Chapter 6: Possible effects of Clofibric acid on gene expression

6.1 Introduction

Aquatic organisms are exposed to a variety of pollutants, all of which have the potential to have a detrimental effect. Clofibric acid, which has also been found to be present in the environment, has so far (within the realms of this thesis) been shown to have effects in fish (some more convincingly so than others) at the physiological and biochemical levels. This chapter attempts to find the mechanistic effects of clofibric acid on adult fathead minnows (if present), by looking at the molecular level.

It was decided that two genes would be considered for examination after exposure of fish to clofibric acid. The decision of which genes to look at was primarily made by identifying those genes already found to be up- or down-regulated by fibrates in humans (there were no data available from fish). It was also important to consider whether the genes had been previously identified in fish (more specifically in the fathead minnow). The chosen genes were Lipoprotein Lipase (LPL) and Glucose-6-Phosphate Dehydrogenase (G6PDH). LPL is a gene involved in lipid metabolism and is a known natural target for fibrates (via PPARα) in humans, and G6PDH is involved in fatty acid synthesis, steroid biosynthesis and defence against xenobiotic toxicity and oxidative stress.

LPL is a glycoprotein, and one of the family of triacylglycerol lipases, which include pancreatic and hepatic lipases. As previously stated, lipoprotein lipase is a key enzyme in the metabolism of lipids (Murthy *et al* 1996). In mammals, the enzyme is synthesised in the parenchymal cells of adipose and several other tissues (excluding the liver), and is secreted into the capillary endothelium, where it is bound to the cell surface and where the lipase acts on triacylglycerols in plasma lipoproteins, to release fatty acids for uptake and

storage or use by other organs such a muscle, for oxidation (Liang *et al*, 2002). In humans, LPL activity is regulated by diet and a number of hormones, including epinephrine, norepinephrine, thyroid stimulating hormone, adrenocorticotrophic hormone (ACTH), growth hormone and glucagons. Liang *et al* (2002) have also shown in the red sea bream, LPL expression seems to also be regulated by the nutritional state of the fish.

Babin and Vernier, (1989) have previously purified trout LPL, and found its properties and molecular weight to be similar to those of the mammalian enzyme. Kwon *et al* (2001) have also previously cloned and sequenced the LPL gene from Rainbow trout. In fish, significant LPL expression occurs in the liver (Oku *et al*, 2002; Liang *et al*, 2002) (unlike the situation in mammals) and also in vitellogenic ovaries (Kwon *et al*, 2001). It has been suggested by Kwon *et al,* (2001) that LPL is involved in the storage and mobilisation of egg yolk constituents during vitellogenesis. The tissue specific mechanisms of LPL gene expression in fish may therefore be different from that in mammals and consequently the literature regarding LPL may not be completely comparable across species.

Peroxisome proliferators-activated receptors (PPARs), as previously mentioned in Chapter 1, were identified in the 1990s in rodents and named after their ability to facilitate peroxisome proliferation. PPARs are key transcriptional factors that catalyze and coordinate different biochemical events in order to achieve energy homeostasis. Fibrates are well known activators of PPARs, specifically PPARα, for the regulation of β-oxidation of fatty acids. They have been shown to control the expression of various genes that are crucial for lipid and glucose metabolism. PPARα activation decreases triglyceride levels by amplifying the expression of lipoprotein lipase (LPL).

Glucose-6-phosphate dehydrogenase (G6PDH), the key enzyme of the pentose phosphate pathway (previously discussed in Section 1.6.1.1), is crucial in the supply of riboses for DNA and RNA synthesis by production of 6-phosphogluconate, particularly in proliferating cells. However, a more important function, the production of NADPH, the major cytoplasmic reducing compound, is now acknowledged (Winzer *et al*, 2002). G6PDH is a regulatory enzyme in NADPH-dependant xenobiotic biotransformation and defences against oxidative stress (Figure 6.1).

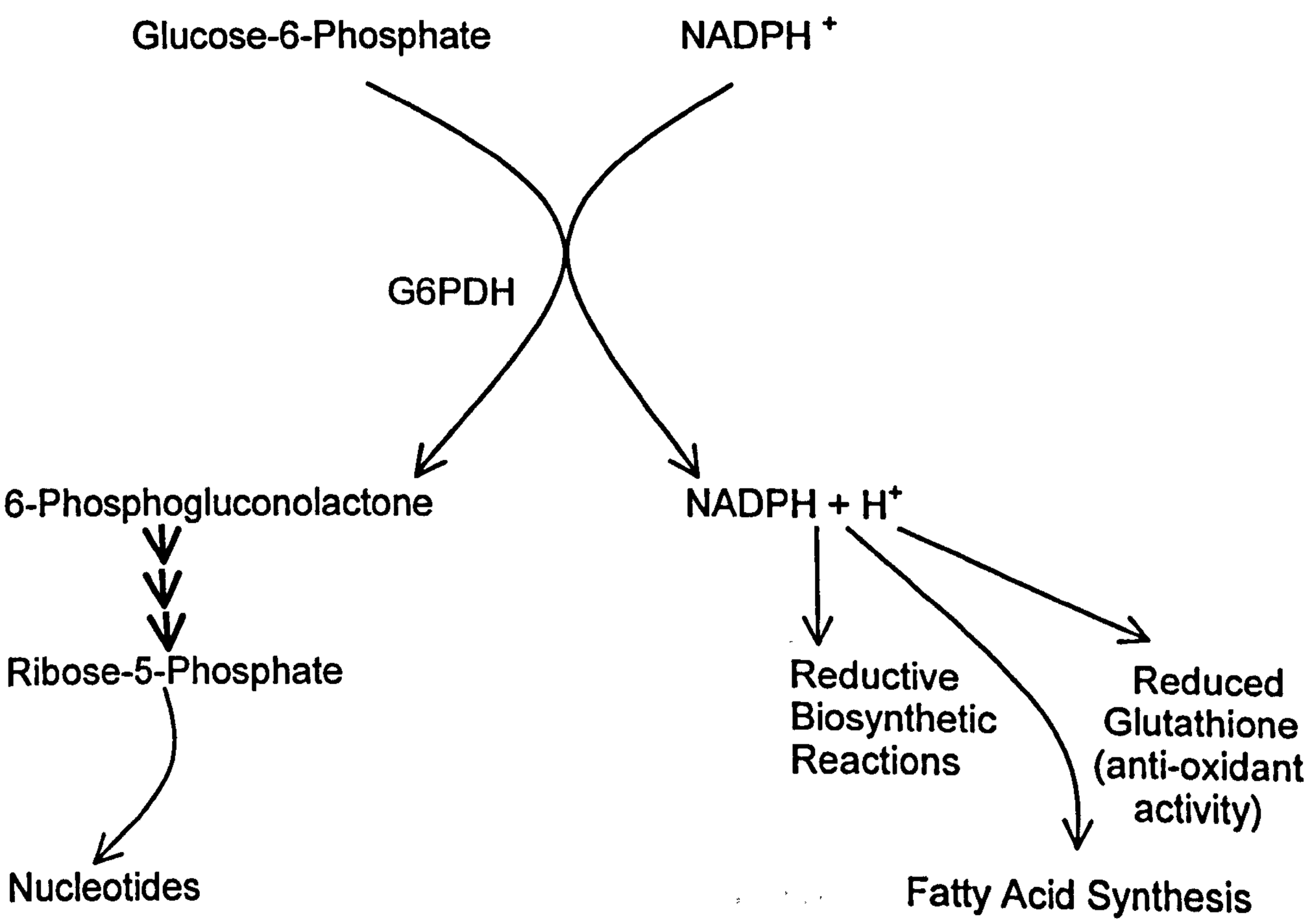

Figure 6.1: The role of glucose-6-phosphate dehydrogenase (G6PDH) in cellular metabolism (adapted from Salati and Amir-Ahmady, 2001).

G6PDH expression is regulated in the liver and adipose tissue by hormonal and nutritional factors. Its activity correlates with the rate of fatty acid biosynthesis. Winzer *et al* (2002) suggest that G6PDH in fish is sensitive to inactivation by xenobiotics in a sex-dependant manner. Physiological

conditions resulting in oxidative stress can also result in changes in G6PDH activity, and its gene expression is essential for protection of the cell against even mild oxidative stress.

6.2 Protocol

The effect of clofibric acid on both LPL and G6DPH mRNA expression levels was investigated. The livers of adult female and male fathead minnow collected from Experiments 4 and 5 (see Chapter 5 for experimental design) were used in this study. Morphometric and biochemical results from these experiments can also be seen in Chapter 5.

6.2.1 Computer analysis of known sequences

Gene sequence searches for fathead minnow LPL, G6PDH and β-actin were carried out using the Entrez search and retrieval system at NCBI (http://www.ncbi.nlm.nih.gov/entrez/). If the specific fathead minnow sequences were not found, sequences from related fish species that were present in the database were aligned using a web-based multiple alignment tool (BCM search launcher - http://searchlauncher.bcm.tmc.edu/multi-align/) so that primers could be designed to the conserved regions (it was important that similar taxa were used in the alignment to ensure a more accurate consensus was achieved). Alignments can be seen in Appendix 2 and 3.

6.2.2 Primers construction

Primers were designed for the lipoprotein lipase and β-actin genes (chosen as a house-keeping gene) by aligning sequences from other species of fish and finding conserved regions. The primers were designed by eye due to

the complexity of the alignments. For LPL, primers were designed for RT-PCR across introns (sequence can be seen in Appendix 2), whereas for β-actin, the only suitable primer binding sites were found within exon 2, so the primers were designed to this region (sequence can be seen in Appendix 3). Although exonic primers potentially lack the specificity of primers designed across introns (as they can also amplify from the genomic gene sequence), genomic contamination was previously shown not to be an issue (see Section 2.3.1.2).

The general rules of primer design were followed. In some primers, when the identity of a base was unclear in the consensus sequence of the alignment, a degenerate base was used to allow for this.

Rules for primer design:

- Keep the G-C content about 50%
- Ensure there is a GC clamp at the 3' end
- Try and design primers between 16-24 base pairs long
- Avoid complementary sequence, both within and between primer pairs (to avoid secondary structure formation)

The primers for the G6PDH gene were designed using PRIMER3 program (part of the Lasergene suite of genetic analysis software) using a submitted sequence for the fathead minnow liver G6PDH gene (Gen Bank Accession number: AF206637), which can be seen in Appendix 1. All primers were then synthesised and purchased from Sigma – Genosys Ltd (Pampisford, UK). They were assessed by PCR-amplification of fathead cDNA at 1, 2 and 3 mM $MgCl_2$ concentrations (screening cycling conditions: 95°C for 15 mins, 35 cycles of 94°C for 30 sec, 58°C for 30 secs, 72°C for 30 secs, and a final 72°C for 5 mins). Primers that failed to amplify were disregarded. Remaining primers

were then screened by amplifying from a fathead mRNA dilution series on the Rotor Gene (Corbett Research, Cams). It was found that the LPL primers were problematic, giving amplification reaction efficiencies greater than 1. It was thought that this might be due to the degeneracy of certain bases within the primer sequence. These primers were then re-synthesised without degeneracy and these were then screened as before. All reaction efficiencies using these non-degenerative primers were less than 1. The optimal primer pair for each gene can be seen in Table 6.1 below.

Table 6.1: Primer pairs used for Real Time RT-PCR for liver samples from Experiments 4 and 5.

Gene	Primer sequence
LPL	FHLPL4r – AAC AGG TGG ATG GAG CGC FHLPL7f – GAC ATC TAC CCC AAT GGA GG
G6PDH	FHGAP3r – ACC CCA TCC CAG CGT TCA TTC FHGAP3f – GAG AAG CCC GCA TCC ACC AG
β-Actin	FHBACT2f – GAT ATG GAG AAG ATC TGG C FHBACT2r – GTT GGC TTT GGG GTT CAG G

6.2.3 Quantification of LPL and G6PDH gene expression by quantitative real time RT-PCR

Livers from control and dosed fathead minnows (*Pimephales promelas*) from both Experiment 4 (exposed to 1mg/l clofibric acid) and Experiment 5 (control, 10µg/l and 1mg/l clofibric acid) were carefully dissected and weighed using RNase free conditions and immediately snap-frozen as previously described in Section 2.1.4. Total RNA extraction was performed on the liver using TRI Reagent, as previously described in Section 2.3.1. The RNA concentrations were determined spectrophotometrically (Genequant,

Pharmacia, Cambridge, UK) and the integrity of the RNA checked by running it on agarose gels (Section 2.3.1.3). After concentrations were determined, all samples were then diluted with double autoclaved MilliQ water to give concentrations of 10ng/µl total RNA in each sample.

Real time RT-PCR was used for relative quantitative determination of LPL, G6DPH and β-actin mRNA expression in adult fathead minnow exposed to clofibric acid (control and dosed), as briefly discussed in Section 2. β-actin was used as a house-keeping gene to allow all other results to be 'normalised' to the level of expression of β-actin.

6.2.3.1 Experiment 4

130 total RNA samples extracted from livers dissected from fathead minnows exposed to clofibric acid in Experiment 4 (Section 2.1.4 and 5.4) were diluted to 10ng/µl using double autoclaved, sterile deionised water.

10µl reactions were run in strips of 4x100µl PCR tubes, where 10ng of RNA was used as a template in each reaction with master mix (Qiagen QuantiTect SyBR Green RT-PCR master mix - consisting of HotStarTaq DNA polymerase, Quantitect SYBR Green RT-PCR buffer (containing TrisCl, KCl, $(NH_4)_2SO_4$, and 5mM $MgCl_2$, pH 8.7 (20°C), dNTP Mix (contains dATP, dCTP, dGTP, and dTTP/dUTP; ultrapure), SYBR Green and ROX florescent dyes, QuantiTect RT mix) and RNase free water (PCR grade)) and 5 picomols of each of the primers. All samples were analysed in duplicate

RNA sample 100 was used as the standard for this experiment and so was diluted using double autoclaved, sterile deionised water to 0.5,2,8,20,50 ng/µl (Figure 2.14). These dilutions were used to produce the standard curve for quantification of all 3 genes.

Amplification and detection of samples and standards were performed on the Rotor Gene 3000 system (Corbett Research, Cambs) using the following thermal cycling conditions:

50°C for 30 minutes	(Reverse transcription)	
95°C for 15 minutes	(Taq activation/initial denaturing step)	
94°C for 10 sec	(denature)	} repeated for 50 cycles
58°C for 10 sec	(annealing)	
72°C for 10 sec	(extension)	

At the end of this cycling, a melt curve was calculated which consisted of measurements of fluorescence at every 1°C from 60-99°C. This curve shows the specificity of the PCR and reveals the presence, if any, of primer-dimer (Figure 2.15).

Quantities of mRNA in the samples were quantified relatively by comparison with a standard curve derived from a dilution series of an RNA reference (sample 100). The level of β-actin in each reaction was used to normalise for the variability in RNA quality or quantity, among the samples. Results are reported as the ratio of LPL/β-actin and G6DPH/β-actin.

