclease protection assay. *Biol Reprod* 58: 1233-1240

Heinis LJ, Knuth ML, Liber K, Sheedy BR, Tunell RL, Ankley GT (1999) Persistence and distribution of 4-nonylphenol following repeated application to littoral enclosures. *Environ Toxicol Chem* 18: 363-375

Herman RL and Kincaid HL (1988) Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* 72: 165-172

Hilakavi-Clarke L, Cho E, Onojafe I, Raygada M, Clarke R (1999) Maternal exposure to genistein during pregnancy increases carcinogen- induced mammary tumorigenesis in female rat offspring. *Oncology Reports* 6: 1089-1095

Hisaw FL (1959) Comparative effectiveness of estrogens on fluid imbibition and growth of the rats uterus. *Endocrinology* 54: 276-289

Hose JE, Cross JN, Smith SG, Diehl D (1989) Reproductive impairment in a fish inhabiting a contaminated coastal environment off Southern California. *Environ Poll* 57: 139-148

Howell WM, Black DA, Bortone SA (1980) Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: Evidence for environmentally-induced masculinization. *Copeia* 4: 676-681

Huggard D, Khakoo Z, Kassam G, Mahmoud SS, Habibi HR (1996) Effect of testosterone on maturational gonadotropin subunit messenger ribonuleic acid levels in the goldfish pituitary. *Biol Reprod* 54: 1184-1191

Imajima T, Shono T, Zakaria O, Suita S (1997) Prenatal phthalate causes cryptochidism postnatally by inducing transabdominal ascent of the testis in fetal rats. *J Ped Surgery* 32: 18-21

Jafri, SIH and Ensor DM (1979) Occurrence of an intersex condition in the roach *Rutilus rutilus* (L). *J Fish Biol* 15: 547-549

Janbakhsh A (1996) The analytical determination of alkylphenols and alkylphenol ethoxylates in rivers and sewage effluents. M.Sc. Thesis, Dept Biol and Chem Sci, University of Essex

Jansen HT, Cooke PS, Porcelli J, Liu T-C, Hansen LG (1993) Estrogenic and antiestrogenic actions of PCBs in the female rat: In vitro and in vivo studies. *Reprod Toxicol* 7: 237-248

Jobling S and Sumpter JP (1993) Detergent compounds in sewage effluent are weakly estrogenic to fish: an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicol* 27: 361-372

Jobling S, Reynolds T, White R, Parker MG, Sumpter JP (1995) A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103: 582-587

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15: 194-202

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

Johnson DC, Sen M, Dey SK (1992) Differential effects of dichlorodiphenyltrichloroethane analogs, chlordecone, and 2,3,7,8-tetrachlorodibenzo-p-dioxin on establishment of pregnancy in the hypophysectomised rat. *Proc Soc Exp Biol Med* 199: 42-48

Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LE Jr (1994) Environmental hormone disruptors: Evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol Appl Pharmacol* 126: 276-285

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM (1995) Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* 375: 581-585

Kellis JT Jr and Vickery LE (1984) Inhibition of human estrogen synthetase (aromatase) by flavones. *Science* 225: 1032-1034

Khan IA and Thomas P (1998) Estradiol-17ß and o,p'-DDT stimulate gonadotropin release in Atlantic Croaker. *Mar Environ Res* 46: 149-152

Khan IA, Hawkins MB, Thomas P (1999) Gonadal stage-dependent effects of gonadal steroids in gonadotropin II secretion in the atlantic croaker (*Micropogonias undulatus*). *Biol Reprod* 61: 834-841

Kime DE (1993) 'Classical' and 'non-classical' reproductive steroids in fish. *Rev Fish Biol and Fisheries* 3: 160-180

Kirchmann H, Aström H, Jonsall G (1991) Organic pollutants in sewage sludge 1. Effects of toluene, napthalene, 2-methylnapthalene, 4-n-nonylphenol, and di-2-ethylhexyl phthalate on soil biological processes and their decomposition in soil. *Swedish J Agri Res* 21: 107-113

Kitahashi T, Ando H, Ban M, Ueda H, Urano A (1998) Changes in the levels of gonadotropin subunit mRNAs in the pituitary of pre-spawning chum salmon. *Zool Sci* 15: 753-760

Kloas W, Lutz I, Einspanier R (1999) Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. *Sci Total Environ* 225: 59-68

Klotz DM, Beckman BS, Hill SM, McLachlan JA, Walters MR, Arnold SF (1996) Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. *Environ Health Perspect* 104: 1084-1089

Knudsen FR, Arukwe A, Pottinger TG (1998) The in vivo effect of combinations of octylphenol, butyl benzyl phthalate and estradiol on liver estradiol receptor modulation and induction of zona radiata proteins in rainbow trout: No evidence of synergy. *Environ Pollution* 103: 75-80

Knudsen FR and Pottinger TG (1999) Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout. *Aquatic Toxicol* 44: 159-170

Kobayashi M and Stacey NE (1990) Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. *Zool Sci* 7: 715-721

Krishnan V and Safe S (1993) Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: Quantitative structure-activity relationships. *Toxicol Appl Pharmacol* 120: 55-61

Krishnan AV, Stathis P, Permuth SF, Tokes L (1993) Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 132: 2279-2286

Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93: 5925-5930

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson J-A (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and ß. *Endocrinology* 138: 863-870

Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-A (1998) Interaction of

estrogenic chemicals and phytoestrogens with estrogen receptor ß. *Endocrinology* 139: 4252-4263

Kupfer D (1975) Effects of pesticides and related compounds on steroid metabolism and function. *Crit Rev Toxicol* 4: 83-124

Lampi P, Vartiainen T, Tuomisto J, Hesso A (1990) Population exposure to chlorophenols, dibenzo-p-dioxins and dibenzofurans after a prolonged ground water pollution by chlorophenols. *Chemosphere* 20: 625-634

Le Menn F and Burzawa-Gérard E (1985) Effect of carp gonadotropin (cGTH) and fraction unabsorbed on Concavalin A-sepharose obtained from cGTH on vitellogenesis in the hypophysectomised marine teleost *Gobius niger*. *Gen Comp Endocrinol* 57: 23-36

Lech JJ, Lewis SK, Ren L (1996) In vivo estrogenic activity of nonylphenol in rainbow trout. *Fund Appl Toxicol* 30: 229-232

Lewis SK and Lech JJ (1996) Uptake, disposition, and persistence of nonylphenol from water in rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica* 26: 813-819

Long JLA, House WA, Parker A, Rae JE (1998) Micro-organic compounds associated with sediments in the Humber rivers. *Sci Total Environ* 210/211: 229-253

Lye CM, Frid CLJ, Gill ME, Cooper DW, Jones DM (1999) Estrogenic alkylphenols in fish tissues, sediments, and waters from the UK Tyne and Tees estuaries. *Environ Sci Technol* 33: 1009-1014

Lygre H, Solheim E, Gjerdet NR, Berg E (1993) Leaching of organic additives from dentures in vivo. *Acta Odontol Scand* 51: 45-51

Lyons G (1995) Phthalates in the Environment. WWF Report (25/7/95), WWF UK, Godalming, Surrey

MacLatchy DL and van der Kraak GJ (1995) The phytoestrogen ß-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol Appl Pharmacol* 134: 305-312

MAFF (1990) Plasticisers: Continuing surveillance. Food Surveillance Paper No. 30. London: HMSO

MAFF (1995) Phthalates in paper and board packaging. Food Surveillance Information Sheet No. 60. London: HMSO

MAFF (1996a) Phthalates in food. Food Surveillance Information Sheet No. 82. London: HMSO

MAFF (1996b) Phthalates in infant formulae. Food Surveillance Information Sheet No. 83. London: HMSO

MAFF (1998) Phthalates in infant formulae - follow-up survey. Food Surveillance Information Sheet No. 168. London: HMSO

Magri M-H, Solar A, Billard R, Reinaud P (1985) Influence of testosterone on precocious sexual development in immature rainbow trout. *Gen Comp Endocrinol* 57: 411-421

Mäkelä S, Poutanen M, Lehtimäki J, Kostian M-L, Santii R, Vihko R (1995) Estrogen-specific 17ß-hydroxysteroid oxidoreductase type 1 (E.C. 1.1.1.62) as a possible target for the action of phytoestrogens. *Proc Soc Exp Biol Med* 208: 51-59

Marcomini A, Capel PD, Lichtensteiger T, Brunner PH, Giger W (1989) Behavior of aromatic surfactants and PCBs in sludge-treated soil and landfills. *J Environ Qual* 18: 523-528

Matta MB, Cairncross C, Kocan RM (1998) Possible effects of polychlorinated biphenyls on sex determination in rainbow trout. *Environ Toxicol Chem* 17: 26-29

Matthiessen P and Logan JWM (1984) Low concentration effects of endosulfan insecticide on reproductive behaviour in the tropical cichlid fish *Sarotherodon mossambicus*. *Bull Environ Contam Toxicol* 33: 575-583

Mazur W and Adlercreutz H (1998) Naturally occurring oestrogens in food. *Pure Appl Chem* 70: 1759-1776

McLeese DW, Zitko V, Sergeant DB, Burridge L, Metcalfe CD (1981) Lethality and accumulation of alkylphenols in aquatic fauna. *Chemosphere* 10: 723-730

McLachlan JA (1997) Synergistic effect of environmental estrogens: report withdrawn. *Science* 277: 462-463

Melamed P, Rosenfeld H, Elizur A, Yaron Z (1998) Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. *Comp Biochem Physiol* 119C: 325-338

Mellanen P, Petänen T, Lehtimäki J, Mäkelä S, Bylund G, Holmbom B, Mannila E, Oikari A, Santti R (1996) Wood-derived estrogens: Studies in vitro with breast cancer cell lines and in vivo in trout. *Toxicol Appl Pharmacol* 136: 381-388

Messina MJ, Persky V, Setchell KDR, Barnes S (1994) Soy intake and cancer risk: A review of the in vitro and in vivo data. *Nutr Cancer* 21: 113-131

Millington AJ, Francis CM, McKeown NR (1964) Wether bioassay of annual pasture legumes. II: The oestrogenic activity of nine strains of *Trifolium subterraneum* L. *Aust J Agric Res* 15: 527

