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\textsubscript{9}H\textsubscript{19}); the ‘4’, or ‘para,’ refers to the position of the alkyl group relative to the hydroxyl group on the phenol ring.
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 \( \mu g/L \) range, but some rivers have been found to carry loads in excess of 100 \( \mu g/L \) 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 \( \mu g/L \) of total extractable 4-NP, and found water samples collected from this river to yield concentrations of up to 180 \( \mu g/L \). 4-NP concentrations in the majority of other sites sampled, however, ranged from <0.2 to 12 \( \mu g/L \) (Blackburn and Waldock,
Ahel et al (1994b) measured 4-NP in Swiss rivers and found concentrations ranging from <1 to 45 \( \mu g/L \). In this case, only one value was found to exceed 10 \( \mu g/L \), and more than 80\% were in the range of 1 to 7 \( \mu g/L \). Sheldon and Hites (1978) found concentrations of only 1 to 2 \( \mu g \) 4-NP/L in rivers in summer, and as little as 0.04 to 1 \( \mu g/L \) 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 \( \mu g/L \). 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 \( \mu g/L \) in the Mersey, and <0.2 to 5.8 \( \mu g/L \) 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 \( \mu g/L \). 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 estrogically 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 ‘β’ 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 β-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:

<table>
<thead>
<tr>
<th>Tank</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Absolute control</td>
</tr>
<tr>
<td>B</td>
<td>MeOH control</td>
</tr>
<tr>
<td>C</td>
<td>1 µg 4-NP/L</td>
</tr>
<tr>
<td>D</td>
<td>10 µg 4-NP/L</td>
</tr>
<tr>
<td>E</td>
<td>100 µg 4-NP/L</td>
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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 β-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-32P]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 β-actin. Membranes were prehybridised at 42°C for 3 hours in hybridisation buffer (50% formamide, 5 x SSC, 5 x Denhardt’s 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'.

132
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.

<table>
<thead>
<tr>
<th>TIME (weeks)</th>
<th>B MeOH CONTROL</th>
<th>C 1 μg/L</th>
<th>D 10 μg/L</th>
<th>E 100 μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.2</td>
<td>1.9</td>
<td>7.8</td>
<td>79.3</td>
</tr>
<tr>
<td>6</td>
<td>&lt;0.2</td>
<td>0.8</td>
<td>10.1</td>
<td>83.5</td>
</tr>
<tr>
<td>12</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>9.2</td>
<td>87.8</td>
</tr>
<tr>
<td>18</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>6.1</td>
<td>91.8</td>
</tr>
</tbody>
</table>

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:

$$\text{coefficient of condition} = \left(\frac{\text{weight}}{\text{length}^3}\right) \times 100$$

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:

\[
GSI = \left(\frac{\text{gonad weight}}{\text{total body weight}}\right) \times 100
\]

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

\[
HSI = \left(\frac{\text{liver weight}}{\text{total body weight}}\right) \times 100
\]

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  17β-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 than 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 / β-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 ** \( p<0.01 \); *** \( p<0.001 \).
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 FSHB mRNA in an individual fish. Figure 4.21B shows these data (arbitrary units) corrected for the level of β-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 ± 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 ≤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 / β-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 LHB mRNA in an individual fish. Figure 4.24B shows these data (arbitrary units) corrected for the level of β-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 \( \mu \text{g} \) 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 \( \mu \text{g} \) 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 \( \mu \text{g} \) 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 levels.
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 FSHβ mRNA levels were suppressed by 4-NP. In contrast, LHβ 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 FSHβ 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.
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