6.2.3.2 Experiment 5

84 total RNA samples previously extracted from livers dissected from fathead minnows exposed to clofibric acid in Experiment 5 (Section 2.1.4 and 5.5) were diluted to 10ng/µl using double autoclaved, sterile deionised water as described previously. In this experiment, however, PCR reactions were carried in 96-well plates in 20µl reactions using the same constituent concentrations as specified previously (Section 6.2.3.1) and again, all samples were analysed in

duplicate. RNA sample 100 from Experiment 4 was again used as the standard for this experiment and so was diluted using double autoclaved, sterile deionised water to give the range 0.19,0.78, 3.125, 12.5, 50, 200 ng/µl. Amplification and detection of samples and standards were performed on the ABI Prism 7900HT Sequence detection system (Applied Biosystems, USA) using thermal cycling conditions as described above. Quantities of mRNA in each of the samples were calculated as described previously.

6.2.4 Statistics

Differences between males and females within control and clofibric acid-exposed experimental groups were analysed using SigmaStat (version 2.03). In cases of normality, differences were determined by a t-test, and where normality test failed, non-parametric statistical tests were used.

6.3 Results

6.3.1 Levels of expression of selected genes in the livers of fish from Experiment 4

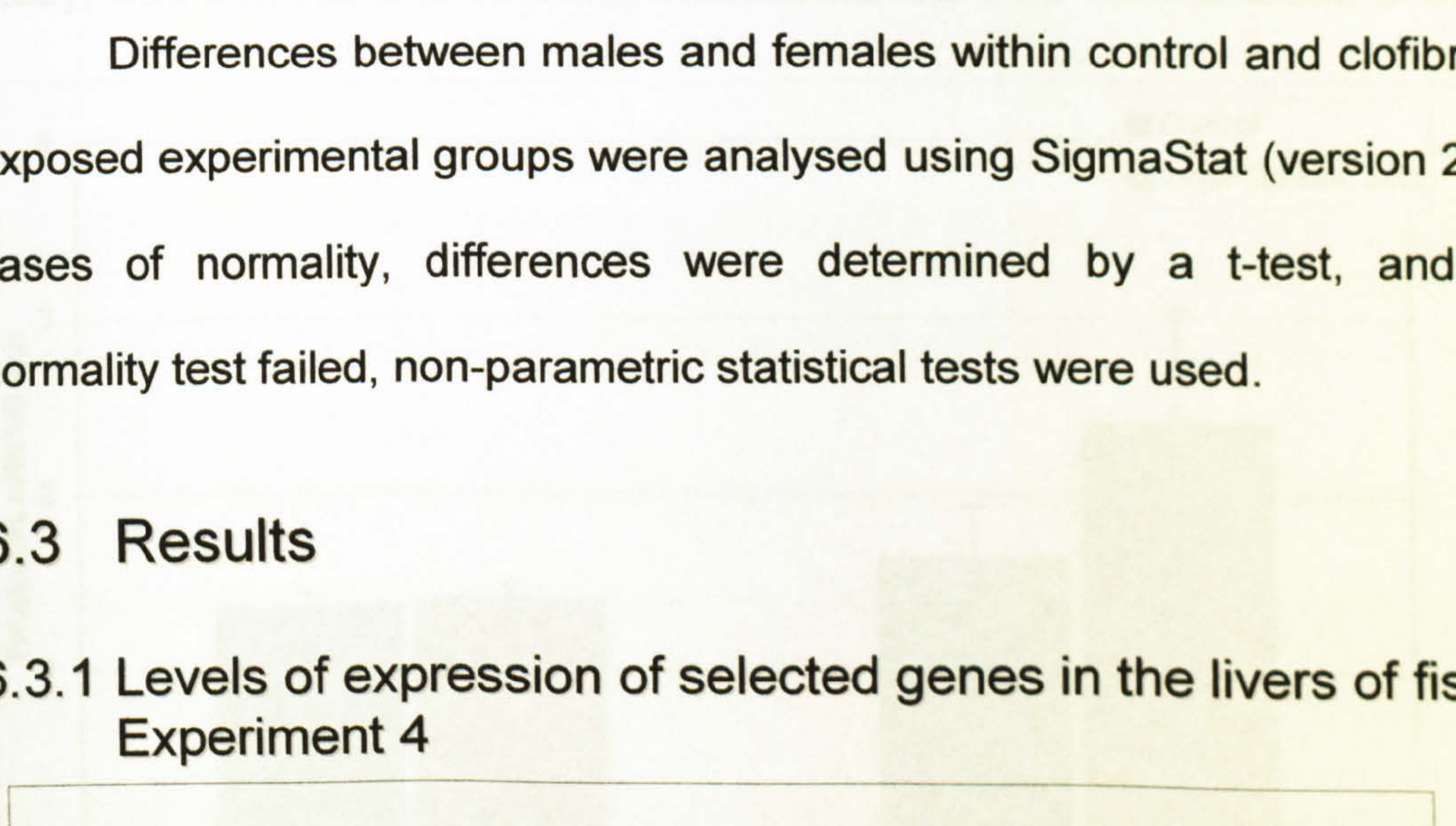

Figure 6.2: Mean relative levels of β-Actin expression in the livers of adult fathead minnow exposed to 1mg/l Clofibric acid from Experiment 4 (p=0.3). Data are expressed as means ± SEM. There was no significant effects of treatment with clofibric acid.

Figure 6.2 shows the levels of β-actin mRNA expression from the livers collected from Experiment 4. It has been shown that gene expression of β-actin (and many other house-keeping genes) may change in the face of physiological challenges (Kazeto *et al*, 2004), thereby negating its usefulness in standardising for loading differences, RNA quality, etc. However, in this experiment, β-actin levels amongst control and dosed fish were shown to be very similar, and were well within the expected individual and procedural variance. Therefore, I feel confident that β-actin expression, at least in this study, was a useful and accurate internal standard for normalisation of the data.

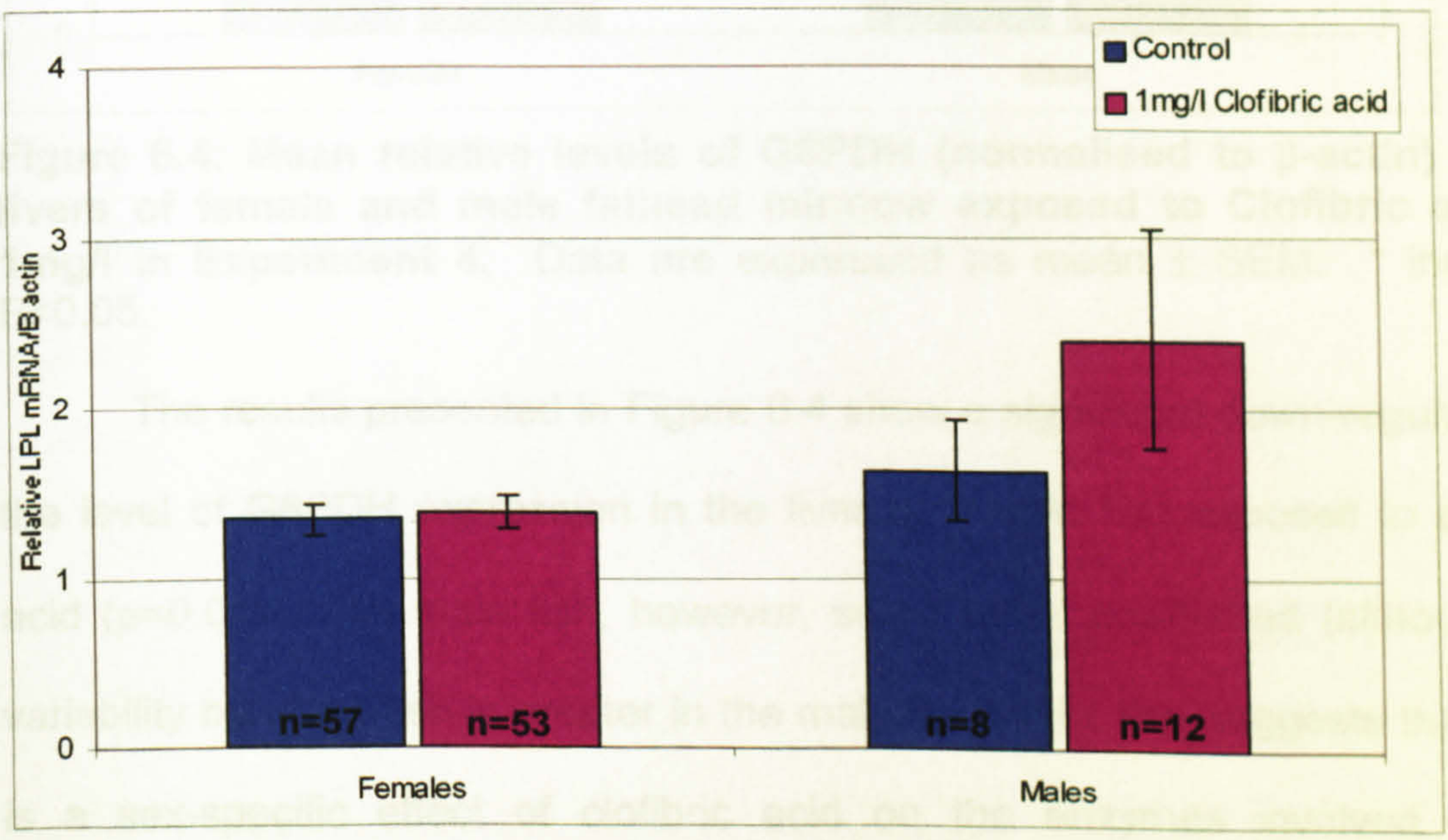

Figure 6.3: Mean relative levels of LPL mRNA expression (normalised to β-actin) in the livers of female and male adult fathead minnow exposed to Clofibric acid at 1mg/l in Experiment 4. Data are expressed as means ± SEM. There was no significant effect of treatment with clofibric acid.

The results presented in Figure 6.3 show the mean levels of lipoprotein lipase mRNA expression normalised to β-actin in the livers. It can be seen that the level of LPL expression in the females seems to be unaffected by exposure to clofibric acid, whereas in the males the levels give the impression that they

are up-regulated (although not significantly). These results indicate that there may be a sex-specific effect on LPL expression after exposure to clofibric acid.

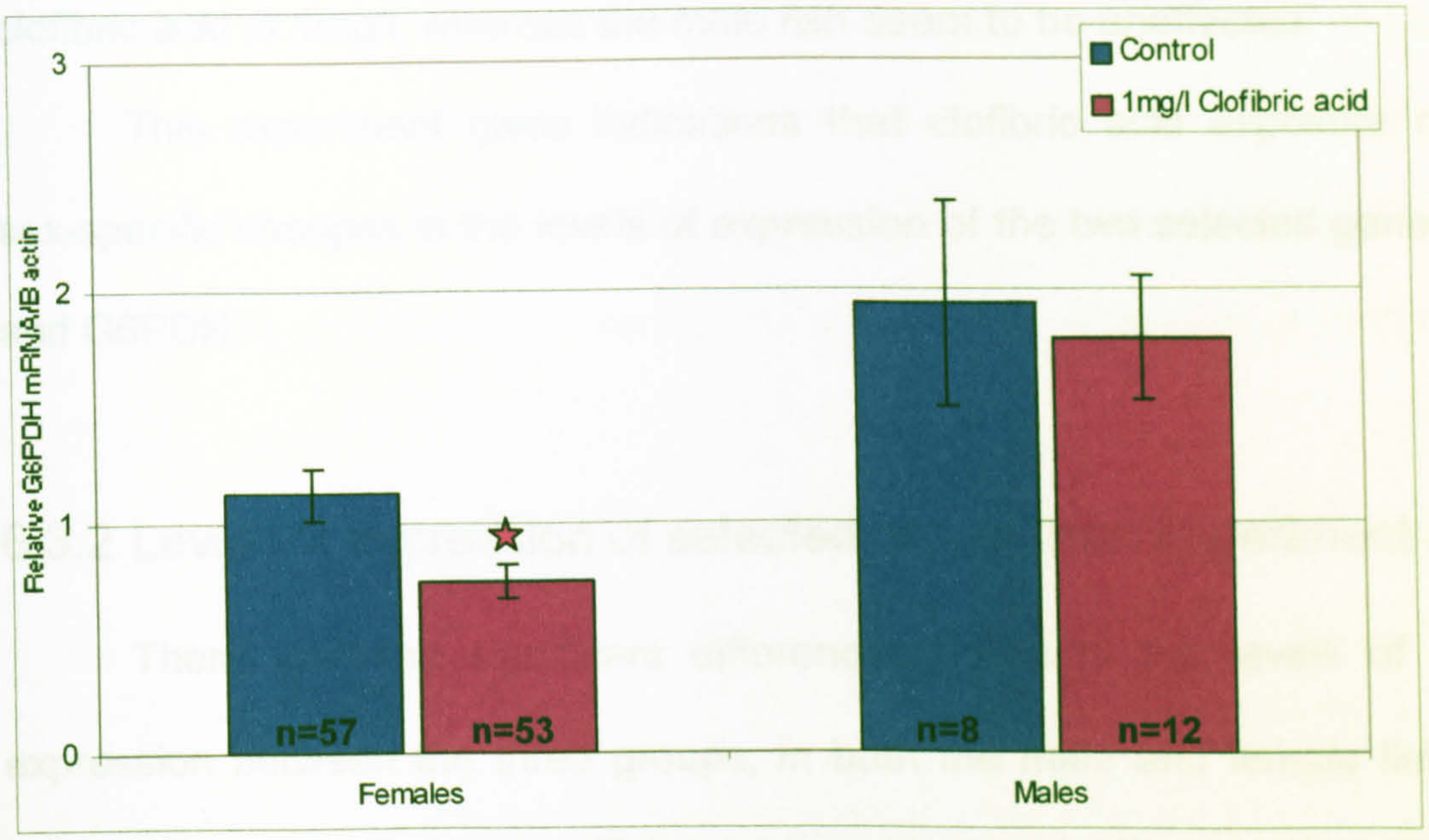

Figure 6.4: Mean relative levels of G6PDH (normalised to β-actin) in the livers of female and male fathead minnow exposed to Clofibric acid at 1mg/l in Experiment 4. Data are expressed as mean ± SEM. * indicates $P<0.05$.

The results presented in Figure 6.4 show a significant down-regulation in the level of G6PDH expression in the liver of female fish exposed to clofibric acid ($p=0.01$). The male fish, however, seem to be unaffected (although the variability between fish is greater in the males). Again, this suggests that there is a sex-specific effect of clofibric acid on the enzymes involved in lipid metabolism in fathead minnow, with the effect in this case on female fish.