Milligan SR, Balasubramanian AV, Kalita JC (1998) Relative potency of xenobiotic estrogens in an acute in vivo mammalian assay. *Environ Health Perspect* 106: 23-26

Mitchell SH and Kennedy S (1992) Tissue concentrations of organochlorine compounds in common seals from the coast of Northern Ireland. *Sci Total Environ* 115: 163-177

Monosson E, Fleming WJ, Sullivan CV (1994) Effects of the planar PCB 3,3'4,4'-tetrachlorobiphenyl (TCB) on ovarian development, plasma levels of sex steroid hormones and vitellogenin, and progeny survival in the white perch (*Morone americana*). *Aquatic Toxicol* 29: 1-19

Mousavi Y and Adlercreutz H (1993) Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. *Steroids* 58: 301-304

Muir DCG, Ford CA, Grift NP, Stewart REA, Bidleman TF (1992) Organochlorine contaminants in narwhal (*Monodon monoceros*) from the Canadian Arctic. *Environ Poll* 75: 307-316

Murk AJ, Boudewijn TJ, Meininger PL, Bosveld ATC, Rossaert G, Ysebaert T, Meire P, Dirksen S (1996) Effects of polyhalogenated aromatic hydrocarbons and related contaminants on common tern reproduction: Integration of biological, biochemical, and chemical data. *Arch Environ Contam Toxicol* 31: 128-140

Mylchreest E, Cattley RC, Foster PMD (1998) Male reproductive tract malformations in rats following gestational and lactational exposure to di-n-butyl phthalate. *Toxicol Sci* 43: 47-60

Mylchreest E, Sar M, Cattley RC, Foster PMD (1999) Disruption of androgen-regulated development by di-n-butyl phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156: 81-95

Nagao T, Saito Y, Usumi K, Kuwagata M, Imai K (1999) Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. *Reprod Toxicol* 13: 303-311

Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV (1997) Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect* 105: 70-76

Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR (1992) Alkylphenol ethoxylates in the environment. *J Am Oil Chem Soc* 69: 695-703

Nelson JA (1974) Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17ß-[^{3}H]estradiol binding to rat uterine receptor. *Biochem Pharmacol* 23: 447-451

Nerin C, Salafranca J, Rubio C, Cacho J (1998) Multicomponent recycled plastics: Considerations about their use in food contact applications. *Food Additives & Contaminants* 15: 842-854

Nesaretnam K, Corcoran D, Dils RR, Darbre P (1996) 3,4,3',4'-Tetrachlorobiphenyl acts as an estrogen in vitro and in vivo. *Mol Endocrinol* 10: 923-936

Nesaretnam K, Darbre P (1997) 3,5,3',5'-Tetrachlorobiphenyl is a weak oestrogen agonist in vitro and in vivo. *J Steroid Biochem Mol Biol* 62: 409-418

Nichols DJ, Daniel TC, Moore PA Jr, Edwards DR, Pote DH (1997) Runoff of estrogen hormone 17ß-estradiol from poultry litter applied to pasture. *J Environ Qual* 26: 1002-1006

Nozaki M, Naito N, Swanson P, Miyata K, Nakai Y, Oota Y, Suzuki K, Kawauchi H (1990a) Salmonid pituitary gonadotrophs I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. *Gen Comp Endocrinol* 77: 348-357

Nozaki M, Naito N, Swanson P, Dickhoff WW, Nakai Y, Suzuki K, Kawauchi H (1990b) Salmonid pituitary gonadotrophs II. Ontogeny of GTH I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). *Gen Comp Endocrinol* 77: 358-367

O'Grady DP, Howard PH, Werner AF (1985) Activated sludge biodegradation of 12 commercial phthalate esters. *Appl Environ Microbiol* 49: 443-445

Olea N, Pulgar R, Perez P, Olea-Serrano A, Rivas A, Novillofertrell A (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ Health Perspect* 104: 298-305

Olsen GW, Bodner KM, Ramlow JM, Ross CE, Lipshultz, LI (1995) Have sperm counts been reduced 50 percent in 50 years? A statistical model revisited. *Fertil Steril* 63: 887-893

Ousterhout J, Struck RF, Nelson JA (1981) Estrogenic activities of methoxychlor metabolites. *Biochem Pharmacol* 30: 2869-2871

Owens JW, Swanson SM, Birkholz DA (1994) Bioaccumulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin, 2,3,7,8-tetrachlorodibenzofuran and extractable organic chlorine at a bleached-kraft mill site in a Northern Canadian river system. *Environ Toxicol Chem* 13: 343-354

Page BD and Lacroix GM (1995) The occurrence of phthalate ester and di(2-ethylhexyl) adipate plasticizers in Canadian packaging and food sampled in 1985-1989: A Survey. *Food Additives & Contaminants* 12: 129-151

Pakdel F, Le Guellec C, Vaillant C, Le Roux MG, Valotaire Y (1989) Identification and estrogen induction of two estrogen receptor (ER) messenger ribonucleic acid in the rainbow trout liver: sequence homology with other ERs. *Mol Endocrinol* 3: 44-51

Palmiter RD and Mulvihill ER (1978) Estrogenic activity of the insecticide kepone on the chicken oviduct. *Science* 201: 356-358

Panter GH, Thompson RS, Beresford N, Sumpter JP (1999) Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. *Chemosphere* 38: 3579-3596

Parkman H & Remberger M (1995) Phthalates in Swedish sediments. Report No. 1167, The Environmental Research Institute (IVL); Stockholm, Sweden

Pastor D, Jover L, Ruiz X, Albaigés J (1995a) Monitoring organochlorine pollution in Audouin's gull eggs: the relevance of sampling procedures. *Sci Total Environ* 162: 215-223

Pastor D, Ruiz X, Barceló D, Albaigés J (1995b) Dioxins, furans and AHH-active PCB congeners in eggs of two gull species from the Western Mediterranean. *Chemosphere* 31: 3397-3411

Paxeus N (1996) Vehicle washing as a source of organic pollutants in municipal wastewater. *Water Sci Technol* 33: 1-8

Pedersen SN, Christiansen LB, Pedersen KL, Korsgaard B, Bjerregaard P (1999) In vivo estrogenic activity of branched and linear alkylphenols in rainbow trout (*Oncorhynchus mykiss*). *Sci Total Environ* 233: 89-96

Petersen JH (1991) Survey of di(2-ethylhexyl) phthalate plasticizer contamination of retail Danish milks. *Food Additives & Contaminants* 8: 701-706

Peterson G and Barnes S (1996) Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. *Cell Growth and Differentiation* 7: 1345-1351

Petit F, LeGoff P, Cravedi J-P, Valotaire Y, Pakdel F (1997) Two complimentary bioassays for screening the estrogenic potency of xenobiotics: Recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *J Mol Endocrinol* 19: 321-335

Piersma AH, Verhoef A, Dortant PM (1995) Evaluation of the OECD 421 reproductive screening test protocol using butyl benzyl phthalate. *Toxicology* 99: 191-197

Piferrer F and Donaldson EM (1989) Gonadal differentiation in Coho salmon, *Oncorhynchus kisutch*, after a single treatment with androgen or estrogen at different stages during ontogenesis. *Aquaculture* 77: 251-262

Pirie D, Steven L, McGrory S, Best G (1996) Survey of hormone disrupting chemicals. Scottish Environmental Protection Agency Report (August 1996), SEPA, Stirling, Scotland

Prat F, Sumpter JP, Tyler CR (1996) Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). *Biol Reprod* 54: 1375-1382

Protiva J, Pihera P, Schwarz V (1984) Biodegradation of the side chain of sterols and their mixture to the intermediate products of steroid drug synthesis. *Cesk Farm* 33: 225-229

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

Purvis IJ, Chotai D, Dykes CW, Lubahn DB, French FS, Wilson EM, Hobden AN (1991) An androgen-inducible expression system for *Saccaromyces cerevisiae. Gene* 106: 35-42

Ramamoorthy K, Wang F, Chen IC, Norris JD, McDonnell DP, Leonard LS, Gaido KW, Bocchinfuso WP, Korach KS, Safe S (1997) Estrogenic activity of a dieldrin/toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells, and yeast-based estrogen receptor assays: No apparent synergism. *Endocrinology* 138: 1520-1527

Rastogi SC (1998) Gas chromatographic analysis of phthalate esters in plastic toys. *Chromatographia* 47: 724-726

Reijnders PJH (1986) Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature* 324: 456-457

Rie M, Lendas K, Callard I (1999) Reproductive changes in the turtle, *Chrysemys picta*, on Cape Cod, Massachusetts: Indices of endocrine disruption? *Biol Reprod* 60(Suppl 1): 194

Rogers HR (1996) Sources, behaviour and fate of organic contaminants during sewage treatment and in sewage sludges. *Sci Total Environ* 185: 3-26

Ronis MJJ and Mason AZ (1996) The metabolism of testosterone by the periwinkle (*Littorina littorea*) in vitro and in vivo: Effects of tributyl tin. *Mar Environ Res* 42: 161-166

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: 241-248

Routledge EJ, Sumpter JP (1997) Structural features of alkylphenolic chemicals associated with estrogenic activity. *J Biol Chem* 272: 3280-3288

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: 1559-1565

Rudel RA, Melly SJ, Geno PW, Sun G, Brody JG (1998) Identification of alkylphenols and other estrogenic phenolic compounds in wastewater, septage, and groundwater on Cape Cod, Massachusetts. *Environ Sci Technol* 32: 861-869

Safe SH (1996) Toxic equivalency factors do not predict the acute toxicities of dioxins in rats. *Human Exp Toxicol* 15: 695-696

Safe SH (1997) Is there an association between exposure to environmental estrogens and breast cancer? *Environ Health Perspect* 105: 675-678

Safe S, Bandiera S, Sawyer T, Robertson L, Safe L, Parkinson A, Thomas PE, Ryan DE, Reik LM, Levin W, Denomme MA, Fujita T (1985) PCBs: Structure-function relationships and mechanism of action. *Environ Health Perspect* 60: 47-56

Safe S, Astroff B, Harris M, Zacharewski T, Dickerson R, Romkes M, Biegel L (1991) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related

compounds as antioestrogens: Characterization and mechanism of action. *Pharmacol Toxicol* 69: 400-409