6.3.1.1 Summary of the results from Experiment 4

In summary, the results obtained from Experiment 4 indicate that the levels of LPL mRNA expression in fathead minnow exposed to 1mg/l clofibric acid are affected in a sex-specific manner. In the males, the levels of LPL mRNA seem to show an upward trend in response to exposure, whereas the female fish seem to be unaffected by this same treatment. The results obtained

for G6PDH expression, on the other hand, tell a different story: the female fish have significantly lower levels of G6PDH mRNA expression when exposed to clofibric acid at 1mg/l, whereas the male fish seem to be unaffected.

This experiment gives indications that clofibric acid exposure causes sex-specific changes in the levels of expression of the two selected genes, LPL and G6PDH.

6.3.2 Levels of expression of selected genes from Experiment 5

There were no significant differences found in the levels of β-actin expression between the three groups, in both the male and female fish, from this study (data not shown). This agrees with the results from Experiment 4, and therefore shows that β-actin is suitable as a house-keeping gene in these studies.

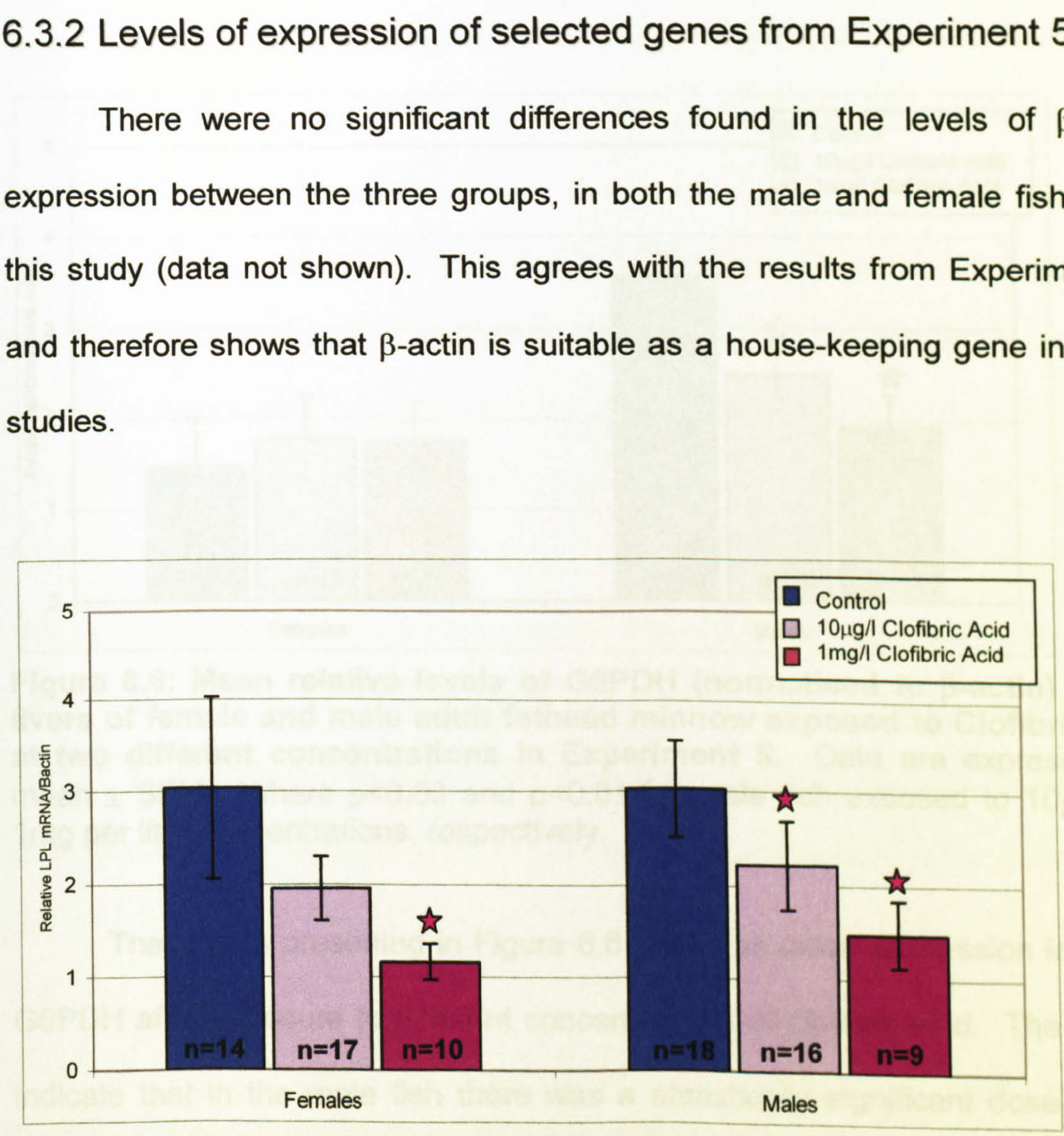

Figure 6.5: Mean relative levels of LPL mRNA expression (normalised to β-actin) in the livers of female and male adult fathead minnow exposed to Clofibric acid at two different concentrations in Experiment 5. Data are expressed as mean ± SEM. Significant differences are shown, where $p<0.03$ (males) and $p<0.01$ (males and females) for the 10µg and 1 mg per litre concentrations, respectively.

The results presented in Figure 6.5 show that there seems to be a dose-dependant decrease on the mean LPL expression levels of both male and female fish in response to clofibric acid exposure for 3 weeks. In the male fish at both doses of clofibric acid, significant differences were seen when compared to the controls ($p<0.03$ and 0.01). In the case of the females, there was a significant difference between the control and highest dose only of clofibric acid ($p<0.01$).

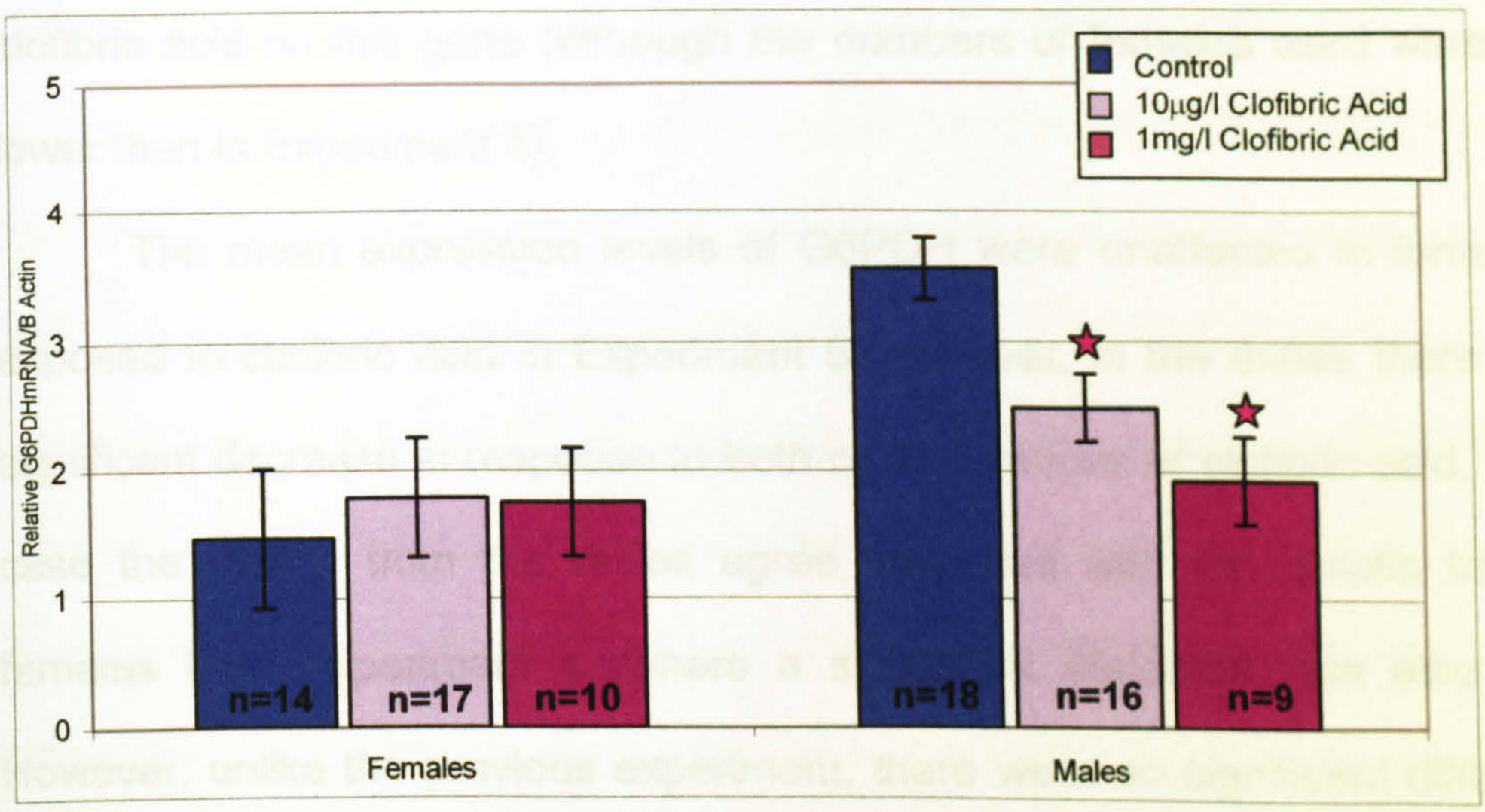

Figure 6.6: Mean relative levels of G6PDH (normalised to β-actin) in the livers of female and male adult fathead minnow exposed to Clofibric acid at two different concentrations in Experiment 5. Data are expressed as mean ± SEM. Where $p<0.03$ and $p<0.01$ for male fish exposed to 10µg and 1mg per litre concentrations, respectively.

The results presented in Figure 6.6 show the mean expression levels of G6PDH after exposure to different concentrations of clofibric acid. The results indicate that in the male fish there was a statistically significant dose-related decrease in mean G6PDH levels with increasing exposure to clofibric acid. In the females, however, no difference was seen with exposure to clofibric acid.

6.3.2.1 Summary of the results from Experiment 5

In summary, the results obtained in Experiment 5 indicate that clofibric acid had significant effects of the levels of expression of LPL in both males and females (with less effect at lower concentrations in the females). These results differ from the results from Experiment 4, where a possible up-regulation was seen in the males and no effect was seen in the females. However, this up-regulation was not significant, and the number of males used in Experiment 5 were higher and therefore may give be a better representation of the effect of clofibric acid on this gene (although the numbers of females used were much lower than in Experiment 4).

The mean expression levels of G6PDH were unaffected in female fish exposed to clofibric acid in Experiment 5, however, in the males there was a significant decrease in response to both concentrations of clofibric acid. In this case the results from the males agree very well with the results from the females from Experiment 4 (where a significant decrease was also seen). However, unlike the previous experiment, there were no significant differences seen in the expression levels in females (although the numbers of fish were lower). The apparent difference in the response of the males may be seen due to the fact that the numbers of fish Experiment 5 were higher than in Experiment 4.

6.4 General summary of the effects of clofibric acid on gene expression in the fathead minnow

The results presented in Chapter 6 indicate there are definite effects of clofibric acid exposure on levels of gene expression in the fathead minnow. It can be seen, however, that there are differences in the results between the two

experiments, although they were both carried out in the same way. The only obvious differences between experiments were in numbers of fish used and the sex ratio within each tank (~5:1 females: males in Experiment 4 and 1:1 in Experiment 5). The importance of this is discussed below.

The LPL results in Experiment 4 indicate that the levels of expression were not significantly different in the males or females exposed to clofibric acid, however, there was an indication in the males of a slight upward trend. On the other hand, in Experiment 5, there were significant and dose-dependant decreases in the males and females exposed to clofibric acid. It is considered that the results from the female fish in Experiment 4 are probably more reliable, as the numbers were higher and therefore there was less variability, whereas with the males, the numbers were lower and may be less reliable than those obtained in Experiment 5. Additionally, these differences in effect on LPL expression between experiments may be explained by the sex ratio of the fish in each experiment. In Experiment 5, there were higher numbers of male fish within each tank and consequently more likelihood of the female fish being encouraged to spawn more regularly, therefore inducing more reproductive cycles and consequently more fluctuations in the vitellogenin production/oogenesis. It has previously been shown that the levels of LPL expression change in the ovary during oogenesis (Kwon *et al*, 2001) and this may be true for LPL of the liver also; this could account for the relatively high variability between fish seen within Experiment 5.

Fibrates have been previously shown to raise LPL expression in rats (Staels and Auwerx, 1992) and female mice (Toda *et al*, 2003), although no induction has been shown in rabbits (Alegret *et al*, 1998), and in fact a decrease in LPL expression has been noted previously in hamster hepatocytes after

exposure to a fibric acid (Guo *et al*, 2001). Alegret *et al* (1998) suggest that the differences seen may be explained by inter-species differences in peroxisome proliferation.

It was noted in both experiments that the levels of expression of G6PDH were higher in males than females, and this agrees with the results of Winzer *et al* (2002), who found that male fish generally show higher G6DPH activity than females. In Experiment 4, the levels of G6PDH were significantly down-regulated in female fish and seemed to be unaffected in male fish exposed to clofibric acid at 1mg/l. However, the results obtained in Experiment 5 show a significant decrease (which was dose-dependant) in the expression levels of G6PDH of male fish, and the female fish in this case seem to be unaffected. Again, as with the LPL results, the numbers of females used in Experiment 4 were higher and consequently results may therefore be more of a true representation of the effects of clofibric acid on the female fish. The males, however, have higher numbers in Experiment 5, and these results also agree with the significant results found in the female and the slight decrease seen in the males from Experiment 4.

G6PDH activity has previously been shown in male rats exposed to clofibric acid to be decreased (Cleary *et al*, 1987), however, they do state that there have been no consistent effects previously seen on G6PDH activity. Winzer *et al* (2002) showed a decrease in the level of G6PDH in female flounder hepatocytes with exposure to xenobiotics, and Bucher, *et al*, (1993) also observed a reduction in G6PDH activity in bullheads exposed to paper mill effluent (however sex was not specified). G6PDH in female flounder has been previously shown to be inhibited by their levels of 17-β-oestradiol, although the mechanisms of action are not clearly identified yet (Winzer *et al*, 2002), and this

inhibitory effect of oestradiol is probably the reason that G6PDH levels are on average lower in females compared to males. It may also explain why no effects were seen in Experiment 5, while a significant effect was seen in Experiment 4: the fish were more likely to be spawning with the presence of more males, which might lead to higher, and more fluctuating 17-β-oestradiol levels. This would lead to more variable G6PDH levels, which could potentially 'mask' any effects of drug exposure in this experiment.

A reduction in G6PDH level increases an organism's susceptibility to xenobiotics, and this may result in the decreased production of NADPH, which is necessary for biosynthetic reactions or biotransformation activities (Winzer *et al*, 2002). This makes females far more susceptible to xenotoxicity, as they also need NADPH for the production of VTG.