Saga T, Oota Y, Nozaki M, Swanson P (1993) Salmonid pituitary gonadotrophs. III. Chronological appearance of GTH I and other adenohypophysial hormones in the pituitary of the developing rainbow trout (*Oncorhynchus mykiss irideus*). *Gen Comp Endocrinol* 92: 233-241

Saligaut C, Linard B, Mananos EL, Kah O, Breton B, Govoroun M (1998) Release of pituitary gonadotropins GTH I and GTH II in the rainbow trout (*Oncorhynchus mykiss*): modulation by estradiol and catecholamines. *Gen Comp Endocrinol* 109: 302-309

Saligaut C, Linard B, Breton B, Anglade I, Bailhache T, Kah O, Jego P (1999) Brain aminergic systems in salmonids and other teleosts in relation to steroid feedback and gonadotropin release. *Aquaculture* 177: 13-20

Santos EM, Rand-Weaver M, Tyler CR (in preparation) Follicle stimulating hormone and its subunit in rainbow trout (*Oncorhynchus mykiss*): Purification, characterisation, and development of specific radioimmunoassays.

Schafer TE, Lapp CA, Hanes CM, Lewis JB, Wataha JC, Schuster GS (1999) Estrogenicity of bisphenol A and bisphenol A dimethacrylate in vitro. *J Biomed Mater Res* 45: 192-197

Scholz N, Diefenbach R, Rademacher I, Linnemann D (1997) Biodegradation of DEHP, DBP, and DINP: Poorly water soluble and widely used phthalate plasticizers. *Bull Environ Contam Toxicol* 58: 527-534

Scott AP (1987) Reproductive endocrinology of fish. *In: Fundamentals of Comparative Vertebrate Endocrinology*. Chester-Jones I, Ingleton PM, Phillips JG (Eds), Plenum Press, New York; pp 223-256

Scott AP and Sumpter JP (1983) A comparison of the female reproductive cycles of autumn-spawning and winter-spawning strains of rainbow trout (*Salmo gairdneri* Richardson). *Gen Comp Endocrinol* 52: 79-85

Scott AP, MacKenzie DS, Stacey NE (1984) Endocrine changes during natural spawning in the white sucker, *Catastomus commersoni.* II. Steroid hormones. *Gen Comp Endocrinol* 56: 349-359

Sekine S, Saito A, Itoh S, Kawauchi H, Itoh S (1989) Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. *Proc Natl Acad Sci USA* 86: 8645-8649

Setchell KDR, Gosselin SJ, Welsh MB, Johnston JO, Balistreri WF, Kramer LW, Dresser BL, Tarr MJ (1987) Dietary estrogens - A probable cause of infertility and liver disease in captive cheetahs. *Gastroenterology* 93: 225-233

Sharman M, Read WA, Castle L, Gilbert J (1994) Levels of di(2-ethylhexyl) phthalate and total phthalate esters in milk, cream, butter and cheese. *Food Additives & Contaminants* 11: 375-385

Sharpe RM and Skakkebaek NE (1993) Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? *The Lancet* 341: 1392-1395

Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP (1995) Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. *Environ Health Perspect* 103: 1136-1143

Sheahan DA, Bucke D, Matthiessen P, Sumpter JP, Kirby MF, Neall P, Waldock M (1994) The effects of low levels of 17α-ethynyloestradiol upon plasma vitellogenin levels in male and female rainbow trout, *Oncorhynchus mykiss,* held at two acclimation temperatures. In: Muller R and Lloyd R (Eds); *Sublethal and chronic effects of pollutants on freshwater fish*; Fishing News Books, Blackwell Scientific, Oxford; pp 99-112

Sheldon LS and Hites RA (1978) Organic compounds in the Delaware river. *Environ Sci Technol* 12: 1188-1194

Shelton DR, Boyd SA, Tiedje JM (1984) Anaerobic biodegradation of phthalic acid esters in sludge. *Environ Sci Technol* 18: 93-97

Shore LS, Gurevitz M, Shemesh M (1993) Estrogen as an environmental pollutant. *Bull Environ Contam Toxicol* 51: 361-366

Siddiqui A, Srivastava SP (1992) Effect of di(2-ethylhexyl) phthalate administration on rat sperm count and on sperm metabolic enzymes. *Bull Environ Contam Toxicol* 48: 115-119

Singh PB and Kime DE (1995) Impact of γ-hexachlorocyclohexane on the in vitro production of steroids from endogenous and exogenous precursors in the spermiating roach, *Rutilus rutilus*. *Aquatic Toxicol* 31: 231-240

Sinjari T, Klasson-Wehler E, Oskarsson A, Darnerud PO (1996) Milk transfer and neonatal uptake of coplanar polychlorinated biphenyl (PCB) congeners in mice. *Pharmacol Toxicol* 78: 181-186

Smeets JMW, Rankouhi TR, Nichols KM, Komen H, Kaminski NE, Giesy JP, van den Berg M (1999a) In vitro vitellogenin production by carp (*Cyprinus carpio*) hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. *Toxicol Appl Pharmacol* 157: 68-76

Smeets JMW, van Holsteijn I, Giesy JP, Seinen W, van den Berg M (1999b) Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. *Toxicol Sci* 50: 206-213

Smith BS (1981a) Reproductive abnormalities in stenoglossan snails related to pollution from marinas. *J Appl Toxicol* 1: 15