In conclusion, it can be seen from these experiments that significant effects of clofibric acid, at different concentrations, occurred on the levels of expression of selected genes. Generally, the effect of clofibric acid seems to be to reduce the expression of LPL and G6PDH in the liver of exposed fish. However, these were not completely reproduced in each experiment, and possible reasons for this have been discussed. The results nevertheless highlight the need for further investigation, taking into account sex ratios, spawning and E2 levels. It may also be informative to look at tissue-specific effects of clofibric acid exposure, as only levels in the liver were measured in these experiments.

Chapter 7: General Discussion

It is now well established that pharmaceuticals and synthetic chemicals from personal care products are being released into the environment. The exact effects that each drug has (both individually and in combination) on ecosystems, biota, and humans, however, are not understood. It is therefore clear that much more research is critically needed.

Prior to the work reported this thesis, there were no known experiments (excluding basic toxicological studies) carried out to look at the effects of clofibric acid or clofibrate on lipid metabolism in fish.

In 2001, Pfluger and Dietrich asked a pertinent question, as to whether clofibric acid can adversely affect cholesterol synthesis, and thus steroidogenesis, and consequently influence endocrine regulation, in aquatic species. Given the complexity of lipid metabolism and its pathways, there may potentially be various mechanisms that could make fish susceptible to exposure to clofibric acid. The results obtained in this thesis suggest that clofibric acid may have complex effects, reflecting several different mechanisms of action at various stages of lipid metabolism. Effects of fibrates on mammals have been seen from the physiological level down to the molecular levels. The chapters within this thesis have presented the first evidence (albeit preliminary) of clofibric acid having effects on both adult and embryo fish, at various levels of organisation.

The studies I conducted involved two different species, and for one of these species, fish of different ages. None of the results I obtained (perhaps with the exception of those concerned with spermatogenesis and some of those involving gene expression) showed major, very pronounced effects of clofibric acid. Further, although some of the effects I observed might have been what I expected if the drug had the same effects (through the same mechanisms) in

fish as it does in mammals, these effects were not always reproducible, raising a question mark as to their robustness. However, my research does show that there are effects of clofibric acid in fish on pathways which were not only previously unexpected (for example, steroidogenesis and spermatogenesis), but which may potentially have a much larger impact on the viability of fish populations. These effects all occurred at concentrations of clofibric acid below those previously shown to have any biological effects on fish.

It may be the case, however, that the seemingly minor effects I found were not statistically significant purely because of the sensitivity of methodologies employed for determining these relatively small differences, rather than them not actually being due to real effects. Consider, for example, the effects of clofibric acid on lipid metabolism in mammals. The effects on lipid levels in humans (a reduction of the concentration of triglycerides by 30-60% and cholesterol by 20-25%) are changes seen in people who have elevated levels in the first place. It is this group of people who take fibrates. Investigating these effects in a different organism, with different levels and route of exposure, and in one whose lipid levels are not elevated above normal by disease, is a completely different scenario - (although it has been shown that in hamsters with normal lipid levels there are significant decreases in both cholesterol and triglyceride levels (Guo *et al* 2001)). In fish, which have been shown to be hyperlipidemic and hypercholesterolemic (when compared with human standards), the effect of clofibric acid on triglyceride and cholesterol levels may be lower than in humans, and as such may be close to or below the sensitivity of the assays employed.

Another point to consider is whether these minor effects of clofibric acid on fish are genuinely minor, or is it possible that the doses I used produced

effects that are just at the bottom of the dose response curve, in which case increasing the dose would increase the magnitude of those effects. And if so, are the higher doses environmentally relevant? Further work is needed to assess these possibilities.

The most significant results from this thesis must be put into context of any relevant previous literature. The reduction in sperm production and steroid synthesis have shown that fish exposed to clofibric acid are deleteriously effected. Similarly to this, studies previously have reported reduced libido and impotence in humans (Martindale, 1999), perhaps due to a reduction in sex steroids, as well as arrested spermatogenesis in dogs and monkeys (Schulman *et al*, 2002). Phthalates (also PPs) have also been shown to induce pathological changes in rodents, and other fibrates have been shown to alter gene expression in Leydig cells of rats, and inhibit progesterone synthesis in human cell lines (Gazouli, *et al*, 2002). In mice Bezafibrate has also been shown to exhibit antiandrogenic properties (Gazouli, *et al*, 2002). The fact that the same results were found in fish and mammals, and even that the potential mechanism (the reduction in testosterone) is the same for fish and mammals, provides much stronger evidence that these are true effects in fish. However, the more expected and well-characterised therapeutic effects that Clofibric acid has in humans and mammals (namely the raising of HDL/reduction in triglyceride and cholesterol, etc) have not been found in this study. The failure to reproduce these effects in fish may highlight true biological differences between fish and mammals, or may be purely due to differences in routes of entry.

Upon reflection, what would I do differently if I were to start my PhD now? I would probably start my studies at the molecular level, using a larger range of concentrations for the exposure experiments – to determine if there were dose-related effects of exposure to clofibric acid. I would also probably more clearly establish the base-line values of the parameters of interest, and their inter-individual variability, before exposing any fish to the drug of interest. For example, more knowledge of the normal levels of cholesterol, triglyceride and lipoproteins in fish, and to what degree and why these change, would have helped a lot in both experimental design and the interpretation of results. More established techniques to measure these parameters would have also been very useful. It is important, however, to remember that assessment of the effects of specific drugs is complicated by their mechanisms of action – which show species-, tissue-, and sometimes cell-specific effects. Consequently, those effects seen in one species may not be seen in others.

It is always very easy to say in hindsight what one would have done differently – however, I believe that the choices I made at the time were correct. They have provided thought-provoking results that can, in the future, be used to take this piece of science further.

I have shown significant effects on a number of very important endpoints, in both adults and embryos, during fixed-term exposures. There is no way of estimating by how much these changes might be magnified over time and successive generations, or what cumulative effect they may have over the entire lifecycle, and consequently what effect they will have on overall fitness. It may be that pronounced effects occur only at high (environmentally unrealistic) concentrations of clofibric acid, but nevertheless much lower (environmentally

realistic) concentrations may still produce significant effects which, although small, could still be important (and adverse).

It is interesting that work designed to ascertain the impact of environmental levels of clofibric acid on fish has highlighted the need to reconsider the impact of these same levels on man (particularly as the drug has been shown to be present in drinking water): what effect will it be having on our young men's sperm counts, and on unborn embryos? Although such statements may seem unnecessarily alarmist to some, others would not dismiss the possibility that low level, but constant, exposure of people might affect some of them (especially in the light of the continued decline of human sperm counts).

In summary, some effects of clofibric acid on fish have been determined, although further, more in-depth studies are required to determine if these effects are reproducible. Multigenerational studies would ideally be carried out, to determine if clofibric acid has any population effects. The fact that effects have been observed when clofibric acid concentrations were well below the previously determined NOEC and LOEC levels, indicates that populations in the wild could well be affected. Long-term exposure to seemingly low concentrations may be very harmful, causing sublethal effects manifested in later generations.

It is important, also, to consider that it may not be exposure to an individual drug which causes a population decline – aquatic organisms are probably never exposed to just one drug (such as clofibric acid), but instead receive simultaneous exposure to a poorly defined, complex mixture of many drugs. I investigated the effects of just one drug, but in the future I hope research will lead to the determination of the effects of the cocktail of drugs fish that are exposed to. These drugs in combination with even more complex

multiple stressors such as disease, predation or competition and habitat destruction, need to be considered, to assess if these individual contributors add to the overall stress a population can be under.

It is clear from the results of the embryo experiment that further work must be carried out to further characterise and quantify the effects that were reported here and ascertain if the effects could be detrimental at the population level. It would be sensible to examine where exactly clofibric acid is located when it is taken up by the egg/embryo. This could be done by using radio-labelled (or dye-conjugated) clofibric acid. Another interesting parameter to look at, in light of the differences in yolk opacity observed, would be the changes (if any) in the lipid profile within the developing embryo yolk; this could be done using gels (similar to the ones used in the adult experiments). Using histological techniques, the development of the spleen could also be examined (i.e. examining changes in sinusoid space, as was done by Handy *et al*, 2002), or by determining white blood cell counts, to see if there is any effect on the immune response with exposure to clofibric acid.

Perhaps the most important endpoints for further study, in view of the results obtained, would be to look at the effects of clofibric acid on gene expression in embryos after exposure to clofibric acid, specifically looking at genes (especially G6PDH) involved in lipid metabolism (for example, acyl CoA, APO E, and A1) and the detoxification pathways (e.g. cytochrome p450 genes). Changes in gene expression in the embryo would be expected to have pronounced effects, as they will be occurring during development, when the embryo is extremely sensitive and gene expression is consequently tightly regulated.

The results from the adult studies also highlight the need for further investigation. It would be interesting to ascertain the reproductive effects of the

lowering of the testosterone and sperm counts, and consequently a pair breeding experiment would be useful to determine if there were any effects on fertility, by measuring the numbers of eggs spawned/fertilised. The sperm quality could be further assessed by measuring sperm motility and viability using the Computer Assisted Sperm Analysis (CASA) system (Hobson Sperm tracker; Kime *et al*, 2001). Additionally, gonads of both sexes could also be examined histological to determine if the decreased sperm count is due to any gross effects on cellular organisation (such as abnormal development of Leydig, Sertoli and germ cells, for example). The effect of clofibric acid on the next generation could also be assessed – by looking at juveniles, which where spawned, hatched, and 'grown on' in the presence of clofibric acid.

It could also be very important to look in more detail at the molecular level. As well as looking at other genes involved in lipid metabolism (see above), it might be very informative to also look at the genes involved in spermatogenesis and steroid metabolism. An extremely powerful way of doing this would be to use DNA microarrays to quantify changes in gene expression using RNA extracted from exposed versus non-exposed fish. In this way, changes in expression of thousands of genes, including whole biochemical pathways, can be assessed at the same time, allowing multiple mechanisms in many different pathways to be identified rapidly and efficiently.

It is true also that the experiments described in this thesis were conducted somewhat unrealistically (in an environmental sense), since only a single compound was assessed. In reality, wildlife are often exposed to multiple concentrations of many pollutants with multiple mechanisms of action. Therefore, once reproducible effects have been established, mixture effects could be assessed.

With respect to pharmaceuticals in the environment, it is now clear that the most important conclusion is that an improvement in the clearance of these drugs from the environment is essential. Although costly, removal/degradation of these contaminants biologically or chemically during wastewater treatment is required, to reduce their concentrations in effluents discharged into the aquatic environment. This, in turn would reduce the long-term and largely unknown cost not only to wildlife, but also ourselves.

Personal Publications

Although none of the research reported in this thesis has currently been published as papers, I intend to write a paper or two, based on the most convincing results, in the near future.

PAPERS

Harries, J.E., **Runnalls, T**, Hill, E., Harris, C.A., Maddix, S, Sumpter, J.P., Tyler, C.R. (2000) Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environmental Science and Technology, 34: 3003-3011.

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MEDIA INTEREST IN MY RESEARCH

Steve Farrar (2004) Cast-off drugs harm wildlife. The Times Higher Education Supplement. 21st May 2004.

POSTERS

Runnalls, T.J. and Sumpter J.P., (2004) Pharmaceuticals in the Environment – the effect of Clofibric acid on fish. SETAC Europe, 14th Annual Meeting, Prague, Czech Republic.

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Tyler, C.R., Hutchinson, T., Harries, J., **Runnalls, T.**, Pounds, N., Maddix, S., van Aerle, R., Harris, C., and Sumpter, J.P. (1999) Reproductive effects of environmental estrogens in the fathead minnow (*Pimephales promelas*). SETAC 20th Annual meeting, Philadelphia, PA.

PHARMACEUTICALS IN THE ENVIRONMENT - the effects of clofibric acid on fish

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Abstract

Pharmaceuticals in the aquatic environment are an emerging issue and the risks they pose are mostly unknown. We have been studying possible effects of clofibric acid, the major metabolite of a lipid-lowering drug, in fish. These were investigated using different experimental approaches and endpoints. When fathead minnow embryos were exposed to clofibric acid, effects were seen on eggshell, time to hatch, hatchability, mortality and viability. Adult fathead minnow were similarly exposed and, in females, significant effects on liver size and HDL cholesterol were observed. The action of clofibric acid on gene expression was also examined: Glucose-6-Phosphate Dehydrogenase (G6PDH) mRNA was significantly down-regulated in females, whilst Lipoprotein Lipase (LPL) mRNA appeared to be up-regulated in males. Vitellogenin (VTG) gene expression decreased, whereas eggshell protein (ZP3) mRNA levels remained unchanged. Clofibric acid also had a significant effect on sperm count in exposed fish; this may be, in part, due to a decrease in plasma testosterone concentration (also reduced in exposed fish). These results show that clofibric acid may have detrimental effects on fish populations.

Introduction

Various classes of prescription drugs have been detected at concentrations up to µg/l levels in sewage, surface and ground water. They include steroids, anti-inflammatory, anti-cancer, lipid regulators, anti-epileptic, antibiotics and painkillers. Little is currently known about the environmental impact of these drugs. As they are developed to have a biological effect, their potential to cause effects within the environment is great.

Clofibric acid (CA) is the major metabolite of the lipid-lowering drugs Clofibrate, Etofibrate and Etofyllinclofibrate. It is non-biodegradable, very persistent and frequently found at µg/l concentrations in the environment. CA was originally detected in the environment as a consequence of the routine analysis for acidic pesticides, and has since been frequently detected in surface waters, ground waters, sewage effluent and even drinking waters.

Materials and Methods

Exposure studies were carried out using both adult and embryo fathead minnow (figure 1) to determine the effects of CA.

➢**Adults** : various assays were employed to determine potential effects of CA (nominal concentrations: 1mg/l and 10 µg/l):

- Serum was run on agarose gels (HYDRAGEL HDL/LDL cholesterol kit - SEBIA) and visualised using a cholesterol specific enzymatic method. Quantification of HDL cholesterol within lipoproteins was achieved using densitometry
- Livers and gonads from exposed and control female fish were used to determine VTG and ZP3 mRNA levels respectively, using Hybridisation Protection Assays (MLT, Cardiff)
- Sperm counts were carried out on male gonads
- Plasma was analysed for levels of testosterone using a radioimmunoassay
- Quantitative real-time RT-PCR was performed on livers samples using Qiagen QuantiTect SYBR green RT-PCR kit to compare expression levels of LPL and G6PDH mRNAs between control and dosed fish

➢**Embryos** : various end points were monitored including time to hatch, hatchability, mortality and viability when exposed to 1mg/l, 1 µg/l and 1 ng/l CA.