Smith BS (1981b) Male characteristics on female mud snails caused by antifouling bottom paints. *J Appl Toxicol* 1: 22

Smith BS (1981c) Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus = Ilyanassa obsoleta*. *J Appl Toxicol* 1: 141-144

So FV, Guthrie N, Chambers AF, Moussa M, Carrol KK (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr Cancer* 26: 167-181

Sohn YC, Yoshiura Y, Kobayashi M, Aida K (1998) Effect of sex steroids on the mRNA levels of gonadotropin I and II subunits in the goldfish *Carassius auratus*. *Fisheries Sci* 64: 715-721

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

Sonnenschein C, Soto AM, Fernandez MF, Olea N, Olea-Serrano MF (1995) Development of a marker of estrogenic exposure in human serum. *Clinical Chemistry* 41: 1888-1895

Soto AM & Sonnenschein C (1985) The role of estrogens on the proliferation of human breast tumour cells (MCF-7). *J Steroid Biochem* 23: 87-94

Soto AM, Justicia H, Wray JW, Sonnenschein C (1991) p-Nonylphenol: an estrogenic xenobiotic released from 'modified' polystyrene. *Environ Health Perspect* 92: 167-173

Soto AM, Lin T-M, Justicia H, Silvia RM, Sonnenschein C (1992) An 'in culture' bioassay to assess the estrogenicity of xenobiotics (E-Screen). In: *Chemically induced alterations in sexual and functional development: The wildlife / human connection* Colborn T & Clement C (eds); Princeton Scientific Publishing, Princeton, NJ; pp 295-309

Soto AM, Chung KL, Sonnenschein C (1994) The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ Health Perspect* 102: 380-383

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Olea-Serrano F (1995) The E-Screen assay as a tool to identify estrogens: An update on estrogenic environmental pollutants *Environ Health Perspect* 103 (Suppl 7): 113-122

Soule HD & McGrath CM (1980) Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Letters* 10: 177-189

Specker JL and Sullivan CV (1993) Vitellogenesis in fishes: Status and perspectives; In: *Perspectives in Comparative Endocrinology*; Davey KG, Peter RE, Tobe SS (Eds); National Research Council of Canada, Ottowa, Canada: 304-315

Spooner N, Gibbs PE, Bryan GW, Goad LJ (1991) The effect of tributyltin upon steroid titres in the female dogwhelk, *Nucella lapillus*, and the development of imposex. *Mar Env Res* 32: 37-49

Stalling DL, Hogan JW, Johnson JL (1973) Phthalate ester residues - their metabolism and analysis in fish. *Environ Health Perspect* 3: 153-157

Staples CA, Peterson DR, Parkerton TF, Adams WJ (1997) The environmental fate of phthalate esters: A literature review. *Chemosphere* 35: 667-749

Staples CA, Weeks J, Hall JF, Naylor CG (1998) Evaluation of aquatic toxicity and bioaccumulation of C8- and C9-alkylphenol ethoxylates. *Environ Toxicol Chem* 17: 2470-2480

Steiner I, Scharf L, Fiala F, Washuttl J (1998) Migration of di(2-ethylhexyl) phthalate from PVC child articles into saliva and saliva simulant. *Food Add Contam* 15: 812-817

Stillman RJ (1982) In utero exposure to diethylstilbestrol: Adverse effects on the reproductive tract and reproductive performance in male and female offspring. *Am J Obstet Gynecol* 142: 905-921

Stringer R, Labounskaia I, Santillo D, Johnston P, Siddorn J, Stephenson A (1997) Determination of the composition and quantity of phthalate ester additives in PVC children's toys. Greenpeace Research Laboratories Technical Note 06/97. University of Exeter, UK

Stumpf M, Ternes TA, Ilaberer K, Baumann W (1996) Determination of natural and synthetic estrogens in sewage plants and river water. *Vom Wasser* 87: 251-261

Subramanian AN, Tanabe S, Tatsukawa R, Saito S, Miyazaki N (1987) Reduction in the testosterone levels by PCBs and DDE in Dall's porpoises of Northwestern North Pacific. *Mar Poll Bull* 18: 643-646

Sugatt RH, O'Grady DP, Banerjee S, Howard PH, Gledhill WE (1984) Shake flask biodegradation of 14 commercial phthalate esters. *Appl Environ Microbiol* 47: 601-606

Sumpter (1985) The purification, radioimmunoassay and plasma levels of vitellogenin from the rainbow trout, *Salmo gairdneri.* In: *Current Trends in Comparative Endocrinology* Lofts B & Holmes WN (eds) Hong Kong University Press, Hong Kong; pp 335-357

Sumpter JP and Scott AP (1989) Seasonal variations in plasma and pituitary levels of gonadotropin in males and females of two strains of rainbow trout (*Salmo gairdneri*). *Gen Comp Endocrinol* 75: 376-388

Sumpter JP and Jobling S (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect* 103 (Suppl 7): 173-178

Suzuki K, Kawauchi H, Nagahama Y (1988a) Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen Comp Endocrinol* 71: 292-301

Suzuki K, Kawauchi H, Nagahama Y (1988b) Isolation and characterization of subunits from two distinct salmon gonadotropins. *Gen Comp Endocrinol* 71: 302-306