Figure1:The fathead minnow (*Pimephales promelas*)**: males are the larger and darker fish, whereas the females are smaller and lighter**

Results and Discussion

➢**Adults:**

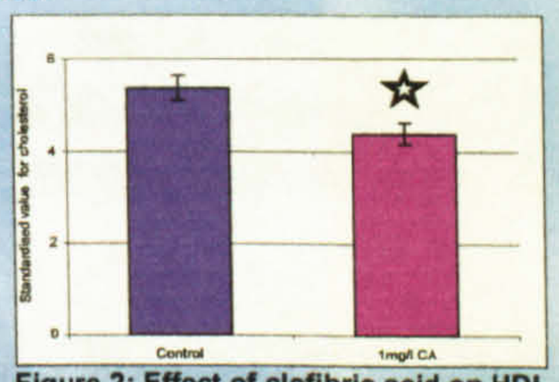

Figure 2: Effect of clofibric acid on HDL cholesterol levels in females (p=0.006)

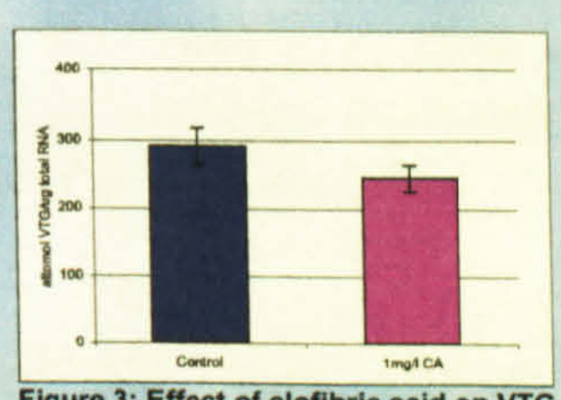

Figure 3: Effect of clofibric acid on VTG mRNA levels in females

➢Significant effects on HDL cholesterol in lipoproteins were observed in females (figure 2)

➢Vitellogenin (VTG) gene expression in females decreased (figure 3), although not significantly, whereas eggshell protein (ZP3) mRNA levels in females remained unchanged (data not shown)

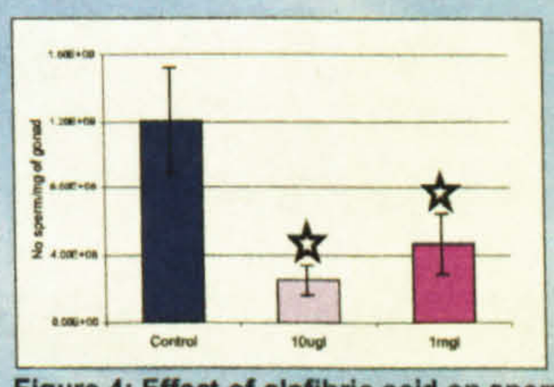

Figure 4: Effect of clofibric acid on sperm counts of males (p=0.01 and 0.04)

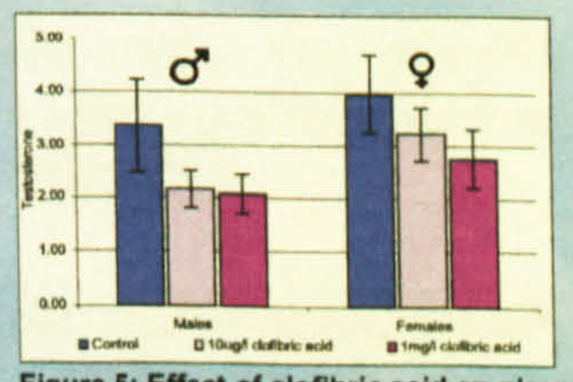

Figure 5: Effect of clofibric acid on plasma testosterone levels in males and females

➢Significant effects on sperm count in exposed fish were seen (figure 4); this may be, at least in part, due to a decrease in plasma testosterone concentration in dosed fish (figure 5)

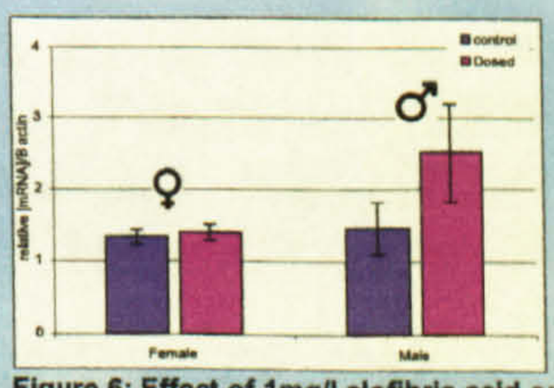

Figure 6: Effect of 1mg/l clofibric acid on LPL mRNA levels

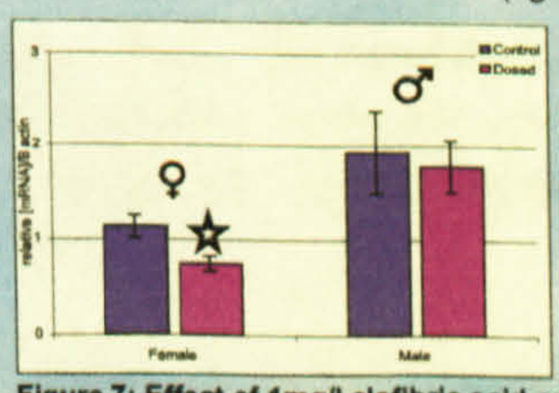

Figure 7: Effect of 1mg/l clofibric acid on G6PDH mRNA levels (p=0.01)

➢Effects on gene expression: LPL mRNA appeared to be up-regulated in males (figure 6), whilst G6PDH mRNA was significantly down-regulated in females (figure 7)

➢Significant effects were seen on HSI in females (data not shown)

➢**Embryos:**

➢Effects were also observed on egg shell, time to hatch, hatchability, mortality and viability (data not shown)

As can be seen, significant effects have been found at relatively low doses (further work needs to be carried out to establish the LOECs for these endpoints)

Conclusions

- Although these are preliminary findings, they indicate a trend, and perhaps a sex-specific effect in some cases. The presence of clofibric acid in the aquatic environment may have effects not only at a physiological level but also at the molecular level.
- In mammals, Clofibrate is known to reduce elevated plasma concentrations of triglycerides by 30-60% and, to a lesser extent, cholesterol (by 20-25%). It has been found to do this by acting through PPAR receptors to alter expression of a number of genes - we have quantified the degree of expression of two of these genes (Lipoprotein Lipase and Glucose-6-Phosphate Dehydrogenase) in fathead minnow and have shown that this mechanism of action may also pertain to fish.
- This work shows that clofibric acid has the potential to have significant effects on all stages of fish development, the cumulative effect of which may have a profound effect on fish fitness when chronically exposed in the natural environment.

Acknowledgements: Molecular Light Technologies, Cardiff for HPA kits, Sue Maddix (Brunel) for radioimmunoassay

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APPENDIX

Appendix 1: Sequence used for designing primers for G6PDH real-time PCR in the PRIMER3 program.

```
LOCUS       AF206637               1412 bp    mRNA    linear   VRT 20-APR-2000
DEFINITION  Pimephales promelas liver glucose-6-phosphate-1-dehydrogenase
            mRNA,partial cds.
ACCESSION   AF206637
VERSION     AF206637.2  GI:7629274
SOURCE      Pimephales promelas
ORGANISM    Pimephales promelas
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi;
            Cypriniformes; Cyprinidae; Pimephales.
REFERENCE   1  (bases 1 to 1412)
AUTHORS     Dasmahapatra,A.K., Wimpee,B.A. and Dellinger,J.
TITLE       G6PD mRNA in fathead minnow liver
JOURNAL      Unpublished
REFERENCE   2  (bases 1 to 516)
AUTHORS     Dasmahapatra,A.K., Wimpee,B.A.B. and Dellinger,J.
TITLE       Direct Submission
JOURNAL     Submitted (18-NOV-1999) School of Allied Health, NIEHS Marine and
            Freshwater Biomedical Sciences Center, University of
            Wisconsin-Milwaukee, 600 East Greenfield Avenue, Milwaukee, WI
            53204, USA
REFERENCE   3  (bases 1 to 1412)
AUTHORS     Dasmahapatra,A.K., Wimpee,B.A.B. and Dellinger,J.
TITLE       Direct Submission
JOURNAL     Submitted (20-APR-2000) NIEHS Marine and Freshwater Biomedical
            Sciences Center, University of Wisconsin-Milwaukee, 600 East
            Greenfield Avenue, Milwaukee, WI 53204, USA
REMARK      Sequence update by submitter
COMMENT     On Apr 20, 2000 this sequence version replaced gi:6601558.
FEATURES             Location/Qualifiers
     source          1..1412
                     /organism="Pimephales promelas"
                     /mol_type="mRNA"
                     /db_xref="taxon:90988"
                     /tissue_type="liver"
     CDS             <1..>1412
                     /note="G6PD"
                     /codon_start=1
                     /product="glucose-6-phosphate-1-dehydrogenase"
                     /protein_id="AAF19030.2"
                     /db_xref="GI:7629275"

translation="LAKKKIYPTLWWLFRDGLLPEQTYFVGFARSDLTVDAIRAACMP
            YMKVVDSDAERLAAFFSRNSYIGGKYVDESSFDNLNTHLLSLPGGAGANRLFYLALPP
            IVYHDVTKNIKHQCMSTKGWNRIIVEKPFGHDLQSSEELSSHLFSLFTEEQIYRIDHY
            LGKEMVQNLMVLRFGNRIFGPIWNRDSVACVVLTFKEPFGTQGRGGYFDDFGIIRDVM
            QNHLLQMLSLVAMEKPASTSSNDVRDEKVKVLKCIEAVSLSDVVLGQYVGDPDGEGDA
            KLGYLDDSTVPKGSTQATFATAVLYVKNERWDGVPFILRCGKALNERKAEVRLQFTDV
            PGDIFDSQCRRNELVVRVQPNEAIYAKMMSKKPGVYFSPEETELDLTYHSRYRDVKLP
            DAYERLILDVFCGQMHFVRSDELREAWRIFTPLLHQIEKEKTPPIKYKYGSRGPAEAD
            ELVQKVGFRYEGTYKWVNPH"
ORIGIN
        1 ctggccaaga agaaaatcta cccaactttg tggtggttgt tcagagacgg tcttctcccc
       61 gaacagacat atttcgtggg ctttgctcgt tccgatctga ccgtggatgc catacgtgcg
      121 gcctgcatgc cctacatgaa ggtggtcgac tctgatgcag agcgcctcgc cgcgttcttc
      181 agtcgaaact cttacatcgg cgggaagtac gtggatgaat cgtctttcga taacctgaac
      241 actcacctgc tgtccctgcc cggaggagcc ggggccaacc ggctcttcta cctggccctg   FHGAP2f
      301 ccgcccatcg tctaccatga tgtcaccaag aacatcaaac atcaatgcat gagcaccaaa
      361 ggctggaaca ggatcatcgt ggagaagccg tttggccatg acttgcagag ctcagaggag
      421 ttatccagtc atctgttttc tctcttcacc gaggaacaga tttaccggat agaccattac
      481 ctaggcaaag agatggtcca gaacctaatg gtgctcaggt ttggaaaccg gatatttggc
FHGAP2r 541 ccca tatgga accgggacag tgtggcatgt gtggttctga ccttcaaaga gccgtttggt
FHGAP1f 601 acccagggcc ggggaggata tttcgatgat tttggtatca ttcgcgatgt catgcagaac
      661 cacctgctgc agatgctctc tctggttgcc atggagaagc ccgcatccac caggctctaat   FHGAP3f
      721 gatgttaggg acgagaaggt gaaggtgttg aagtgcattg aagcagtctc tctgtcagac
      781 gtggtcttgg gtcagtacgt gggagatcca gatggagaag gagatgctaa acttggttat
      841 ctagatgact caactgtccc taaaggctcc actcaagcta catttgccac tgcagtgctt
```

FHGAP3r FHGAP1rb

```
FHGAP1r
       901 tatgtaaaga atgaacgctg ggatggggtt cccttcatcc tccggtgtgg aaaggctctg
       961 aatgagagga aggcagaggt gaggctgcag ttcaccgacg ttcctggaga catctttgac
      1021 tctcagtgca ggcggaacga gttggtggtc cgcgtgcagc ccaacgaggc catctacgcc
      1081 aagatgatga gcaagaaacc tggagtctac ttcagtccgg aagagacgga gctggacctg
      1141 acttaccaca gcagatacag ggatgttaag ctccctgatg cttatgagcg actgatttg
      1201 gatgtctttt gtggtcagat gcattttgtt cgcagtgacg aattgaggga agcctggaga
      1261 atcttcactc ctcttcttca tcagatcgag aaagagaaga cgccacccat caaatacaaa
      1321 tacgggagtc gtggtcctgc agaggctgac gagctggtgc agaaggtggg ctttcgctat
      1381 gagggtacat acaagtgggt caacccacac ag
```

Where: intron/exon boundies; reverse primers; forward primers; over lap between primers.

Appendix 2: LPL sequence alignment using the ClustalW Multiple sequence alignment on the BCM search launcher.