Swanson P (1991) Salmon gonadotropins: reconciling old and new ideas; In: Scott AP, Sumpter JP, Kime DE, Rolfe MS (Eds) *Proceedings of the 4th International Symposium on the Reproductive Physiology of Fish*, Norwich, UK; pp 2-7

Swanson P, Suzuki K, Kawauchi H, Dickhoff WW (1991) Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol Reprod* 44: 29-38

Sweeting R (1981) Hermaphrodite roach in the River Lee; Internal Report, Thames Water, Lea Division, Herts, UK

Tanghe T, Devriese G, Verstraete, W (1998) Nonylphenol degradation in lab scale activated sludge units is temperature dependent. *Water Res* 32: 2889-2896

Tchoudakova A, Pathak S, Callard GV (1999) Molecular cloning of an estrogen receptor beta subtype from the goldfish, *Carassius auratus*. *Gen Comp Endocrinol* 113: 388-400

Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken R-D, Servos M (1999) Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. *Sci Total Environ* 225: 81-90

Thibaut R, Debrauwer L, Rao D, Cravedi JP (1998) Disposition and metabolism of [H-3]-4-n-nonylphenol in rainbow trout. *Mar Env Res* 46:521-524

Thomas DJ, Tracey B, Marshall H, Nostrom RJ (1992) Arctic terrestrial ecosystem contamination. *Sci Total Environ* 122: 135-164

Thuren A and Larsson P (1990) Phthalate esters in the Swedish atmosphere. *Environ Sci Technol* 24: 554-559

Toppari J, Larsen JChr, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, MacLachlan JA, Meyer O, Müller J, Rajpert-de Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE (1996) Male reproductive health and environmental xenoestrogens. *Environ Health Perspect* 104(Suppl 4): 741-803

Tremblay L and van der Kraak G (1999) Comparison between the effects of the phytosterol ß-sitosterol and pulp and paper mill effluents on sexually immature rainbow trout. *Environ Toxicol Chem* 18: 329-336

Trinh KY, Wang NC, Hew CL, Crim LW (1986) Molecular cloning and sequencing of salmon gonadotropin ß subunit. *European J Biochem* 159: 619-624

Tyler CR & Sumpter JP (1990) The development of a radioimmunoassay for carp, *Cyprinus carpio*, vitellogenin. *Fish Physiol Biochem* 8: 129-140

Tyler CR and Sumpter JP (1996) Oocyte growth and development in teleosts. *Reviews in Fish Biol and Fisheries* 6: 287-318

Tyler CR, Sumpter JP, Kawauchi H, Swanson P (1991) Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. *Gen Comp Endocrinol* 84: 291-299

Tyler CR, van der Eerden, B, Jobling S, Panter G, Sumpter JP (1996) Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. *J. Comp. Physiol. B* 166: 418-426

Tyler CR, Pottinger TG, Coward K, Prat F, Beresford N, Maddix S (1997) Salmonid follicle-stimulating hormone (GTH I) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhynchus mykiss*. *Biol Reprod* 57: 1238-1244

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

Tyler CR, Beresford N, van der Woning M, Sumpter JP, Thorpe K (2000) Metabolism and environmental degradation of pyrethroid insecticides produce compounds with endocrine activities. *Environ Toxicol Chem* 19: 801-809

Ungerer J and Thomas P (1996) Transport and accumulation of organochlorines in the ovaries of atlantic croaker (*Micropogonias undulatus*). *Mar Env Res* 42: 167-171

van Baal J, Hassing GAM, Goos HJTh, Schulz RW (2000) Modulatory effects of 4-nonylphenol on LH production in the african catfish pituitary. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (Eds) *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*; Bergen, Norway; p 373

van Bohemen ChG and Lambert JGD (1981) Estrogen synthesis in relation to estrone, estradiol, and the vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, *Salmo gairdneri* . *Gen Comp Endocrinol* 45: 105-114

van Bohemen ChG, Lambert JGD, Peute J (1981) Annual changes in plasma and liver in relation to vitellogenesis in the female rainbow trout, *Salmo gairdneri* . *Gen Comp Endocrinol* 44: 94-107

van Bohemen ChG, Lambert JGD, Goos HJTh., 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 den Hurk R and Slof GA (1981) A morphological and experimental study of gonadal sex differentiation in the rainbow trout, *Salmo gairdneri*. *Cell Tissue Res* 218: 487-497

van Lierop JBH (1997) Enforcement of food packaging legislation. *Food Additives & Contaminants* 14: 555-560

van Putten LJA, Peute J, van Oordt PGWJ, Goos HJTh, Breton B (1981) Glycoprotein gonadotropin in the plasma and its cellular origin in the adenohypophysis of sham-operated and ovariectomized rainbow trout, *Salmo gairdneri*. *Cell Tissue Res* 218: 439-448

Varineau PT, Williams JB, Naylor CG, Cady C, Serak K, Yunick RP (1996) The biodegradation of a 14C ring-labeled nonylphenol ethoxylate in

river water and activated sludge. *Proceedings*, 17th Annual Meeting, Society of Environmental Toxicology and Chemistry, Washington, DC, USA, November 17-21, p 102

Vinkelsoe J, Jensen GH, Johansen E, Carlsen I, Rastogi SC (1997) Migration of phthalates from teething rings. Danish EPA 15.4.97

Vonier PM, Crain DA, McLachlan JA, Guillette LJ Jr, Arnold SF (1996) Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. *Environ Health Perspect* 104: 1318-1322

Wakeling AE and Valcaccia B (1983) Antiestrogenic and anti-tumour activities of a series of non-steroidal antiestrogens. *J Endocrinol* 99: 455-464

Wannemacher R, Rebstock A, Kulzer E, Schrenk D, Bock KW (1992) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on reproduction and oogenesis in zebrafish (*Brachydanio rerio*). *Chemosphere* 24: 1361-1368

Warhurst AM (1995) An environmental assessment of alkylphenol ethoxylates and alkylphenols. Friends of the Earth: Edinburgh

Wauchope RD, Buttler TM, Hornsby AG, Augustijn-Beckers PWM, Burt JP (1992) SCS/ARS/CES pesticides properties database for environmental decision making. *Rev Environ Contam Toxicol* 123: 1-157

Weil C, Bougoussa-Houadec M, Gallais C, Itoh S, Sekine S, Valotaire Y (1995) Preliminary evidence suggesting variations of GTH I and GTH II mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss. Gen Comp Endocrinol* 100: 327-333

Weil C, Carré F, Blaise O, Breton B, Le Bail P-Y (1999) Differential effect of insulin-like growth factor I on in vitro gonadotropin (I and II) and growth hormone secretions in rainbow trout (*Oncorhynchus mykiss*) at different stages of the reproductive cycle. *Endocrinology* 140: 2054-2062

Welch RM, Levin W, Conney AH (1969) Estrogenic action of DDT and its analogs. *Toxicol Appl Pharmacol* 14: 358-367

Wester PW, Canton JH, Bisschop A (1985) Histopathological study of *Poecilia reticulata* (guppy) after long-term ß-hexachlorocyclohexane exposure. *Aquat Toxicol* 6: 271-296

Wester PW, Canton JH (1986) Histopathological study of *Oryzias latipes* (medaka) after long-term ß-hexachlorocyclohexane exposure. *Aquat Toxicol* 9: 21-45

Wheeler TF, Heim JR, LaTorre MR, Blair Janes A (1997) Mass spectral characterisation of p-nonylphenol isomers using high-resolution capillary GC-MS. *J Chromatogr Sci* 35: 19-30

White R, Jobling S, Hoare SA, Sumpter JP, Parker MG (1994) Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135: 175-182

Whitten PL and Naftolin F (1998) Reproductive actions of phytoestrogens. *Baillieres Clinical Endocrinol Metab* 12: 667-690

Wiig Ø, Derocher AE, Cronin MM, Skaare JU, (1998) Female pseudohermaphrodite polar bears at Svalbard. *J Wildlife Diseases* 34: 792-796

Wildbrett G (1973) Diffusion of phthalic acid esters from PVC milk tubing. *Environ Health Perspect* 3: 29-35

Wilson EM and French FS (1976) Binding properties of androgen receptors - evidence for identical receptors in rat testis, epididymis and prostate. *J Biol Chem* 251: 5620-5629

Wine RN, Li L-H, Hommel Barnes L, Gulati DK, Chapin RE (1997) Reproductive toxicity of di-n-butyl phthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ Health Perspect* 105: 102-107

Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N (1993) Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* 85: 648-652

Yadetie F, Arukwe A, Goksoyr A, Male R (1999) Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. *Sci Total Environ* 233: 201-210

Yam KM, Yoshiura Y, Kobayashi M, Ge W (1999) Recombinant goldfish activin B stimulates gonadotropin-Iß but inhibits gonadotropin-IIß expression in the goldfish, *Carassius auratus*. *Gen Comp Endocrinol* 116: 81-89

Yan H, Ye C, Yin C (1995) Kinetics of phthalate ester biodegradation by *Chlorella pyrenoidosa*. *Environ Toxicol Chem* 14: 931-938

Zacharewski T (1997) In vitro bioassays for assessing estrogenic substances. *Environ Sci Technol* 31: 613-623

Zacharewski T (1998) Identification and assessment of endocrine disruptors: Limitations of in vivo and in vitro assays. *Environ Health Perspect* 106(Suppl. 2): 577-582

Zacharewski TR, Meek MD, Clemons Jd, Wu ZF, Fielden MR, Matthews JB (1998) Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. *Toxicol Sci* 46: 282-293

Zeleznik AJ, Hutchison JS, Schuler HM (1985) Interference with the gonadotropin-suppressing actions of estradiol in macaques overrides the selection of a single preovulatory follicle. *Endocrinology* 117: 991-999

Zilberstein Y, Cohen Y, Gur G, Rosenfeld H, Elizur A, Yaron Z (2000) Nonylphenol as a xenoestrogen in tilapia: Hypophyseal effects. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (Eds) *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*; Bergen, Norway; p 374

Zucker E (1985) Hazard Evaluation Division, Standard Evaluation Procedure: Acute toxicity test for freshwater fish. EPA-540/9-85-006; Environmental Protection Agency (USA), Washington, DC 20460, USA