Including bream genomic sequence did not give good alignment (compared to β-actin), possibly because of the large number of exons, or diversity between genes. Intron/exon boundaries, therefore, are from the submitted genomic sequence for bream (AB054063) and are marked on the bream cDNA sequence ([]). Marked on the alignment below are the primers binding sites for the original degenerate primer pairs, non-degenerate primers were subsequently designed and screened from these.

```
                          1            15 16           30 31           45 46           60 61           75 76           90
1 rainbowtrout1           -----------CCAC TCTTATCACAAATCA GATTTACTTTTAGAC CAGAGCAGTGGTCCT ACTGACCTATACAAT TGGATCTACCACTCC     79
2 rainbowtrout2           CATAAGAAACTCCAC TCTTATCACAAATCA GATTTACTTTTAGAC CAGAGCAGTGGTCCT ACTGACCTATACAAT TGGATCTACCACTCC     90
3 zebrafish1              --------------- --------------- --------------- --------------- --------------- ---------------      0
4 zebrafish2              --------------- --------------- --------------- --------------- --------------- ---------------      0
5 catfish                 --------------- --------------- --------------- --------------- --------------- ---------------      0
6 atlanticsalmon2         --------------- --------------- --------------- --------------- --------------- ---------------      0
7 bream                   -----GACGCACTGA ACTCAGTCTGAGAGG GAAATATACAGTCTT CTGCAAAGAGATCCG ACTGATTTAATTAAC TGCTGAAGCGATTCT     85
8 atlanticsalmon1         --------------- --------------- --------------- --------------- --------------- ---------------      0
9 winterflounder1         --------------- --------------- --------------- --------------- --------------- ---------------      0
10 winterflounder2        --------------- --------------- --------------- --------------- --------------- ---------------      0

                          91          105 106          120 121          135 136          150 151          165 166          180
1 rainbowtrout1           TAAACCAACATACAA GAACATTTTCATTCG GGACTTGACGGAGAA ACCTCTGGATCAACA ATAATATGGGAAAAG AAAATACCTTTTTGG     169
2 rainbowtrout2           TAAACCAACATACAA GAACATTTTCATTCG GGACTTGACGGAGAA ACCTCTGGATCAACA ATAATATGGGAAAAG AAAATACCTTTTTGG     180
3 zebrafish1              --------------- --------------- --------------- --------------- --------------- ---------------      0
4 zebrafish2              --------------- --------------- --------------- --------------- --------------- ---------------      0
5 catfish                 --------------- --------------- --------------- --------------- --------------- ---------------      0
6 atlanticsalmon2         --------------- --------------- --------------- --------------- --------------- ---------------      0
7 bream                   TCTGTGAAAGGACA- -AACCTGTGAA---- AGAAATGAAAGCGTG GCGAGTTGTGTTTCT GTACTTTCTGGTGTT GAA-TGCAGTCGTGC     168
8 atlanticsalmon1         --------------- --------------- --------------- --------------- --------------- ---------------      0
9 winterflounder1         --------------- --------------- --------------- --------------- --------------- ---------------      0
10 winterflounder2        --------------- --------------- --------------- --------------- --------------- ---------------      0

                          181          195 196          210 211          225 226          240 241          255 256          270
1 rainbowtrout1           TCACCGTTTGGATAA TTCTGGCAAATATCT GTGTATCTTTTTCAA GTACACCAGAACAAA CACTTTTTGGTAACA GCAACTCCACTGAAT     259
2 rainbowtrout2           TCACCGTTTGGATAA TTCTGGCAAATATCT GTGTATCTTTTTCAA GTACACCAGAACAAA CACTTTTTGGTAACA GCAACTCCACTGAAT     270
3 zebrafish1              --------------- --------------- --------------- --------------- --------------- ---------------      0
4 zebrafish2              --------------- --------------- --------------- --------------- --------------- ---------------      0
5 catfish                 --------------- --------------- --------------- --------------- --------------- ---------------      0
6 atlanticsalmon2         --------------- --------------- --------------- --------------- --------------- ---------------      0
7 bream           AGCATGTGACGTCCC TGGAAGAAGAGCTCT CCGATTCTATTTTTG] [GTA----ACTTCCTC GACCCTCTGAAA--- --GACTTGATTGAAC     249
8 atlanticsalmon1         --------------- --------------- --------------- --------------- --------------- ---------------      0
9 winterflounder1         --------------- --------------- --------------- --------------- --------------- ---------------      0
10 winterflounder2        --------------- --------------- --------------- --------------- --------------- ---------------      0

                          271          285 286          300 301          315 316          330 331          345 346          360
1 rainbowtrout1           GGCTTGAGGACTACA CAGACATTGTATCCA AGTTCTCCCTGAGAA CTGCTGAGATACCGG ATGATGACTTGTGCT ACATCGTTCCCGGCC     349
2 rainbowtrout2           GGCTTGAGGACTACA CAGACATTGTATCCA AGTTCTCCCTGAGAA CTGCTGAGATACCGG ATGATGACTTGTGCT ACATCGTTCCCGGCC     360
3 zebrafish1              --------------- --------------- --------------- --------------- --------------- ---------------      0
4 zebrafish2              --------------- --------------- --------------- --------------- --------------- ---------------      0
5 catfish                 --------------- --------------- --------------- --------------- --------------- ---------------      0
6 atlanticsalmon2         --------------- --------------- --------------- --------------- --------------- ---------------      0
7 bream                   ACAAGGATGACGCCA ATCAAACCGTTGCCA AATTCTCCCTCCGGA AACCGTCCCATCCTG ATGACGACCTGTGCT ACATCGTTCCTGGCA     339
8 atlanticsalmon1         --------------- --------------- --------------- --------------- --------------- ---------------      0
9 winterflounder1         --------------- --------------- --------------- --------------- --------------- ---------------      0
10 winterflounder2        --------------- --------------- --------------- --------------- --------------- ---------------      0

                          361          375 376          390 391          405 406          420 421          435 436          450
1 rainbowtrout1           AGCCCTCAACCATCC CAAAGTGTGAATTCA ACCCTGGGTATAAGA CGTTCGTGGTCATTC ATGGATGGACGGTCA CGGGGCTGTTTGAGA     439
2 rainbowtrout2           AGCCCTCAACCATCC CAAAGTGTGAATTCA ACCCTGGGTATAAGA CGTTCGTGGTCATTC ATGGATGGACGGTCA CGGGGCTGTTTGAGA     450
3 zebrafish1              --------------- --------------- --------------- --------------- --------------- ---------------      0
4 zebrafish2              --------------- --------------- --------------- --------------- --------------- ---------------      0
5 catfish                 --------------- --------------- --------------- --------------- --------------- ---------------      0
6 atlanticsalmon2         --------------- --------------- --------------- --------------- --------------- ---------------      0
7 bream           AACCTGACTCCCTGG CAGCCTGCACCTTCA ACAGCTCCTCCAAAA CCTTCCTAGTGATCC ACGGATGGACG][TTGA GCGGCATGTTTGAAA     429
8 atlanticsalmon1         --------------- --------------- --------------- --------------- --------------- ---------------      0
9 winterflounder1         --------------- --------------- --------------- --------------- --------------- ---------------      0
10 winterflounder2        --------------- --------------- --------------- --------------- --------------- ---------------      0

                                                                                                          FHLPL-1f
                          451          465 466          480 481          495 496          510 511          525 526          540
1 rainbowtrout1           GCTGGGTCCCTAAGC TGGTGACAGCGCTGT ACAAGAGGGAGCCCA AGGCCAACGT[ATTG TGGTGGACTGGCTGA CACGGGCGCAGCAGC     529
2 rainbowtrout2           GCTGGGTCCCTAAGC TGGTGACAGCGCTGT ACAAGAGGGAGCCCA AGGCCAACGT[ATTG TGGTGGACTGGCTGA CACGGGCGCAGCAGC     540
3 zebrafish1              --------------- --------------- --------------- ---------[---- ---------------] --------------      0
4 zebrafish2              --------------- --------------- --------------- ---------[---- ---------------] --------------      0
5 catfish                 --------------- --------------- --------------- ---------[---- ---------------] --------------      0
6 atlanticsalmon2         --------------- --------------- --------------- ---------[---- ---------------] --------------      0
7 bream                   GCTGGGTGGCGAAGC TGGTGTCAGCGCTGT ACGAGAGAGAGCAGA CGGCCAACGT[ATCG TGGTGGACTGGCTCA CCTCGGCACAGAACC     519
8 atlanticsalmon1         --------------- --------------- --------------- ---------[---- ---------------] --------------      0
9 winterflounder1         --------------- --------------- --------------- ---------[---- ---------------] --------------      0
10 winterflounder2        --------------- --------------- --------------- ---------[---- ---------------] --------------      0

                          541          555 556          570 571          585 586          600 601          615 616          630
1 rainbowtrout1           ACTACCTCACCTCCG CTGCCAACACCAAGC TGGTGGGCAAAGACG TGGCCAAGTTTGTTA ATTGGCTGCAGAAAA CACTCGACTATCCCT     619
2 rainbowtrout2           ACTACCTCACCTCCG CTGCCAACACCAAGC TGGTGGGCAAAGACG TGGCCAAGTTTGTTA ATTGGCTGCAGAAAA CACTCGACTATCCCT     630
3 zebrafish1              --------------- --------------- --------------- --------------- --------------- ---------------      0
4 zebrafish2              --------------- --------------- --------------- --------------- --------------- ---------------      0
5 catfish                 --------------- --------------- --------------- --------------- --------------- ---------------      0
6 atlanticsalmon2         --------------- --------------- --------------- --------------- --------------- ---------------      0
7 bream           ACTACGTGGTGGCTG CTCAGAACACCAAAG CAGTGGGACAGGAGA TCGCTCGCTTCATCG ACTGGATCGAG][GAAA CCACCAACATGCCTC     609
8 atlanticsalmon1         --------------- --------------- --------------- --------------- --------------- ---------------      0
9 winterflounder1         --------------- --------------- --------------- --------------- --------------- ---------------      0
10 winterflounder2        --------------- --------------- --------------- --------------- --------------- ---------------      0
```

```
                      631         645 646         660 661         675 676         690 691         705 706         720
 1 rainbowtrout1      GGGAGAAGATACATC TGCTGGGCTACAGTC TGGGTGCTCATGTAG CTGGCATCGCTGGTC TCCTCACCAACCACA AAGTCAGCAGGATCA     709
 2 rainbowtrout2      GGGAGAAGATACATC TGCTGGGCTACAGTC TGGGTGCTCATGTAG CTGGCATCGCTGGTC TCCTCACCAACCACA AAGTCAGCAGGATCA     720
 3 zebrafish1         --------------- --------------- --------------- --------------- --------------- ---------------       0
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream              TTGAGAACATCCACC TGATTGGCTACAGCC TCGGCGCTCATGTGG CAGGATTCGCCGGAA GCCATGCAACCAATA AAGTGGGAAGAATCA     699
 8 atlanticsalmon1    --------------- --------------- --------------- --------------- --------------- ---------------       0
 9 winterflounder1    --------------- --------------- --------------- --------------- --------------- ---------------       0
10 winterflounder2    --------------- --------------- --------------- --------------- --------------- ---------------       0

                                                                                                                FHLPL-2f
                      721         735 736         750 751         765 766         780 781         795 796         810
 1 rainbowtrout1      CAGGTTTGGACCCGG CCGGCCCGACCTTTG AGTTTGCTGACGCCC AGAGCACCCTGTCCC CCGACGATGCTCTCT TCGTGGACGTCCTGC     799
 2 rainbowtrout2      CAGGTTTGGACCCGG CCGGCCCGACCTTTG AGTTTGCTGACGCCC AGAGCACCCTGTCCC CCGACGATGCTCTCT TTGTGGACGTCCTGC     810
 3 zebrafish1         --------------- --------------- --------------- --------------- --------------- ---------------       0
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream                    CAG][GTCTGGACCCAG CTGGTCCTGACTTTG AGGGGATGCACGCCC ACAGGCGTCTCTCCC CCGACGACGCTCACT TTGTGGACGTCCTCC     789
 8 atlanticsalmon1    --------------- --------------- --------------- --------------- --------------- ---------------       0
 9 winterflounder1    --------------- --------------- --------------- --------------- --------------- ---------------       0
10 winterflounder2    --------------- --------------- --------------- --------------- --------------- ---------------       0

                                                                                                  FHLPL-3f
                      811         825 826         840 841         855 856         870 871         885 886         900
 1 rainbowtrout1      ACACCAACACCCGGG GCTCCCCAGACCGCA GCATCGGCATCCAGA GACCCGTGGGCCACG TGGACATCTACCCCA ACGGAGCAACCTTCC     889
 2 rainbowtrout2      ACACCAACACCCGGG GCTCCCCAGACCGCA GCATCGGCATCCAGA GACCCGTGGGCCACG TGGACATCTACCCCA ACGGAGCAACCTTCC     900
 3 zebrafish1         --------------- --------------- --------------- --------------- --------------- ---------------       0
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream              ACACCTTCACCCGCG GCTCCCTGGGTCTCA GCATCGGGATCCAGC AGCCAGTCGGCCATG TTGACATTTACCCCA ATGCAGCCAGCTTCC     879
 8 atlanticsalmon1    --------------- --------------- --------------- --------------- --------------- ---------------       0
 9 winterflounder1    --------------- --------------- --------------- --------------- --------------- ---------------       0
10 winterflounder2    --------------- --------------- --------------- --------------- --------------- ---------------       0

                      901         915 916         930 931         945 946         960 961         975 976         990
 1 rainbowtrout1      AGCCAGGCTGTGATC TGCAGAACACCATGA TGATGATCGCCACCA CCGGCATCCGTAATA TGGACCAGCTTGTGA AGTGCTCCCATGAGC     979
 2 rainbowtrout2      AGCCAGGCTGTGATC TGCAGAACACCATGA TGATGATCGCCACCA CCGGCATCCGTAATA TGGACCAGCTTGTGA AGTGCTCCCATGAGC     990
 3 zebrafish1         --------------- --------------- --------------- --------------- --------------- ---------------       0
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream          AGCCAGGCTGCAACC TCAGGGGGGCCCTGG AGAAGATCGCTAACT TTGGGATATTTG][CCA TCACAGACGCAGTCA AGTGCGAACACGAGC     969
 8 atlanticsalmon1    --------------- --------------- --------------- --------------- --------------- ---------------       0
 9 winterflounder1    --------------- --------------- --------------- --------------- --------------- ---------------       0
10 winterflounder2    --------------- --------------- --------------- --------------- --------------- ---------------       0

                                FHLPL-1r                FHLPL-2r
                      991         1005 1006        1020 1021        1035 1036        1050 1051        1065 1066        1080
 1 rainbowtrout1      GCTCCATCCACCTGT TTATCGACTCACTGG TGAACGCAGCAGAGC ATCAGACCATGGCCT ACCGCTGCAGCTCCA AGGAGGCCTTCATGA    1069
 2 rainbowtrout2      GCTCCATCCACCTGT TTATCGACTCACTGG TGAACGCAGCAGAGC ATCAGACCATGGCCT ACCGCTGCAGCTCCA AGGAGGCCTTCATGA    1080
 3 zebrafish1         --------------- --------------- --------------- --------------- --------------- ---------------       0
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream              GCTCGATCCACCTGT TCATCGACTCCCTGC TGAACG-AGCAG--G AGGCGGCCAAGGCTT ACAGATGCGGCAGCA GCGACATGTTTAACC    1056
 8 atlanticsalmon1    --------------- --------------- --------------- --------------- --------------- ---------------       0
 9 winterflounder1    --------------- --------------- --------------- --------------- --------------- ---------------       0
10 winterflounder2    --------------- --------------- --------------- --------------- --------------- ---------------       0

                      1081        1095 1096        1110 1111        1125 1126        1140 1141        1155 1156        1170
 1 rainbowtrout1      AGGGCATGTGCCTCA ACTGCCGTAAGAACC GCTGCAACAAGGTGG GCTACGGTGTCAACA AGGTCCGCCTGCCCC GGAACACCAAGATGT    1159
 2 rainbowtrout2      AGGGCATGTGCCTCA ACTGCCGTAAGAACC GCTGCAACAAGGTGG GCTACGGTGTCAACA AGGTCCGCCTGCCCC GGAACACCAAGATGT    1170
 3 zebrafish1         --------------- --------------- --------------- --------------- --------------- ---------------       0
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream              GCGGCATGTGTCTCA GTTGCCGCAAGGGCC GCTGCAACACGGTGG GCTACGACATCAGCA AGGTCCGCAAGGCGC GCAACGTTCAGATGT    1146
 8 atlanticsalmon1    ------------TCA GCTGCCGCAGGAACC ACTGTAACACCGTGG GCTATGATATCAGCA AGGTTCGCAAGACAC GGAGCGTCAAACTGT      78
 9 winterflounder1    --------------- --------------- --------------- --------------- --------------- ---------------       0
10 winterflounder2    --------------- --------------- --------------- --------------- --------------- ---------------       0

                      1171        1185 1186        1200 1201        1215 1216        1230 1231        1245 1246        1260
 1 rainbowtrout1      ACCTCAAGACCCGCG AGATGATGCCCTTCA AACTTTTCCATTACC AAGTGAAGGTGCATT TCTTCAGC-AGTGAG AAACTGGCCTACACT    1248
 2 rainbowtrout2      ACCTCAAGACCCGTG AGATGATGCCCTTCA AACTTTTCCATTACC AAGTGAAGGTGCATT TCTTCAGC-AGTGAG AAACTGGCCTACACT    1259
 3 zebrafish1         --------------- --------------- --------------- -----ACGACGCAGC GAGTCAGTGAGCGAG AACGCCGCATA-ACT      39
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            --------------- --------------- --------------- --------------- --------------- ---------------       0
 6 atlanticsalmon2    --------------- --------------- --------------- --------------- --------------- ---------------       0
 7 bream          ACACCAAGACGAGAG CCTCAATGCCTTTCA GAG][TTTACCACTACC AGCTGAAGATCCACT TCTCCAGT-AAAGTG AACCGCTCAGAGATG    1235
 8 atlanticsalmon1 ACACCAAGACCAGGG CCTCCATGCCGTTCA GAGTCTATCACTACC AGGTGAAGATCCACT TCTCCAGC-AAGGTG AACAGGTCTGAGATG     167
 9 winterflounder1 --------------- --------------- -----------ACG CTGGACAGAGCCCAG AGTCCACGTTTCCAG AAATAACCAGAAAAC      48
10 winterflounder2 --------------- --------------- -----------ACG CTGGACAGAGCCCAG AGTCCACGTTTCCAG AAATAACCAGAAAAC      48

                      1261        1275 1276        1290 1291        1305 1306        1320 1321        1335 1336        1350
 1 rainbowtrout1      GAGCAGCCCATGAAA ATCTCTCTGTACGGA ACCCATGATGAGAAG ATTGA-CAT--ACCT TACACCA-TGCCCTT CCTGAACACCAACAG    1334
 2 rainbowtrout2      GAGCAGCCCATGAAA ATCTCTCTGTACGGA ACCCATGATGAGAAG ATTGA-CAT--ACCT TACACCA-TGCCCTT CCTGAACACCAACAG    1345
 3 zebrafish1         TCGTATAGCATA--- ----CATTATACG-A AGTTATCAGTCGACG GT--A-CCG--GACA TATGCCC-GGGAATT C--GGCCATTACGGC     113
 4 zebrafish2         --------------- --------------- --------------- --------------- --------------- ---------------       0
 5 catfish            ------------AG ATTTCCCTGTATGGC TATTCTGGGGAGAAG GAGAA-CAT--CCCC TACATTA-TGCCTGC TTTGAAAACCAACAC      73
 6 atlanticsalmon2    --------------- --------------- --GCACGAGGCGTAG TTGGA-CA---ACTT TACAAAAATAGACTT TT-----ACAAGCGG      49
 7 bream          GAGCCGTCACTCACC GTCTCCCTGTACGGG ACAAACGGAGAGGCT GAAAA-CCTGGAGCT TAAACT][GAAGGAGAA AATTGCGACAAATAA    1324
 8 atlanticsalmon1 GAGCCTTCACTGACA GTCTCCCTGATTGGA ACCAAAGGAGAGGTT GAAGA-CCTCAAACT GACACTAAAGGAGAA GATAACAACCAATAA     256
 9 winterflounder1 TTCCAGACGCTGAAA --CCTCCAGCTCA-T CCCAACCGTGCGCTG ATCAATCCTCCTCCA AACGAAAAAGTGCTC G-----CATCAAGAG     130
10 winterflounder2 TTCCAGACGCTGAAA --CCTCCAGCTCA-T CCCAACCGTGCGCTG ATCAATCCTCCTCCA AACGAAAAAGTGCTC G-----CATCAAGAG     130
```

```
                    1351       1365 1366       1380 1381       1395 1396       1410 1411       1425 1426       1440
 1 rainbowtrout1    CACTGTGTCATTCCT GCTGACCACTGACGT GGACGTTGGGGAGCT GCTTATGGTCAAGCT GCGCTGG---GAGAA AGATGCCT-----AT   1416
 2 rainbowtrout2    CACTGTGTCATTCCT GCTGACCACTGACGT GGACGTTGGGGAGCT GCTTATGGTCAAGCT GCGCTGG---GAGAA AGATGCCT-----AT   1427
 3 zebrafish1       CGGGGTGTCTTTCCT GTTGACCACGGATGC AGACATCGGAGAGCT GCTGATGGTTAAACT TCTCTGG---GAGAA AGACACGC-----TC    195
 4 zebrafish2       -GGGGTGTCTTTCCT GTTGACCACGGATGC AGACATCGGAGAGCT GCTGATGGTTAAACT TCTCTGG---GAGAA AGACACGC-----TC     81
 5 catfish          CACCATCTCTTTCTT ACTCACCACGGATGT AGACATTGGTGAGCT GCTGATGGTGAAGCT GCTCTGG---GAGAA GGACACCA-----TC    155
 6 atlanticsalmon2 TAAAATA-CATTCCA GCGGATTAAAGTGAC AAACAATTTTCTTTT TCATTTGG--AAAGT TCTTT-----CATTA AATTGA-------A-    123
 7 bream            GACACATTCATTCCT GCTGGTGACAGAGAA AGACATCGGAGATCT CCTGATGCTGAAGTT TAAATGGGAGGAGAC AAACGGTTGGTCAAC   1414
 8 atlanticsalmon1 GACCCATTCCTTCCT GCTGGTAGCGGAGAA AGACATTGGTGACCT CCTGATGGTGAAGTT CAAGTGGGAGGAGTC TACTAGCTGGTCTGC    346
 9 winterflounder1 GAGGTAGTGCTCCTC GTTGATCCATGTTG- -------GGTCCCCC GC----GGTGACGGT GCATTGGTTGTTGGA AG-TGCCG-----AG    202
10 winterflounder2 GAGGTAGTGCTCCTC GTTGATCCATGTTG- -------GGTCCCCC GC----GGTGACGGT GCATTGGTTGTTGGA AG-TGCCG-----AG    202

                    1441       1455 1456       1470 1471       1485 1486       1500 1501       1515 1516       1530
 1 rainbowtrout1    TTC----------A GCTGGT-----CTGA CATTT---GGGGCAA CAA--AAACT----T CCACATC-CGCAAAA TGCGTGTCAAGGCCG   1480
 2 rainbowtrout2    TTC----------A GCTGGT-----CTGA CATTT---GGGGAAA CAA--AAACT----T CCACATC-CGCAAAA TGCGTGTCAAGGCCG   1491
 3 zebrafish1       ATC----------A GCTGGC-----CTTG G---T---GGAACTC CGA--CACCT----T CCACATT-CGCAAAC TGCGCATCAAGTCTG    256
 4 zebrafish2       ATC----------A GCTGGC-----CTTG G---T---GGAACTC CGA--CACCT----T CCACATT-CGCAAAC TGCGCATCAAGTCTG    142
 5 catfish          CTC----------A GTTGGC-----CCTG G---T---GGAATCC AGA--CACTT----T CCACATT-CGCAAGC TGCGTGTCAAATCTG    216
 6 atlanticsalmon2 -TC----------A ACTGTA-----CTAT G-------GGAAAAG AAAT-CACCTGGT-T GATCATA-ATTGGGA TTTGTATCAAGTCGT    186
 7 bream            CTCCAACATGCTGAA GATGGTTTCTTCTTG GTGGTCCGGTGACTC AGACGGTGCTAACAT GGAGGTT-CACAAAA TCCGCATTCGCGCAG   1503
 8 atlanticsalmon1 CTCGTTCATGCTCAA CATGGTGTCTTCCTG GTGGTCAGGTGACTC TGCGGAGTCTGAAGT GGAGGTC-CACAAAA TACGCATCCGAGTCG    435
 9 winterflounder1 TTGTT---------A CATGGGCAGC-CTTT T---TCTAGGTTTCA CTTCCCAGCC-ATCA CGACATTTCACAAAC GTTATGGCCTCTCCT    278
10 winterflounder2 TTGTT---------A CATGGGCAGC-CTTT T---TCTAGGTTTCA CTTCCCAGCC-ATCA CGACATTTCACAAAC GTTATGGCCTCTCCT    278

                    1531       1545 1546       1560 1561       1575 1576       1590 1591       1605 1606       1620
 1 rainbowtrout1    ---GGGAGACT-CAA T-CCAGGGTGAT-CT TCAGCGCTAAAGA-- ----TGG--AGAGTA T---------GCCTA CCTCAT--CAGAGGA   1545
 2 rainbowtrout2    ---GGGAGACT-CAA T-CCAGGGTGAT-CT TCAGCGCTAAAGA-- ----TGG--AGAGTA T---------GCCTA CCTCAT--CAGAGGA   1556
 3 zebrafish1       ---GAGAGACA-CAG T-CAAAAATCAT-CT TTAGTGCAAAAGA-- ----AAG--TGAATT T---------TCCTA CCTTTC--CCGTGGA    321
 4 zebrafish2       ---GAGAGACA-CAG T-CAAAAATCAT-CT TTAGTGCAAAAGA-- ----AAG--TGAATT T---------TCCTA CCTTTC--CCGTGGA    207
 5 catfish          ---GGGAAACA-CAG T-CCAGAGTGAT-CT TCATTGCCAAGGA-- ----AGG--TGAGTT T---------GCCTA CCTCAC--CCGTGGA    281
 6 atlanticsalmon2 CATGTGAGTCT-CTC TTCAACACTGAAACT CCTGCTCTCAGCA-- ----ATGGTAAAGAC T---------GGATA CTGGACTACACTGAC    260
 7 bream         ---GCGAGACC-CAA C-AAAA][GATGGT-GT TCTGTGTCAAAGACC CTGAAAGTCAGAAGT TAACACAAGAGGTCA CCTTTGTTAAATGTA   1587
 8 atlanticsalmon1 ---GGGAGACG-CAG A-AAAAGATGGA-GT TCTGCATTAAAGATC CTCATGCTCTGAGCT TACAACAGGAAGTTA CGTTTGTAAAATGCA    519
 9 winterflounder1 --GGTGAGATTTCAG TTTTTGAAGGGT-CT TGAGC---ACAGA-- ----AAG--TAAATT TCTTCTGCGTTTCTC CCGCTTT-CACACGA    353
10 winterflounder2 --GGTGAGATTTCAG TTTTTGAAGGGT-CT TGAGC---ACAGA-- ----AAG--TAAATT TCTTCTGCGTTTCTC CCGCTTT-CACACGA    353

                    1621       1635 1636       1650 1651       1665 1666       1680 1681       1695 1696       1710
 1 rainbowtrout1    AAA--------GACGA CGTAGTCTTTGTCAA ATC--AAAAGAGGA- --CAACATGAGC--- ---CGGAAAGAGAAA AC---GATGCACAGA   1614
 2 rainbowtrout2    AAA--------GACGA CGTAGTCTTTGTCAA ATC--AAAAGAGGA- --CAACATGAGC--- ---CGGAAAGAGAAA AC---GATGCACAGA   1625
 3 zebrafish1       GGT--------GAGGC GGCCGTCTTCGTGAA AGA--CAAAGAGGC- --CCAGTCGAGC--- ---CGCAAAAACCAG AG---ATTGCACAAG    390
 4 zebrafish2       GGT--------GAGGC GGCCGTCTTCGTGAA AGA--CAAAGAGGC- --CCAGTCGAGC--- ---CGCAAAAACCAG A--------------    265
 5 catfish          GGG--------GAAGC TTCCATGTTTGTCAA AGA--CAAAGAAGC- --TCAGTCAAGC--- ---CACAAAAATTAA AG---GTAGCATT--    348
 6 atlanticsalmon2 ATA--------GAGAC CAAGTTCTCAGTGCG TTC--AGTTGAGGAG --CCAGAGGAGG--- ---ATCTGTGTTACC TG---GTGCCAGGCA    330
 7 bream          AGGATGCATGGAGGA CGAACTCAAAACAAA CTCCTAAAAG][AGTAA CTCTGGAGAAGCACT GACCTCGAAGAAAAG ACCCCACTCCACCGT   1677
 8 atlanticsalmon1 A----------GGA CGAA-TGGAGGAAAA CTTCAAAAAGAGTGA A--------------- --------------- ---------------    553
 9 winterflounder1 AT--------GCGCC GGACTTCCAGGACAG GTT---TGGGGGGTT TGGCGTTCCAGGCC- ---CAGAAGGACTTC TTCACGCTCTTCCAC    428
10 winterflounder2 AT--------GCGCC GGACTTCCAGGACAG GTT---TGGGGGGTT TGGCGTTCCAGGCC- ---CAGAAGGACTTC TTCACGCTCTTCCAC    428

                    1711       1725 1726       1740 1741       1755 1756       1770 1771       1785 1786       1800
 1 rainbowtrout1    CTCAAGATGCAG--- GGAAG--CCACTT-C AAGAATAACATTGCA TGAAGCAACACTTGA GTAGAGTGCACACTG --AACCACACAGCCA   1696
 2 rainbowtrout2    CTCAAGATGCAG--- GGAAG--CCACTT-C AAGAATAACATTGCA TGAAGCAACACTTGA GTAGAGTGCACACTG --AACCACACAGCCA   1707
 3 zebrafish1       ATGAAGATGCAC--- GGCAG--TTCATT-C AAACAGAACACCGAG TAAAGACCCACGTCA --------------- --AAATCCACCTCC-    456
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------    265
 5 catfish          -TTGACTTATTT--- CCAAC--TTACTGAC ACACAGTAGCATCAC AAAAGAGTC------ --------------- ---------------    396
 6 atlanticsalmon2 AAAAGGATACA---- GTCAC--ACAGTG-- TGGCTTCAAAATCAG CTTACCTACGTTCGC ---CATCATACATGG --CTGGTCGGTGGCA    407
 7 bream            -TGAACATCCCTCCG TCCAT--TCAAGCAC TTAGTAAATAATTTA TACACCTGTAACTTA TTTTACTTCTCAAA- --ACTCTCACCGCCT   1761
 8 atlanticsalmon1 --------------- --------------- --------------- --------------- --------------- ---------------    553
 9 winterflounder1 ATGGAGCTCCAGGAC TCATCGTTTCCTTCC CAGCTCAGACTGATC TTCAGCAGGTCCCCA ATCTCCTTCTCGGTA AAAACCAGGAAGGTG    518
10 winterflounder2 ATGGAGCTCCAGGAC TCATCGTTTCCTTCC CAGCTCAGACTGATC TTCAGCAGGTCCCCA ATCTCCTTCTCGGTA AAAACCAGGAAGGTG    518

                    1801       1815 1816       1830 1831       1845 1846       1860 1861       1875 1876       1890
 1 rainbowtrout1    AAGCT-TGATGGA-G CCTGGGAGAATACAC TGG---AGAGAAGAT AGGGGATGTTAGT-C TCTGACCTGTGCTGT ATGT--------TCTG   1773
 2 rainbowtrout2    AAGCT-TGATGGA-G CCTGGGAGAATACAC TGG---AGAGGAGAT AGGGGATGTTAGT-C TCTGACCTGTGCTGT ATGT--------TCTG   1784
 3 zebrafish1       -------GCTGGG-A TCTGGCACAG-AATC TGC---CAGGGAAAA CTGGAATGGTAGA-A TCTGACCTTTCCCGT ATATA------TTCGG    528
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------    265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------    396
 6 atlanticsalmon2 GGTCT-GTTTGAG-A GCTGGATCCACAAGC TGG---T-AGCGGCC CTG---TTTGAGC-G TGTGCCCAATGCCAA CGTTA------TAGTG    482
 7 bream            TGTAT-ATATCCACA AAAGTATGTTTGTTA TTT---AAAAAAAAA CATCTATGTGAATAC TGTAAAATATTTTAT AAAGAGCTGTTTTTA   1847
 8 atlanticsalmon1 --------------- --------------- --------------- --------------- --------------- ---------------    553
 9 winterflounder1 TNGGTCAGGTNCAGA CCGATGACGTTTTCA TGGCTGAACACAGAT AAANCAGGTTAGG-- TCAGATGTGTGCTCA ANGTGGATTATNGTA    606
10 winterflounder2 TNGGTCAGGTNCAGA CCGATGACGTTTTCA TGGCTGAACACAGAT AANC-AGGTTAGG-- TCAGATGTGTGCTC- ---------------    589

                    1891       1905 1906       1920 1921       1935 1936       1950 1951       1965 1966       1980
 1 rainbowtrout1    CCTCCCAACCTGTGT C-ATCTCATCTCCAC C-CGCAATCACTTCA TCC--CACTGTGCTG GGTG--TCGGCTAAC GTGCT-TTTTCTTCT   1856
 2 rainbowtrout2    CCTCCCAACCTGTGT C-ATCTCATCTCCAC C-CGCAATCACTTCA TCC--CACTGTGCTG GGTG--TCGGCTAAC GTGCT-TTTTCTTCT   1867
 3 zebrafish1       ACTAACAACCTTCAC CCAACCCAAGTCCAG G-AGCACATTCTGCA AGA--AACCGCATCT TGAA--AATGTTACT GC----TGTGCAGTT    609
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------    265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------    396
 6 atlanticsalmon2 GTGGACTGGCTGGAC CGGGCCCAGCACCAC TACCCCAACTCTGCT GCCAACACCAAGCTG GTTG--GA-GAGGAT GTGGC-CAGGCT--C    566
 7 bream            TGGTAGTAACTGTAT GTATGCCGTCTTCTT TGGTGCATCTTTTGT GTC--CAAAAGGTTG GATACTGCGCCTCAC ATACTGAGAGGTACA   1935
 8 atlanticsalmon1 --------------- --------------- --------------- --------------- --------------- ---------------    553
 9 winterflounder1 ATTCTGCAT------ --------------- --------------- --------------- --------------- ---------------    615
10 winterflounder2 --------------- --------------- --------------- --------------- --------------- ---------------    589

                    1981       1995 1996       2010 2011       2025 2026       2040 2041       2055 2056       2070
 1 rainbowtrout1    ATTAACCTTCATCA- -TGGAGAGTATTACA ATTGCTTTTTTTTTA ---TGATTTTGACTT GGTCTTACGTTGCTA CATTATCTTTTTAAT   1941
 2 rainbowtrout2    ATTAACCTTCATCA- -TGGAGAGTATTACA ATTGCTTTTTTTTTA A--TGATTTTGACTT GGTCTTACGTTGCTA CATTATCTTTTTAAT   1953
 3 zebrafish1       ATAAAACAAAAAAA- -TATATATTTTTGGA AAGATCACAGGTCTG ----GCT-------- --------------- ---------------    655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------    265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------    396
 6 atlanticsalmon2 ATCAACTGGCTGGA- -GGTGGATCTGAAGT ATGACCTG------- --------------- --------------- ---------------    602
 7 bream            ACCAACTGGATTTAC TTTCATATTTCTGGA GGTGCTGATGTCCCA TTGTGTAAAATACCT CAAGTTAAATTTCAC AATCAACCACTGTGA   2025
 8 atlanticsalmon1 --------------- --------------- --------------- --------------- --------------- ---------------    553
 9 winterflounder1 --------------- --------------- --------------- --------------- --------------- ---------------    615
10 winterflounder2 --------------- --------------- --------------- --------------- --------------- ---------------    589
```

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                    2071       2085 2086       2100 2101       2115 2116       2130 2131       2145 2146       2160
 1 rainbowtrout1    CCTTTCATCTCAACT GTGTTT--ATGTATA CCTACTAAATCAAAT AAATTAATTCCTAAC GCCATAAAAAAAAAA AAAAAAA-------  2022
 2 rainbowtrout2    CCTTTCATCTCAACT GTGTTT--ATGTATA C-------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            GGAATAATGTCCTCT TTGTGTCCATGGAGG TCTGCAAAGGGGAAC ATGAGAAATTCTTCT TCATAGTAAGATGAA GGTGTTTTATGCAAT  2115
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2161       2175 2176       2190 2191       2205 2206       2220 2221       2235 2236       2250
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            AGGGCCCACATTATT TACCTTTATGTGTTA TCTAACCTGACAGCA TTCACCACTGTGGCT CTCAGTACTGTATGT GACTGTGGGACCTGT  2205
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2251       2265 2266       2280 2281       2295 2296       2310 2311       2325 2326       2340
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            ATCAGTGTGCCTTAT ACTGTAAACAGTCAC CAGAAGTAAGCTGTT GCTGTTTGTCTTAAC TTAGCGCTTTGTTGC TGTTTTTCACCTCGG  2295
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2341       2355 2356       2370 2371       2385 2386       2400 2401       2415 2416       2430
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            CATGCTTTATTGTTG TTATTCATTTAACTG TGTGACTTTTACTGA AAACGTCACCCATGA AAAAGATGTTTTAAT CTCCTGTGCAAGATG  2385
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2431       2445 2446       2460 2461       2475 2476       2490 2491       2505 2506       2520
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            TTACTTTGAAGCTGT TACGGTATAGAAGAC ATTTTGTCTACCATG GTTTTATTAAATTGG TGAAATTAGATGAGA GGCGTGATGCTCAGT  2475
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2521       2535 2536       2550 2551       2565 2566       2580 2581       2595 2596       2610
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            GCGAGGTGGAAAGCA CAGTTCATATCAGTG TTGTCATTTATTTGT ACTGTCTGTCGTTAG TCACCTTTGACTTCT GTGCATTTAGCTTTT  2565
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2611       2625 2626       2640 2641       2655 2656       2670 2671       2685 2686       2700
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            TAAAATCTTTTATTC ATAATTACATTTCCA GTGCTGTCAGCAATG TCTCAACCTCGGCTC TTTAGAGATCGGTAC TGTAATCATTGTGAC  2655
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2701       2715 2716       2730 2731       2745 2746       2760 2761       2775 2776       2790
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            TTCATTTCAACTGAC TTGAAAAATGTGGAA ATGTTTGACTTTTGC ACTGAAAACAGGGGA CGAGATTGCTTAACC AAGTTACTTGTACAG  2745
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589
```

```
                    2791       2805 2806       2820 2821       2835 2836       2850 2851       2865 2866       2880
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            TTATGTAAATACAAC AATTACAGATGATAG ATTTTGTAGTATTCA GCACTGTTAGTTGTA CATTTCTACCTTTGG AATTTAATCTGTAAA  2835
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2881       2895 2896       2910 2911       2925 2926       2940 2941       2955 2956       2970
 1 rainbowtrout1    --------------- --------------- --------------- --------------- --------------- ---------------  2022
 2 rainbowtrout2    --------------- --------------- --------------- --------------- --------------- ---------------  1982
 3 zebrafish1       --------------- --------------- --------------- --------------- --------------- ---------------   655
 4 zebrafish2       --------------- --------------- --------------- --------------- --------------- ---------------   265
 5 catfish          --------------- --------------- --------------- --------------- --------------- ---------------   396
 6 atlanticsalmon2  --------------- --------------- --------------- --------------- --------------- ---------------   602
 7 bream            ATCTATAATCTTTTG TACATATTTGTAAAT ATTCGCTGTACTGTA TACATTCTTTAATAA ACCAAAAGTGCCATC CAAAAAAAAAAAAAA  2925
 8 atlanticsalmon1  --------------- --------------- --------------- --------------- --------------- ---------------   553
 9 winterflounder1  --------------- --------------- --------------- --------------- --------------- ---------------   615
10 winterflounder2  --------------- --------------- --------------- --------------- --------------- ---------------   589

                    2971       2985 2986       3000
 1 rainbowtrout1    --------------- ------    2022
 2 rainbowtrout2    --------------- ------    1982
 3 zebrafish1       --------------- ------     655
 4 zebrafish2       --------------- ------     265
 5 catfish          --------------- ------     396
 6 atlanticsalmon2  --------------- ------     602
 7 bream            AAAAAAAAAAAAAAA AAAAAA    2946
 8 atlanticsalmon1  --------------- ------     553
 9 winterflounder1  --------------- ------     615
10 winterflounder2  --------------- ------     589
```

Where: reverse primers; forward primers; over lap between primers.

The LPL Primers FHLPL4r and 7f were non-degenerate primers based on the primers FHLPLr1 and FHLPLf3 respectively, which can be seen on the above alignment.

Accession numbers for sequences for different species used: Rainbow trout 1: 4584059, Rainbow trout 2: 14582900, zebrafish 1: CB352163.1, zebrafish 2: CB352334.1, Catfish: 18460110, Atlantic salmon 1: 15280954, Atlantic salmon 2: 40541657, Bream: AB054062, Winter flounder 1: CF195532, Winter flounder 2: CF195573.

Appendix 3: β-actin sequence alignment using the ClustalW Multiple sequence alignment on the BCM search launcher.

The genomic sequence of Chinese minnow was included because a) it is closely related to fathead minnow and b) to identify intron/exon boundaries.

```
                                1               15 16              30 31              45 46              60 61              75 76              90
1 yellow_perch                  --------------- --------------- --------------- --------------- --------------- ---------------          0
2 gilthead_bream                --------------- --------------- --------------- --------------- --------------- ---------------          0
3 grass_carp                    --------------- --------------- --------------- --------------- --------------- ---------------          0
4 Chinese_minnow                --------------- --------------- --------------- --------------- --------------- ---------------          0
5 Jap_Silver_carp               --------------- --------------- --------------- --------------- --------------- ---------------          0
6 zebrafish2                    --------------- --------------- ---------GGGGAT TGTGAGTTTTCAGTG CACGCTGAGAAGATC TTCACT-CCCCTTGT         50
7 zebrafish1                    GGGAAGCAGTGGTAT CAACGCAGCGTGCCC ATTACGGCTGGGGAT TGTGAGTTTTCAGTG CACGCTGAGAAGATC TTCACTTCCCCTTGT         90

                                91              105 106             120 121             135 136             150 151             165 166             180
1 yellow_perch                  --------------- ------------AT GGATGACGAAATCGC CGCCCTCGTTGTTGA CAACGGATCCGGTAT GTGCAAAGCTGGCTT         62
2 gilthead_bream                --------------- --------------- --------------- --------------- --------------- ---------------          0
3 grass_carp                    --------------- ------------AT GGATGATGAAATTGC CGCACTGGTTGTTGA CAACGGATCCGGTAT GTGCAAAGCCGGATT         62
4 Chinese_minnow                --------------- ------------AT GGATGATGAAATTGC CGCACTGGTTGTTGA CAACGGATCCGGTAT GTGCAAAGCCGGATT         62
5 Jap_Silver_carp               --------------- --------------- --------------- --------------- --------------- ---------------          0
6 zebrafish2                    TCACAATAACCTACT -AATACACAGCC-AT GGATGAGGAAATCGC TGCCCTGGTCGTTGA CAACGGCTCTGGTAT GTGCAAAGCCGGTTT        138
7 zebrafish1                    TCACAATAACCTACT TAATACCCAGCCCAT GGATGAGGAAATCGC TGCCCTGGTCGTTGA TAACGGCTCCGGTAT GTGCAAAGCCGGTTT        180

                                181             195 196             210 211             225 226             240 241             255 256             270
1 yellow_perch                  TGCAGGAGATGATGC TCCGCGTGCTGTGTT CCCCTCCATTGTTGG ACGTCCAAGACATCA GG------------- ---------------        124
2 gilthead_bream                --------------- --------------- --------------- --------------- --------------- ---------------          0
3 grass_carp                    CGCTGGAGATGATGC TCCCCGTGCTGTCTT CCCATCCATCGTGGG TCGCCCCAGACATCA GG------------- ---------------        124
4 Chinese_minnow                CGCTGGAGATGATGC TCCCCGAGCTGTCTT CCCATCCATCGTGGG TCGCCCCAGACATCA GGTGAGAAGCGGATG ATAAATCGATTTAGG        152
5 Jap_Silver_carp               -------------CC TCCCCGTGCTGTCTT CC-ATCC-TCGTGGG TCGCCCCAGAC-TCA GG------------- ---------------         46
6 zebrafish2                    TGCTGGAGATGATGC CCCTCGTGCTGTTTT CCCCTCCATTGTTGG ACGACCCAGACATCA GG------------- ---------------        200
7 zebrafish1                    TGCTGGAGATGATGC CCCTCGTGCTGTTTT CCCCTCCATTGTTGG ACGACCCAGACATCA GG------------- ---------------        242

                                271             285 286             300 301             315 316             330 331             345 346             360
1 yellow_perch                  --------------- --------------- --------------- --------------- --------------- ---------------        124
2 gilthead_bream                --------------- --------------- --------------- --------------- --------------- ---------------          0
3 grass_carp                    --------------- --------------- --------------- --------------- --------------- ---------------        124
4 Chinese_minnow                GTTATCTGTAATGAG AATTATTTTCGTACT TAAAAGTGAGTTCAT TTCTAGTTCCTAAAC ATTTTACAAAAATTA ACATTGCTTTCTTTG        242
5 Jap_Silver_carp               --------------- --------------- --------------- --------------- --------------- ---------------         46
6 zebrafish2                    --------------- --------------- --------------- --------------- --------------- ---------------        200
7 zebrafish1                    --------------- --------------- --------------- --------------- --------------- ---------------        242

                                                                                                                                  FHbact-f1
                                361             375 376             390 391             405 406             420 421             435 436             450
1 yellow_perch                  -------GTGTGATG GTTGGCATGGGCCAG AAAGATAGCTATGTT GGTGATGAGGCACAG AGCAAAAGGGGTATC CTGACCCTGAAGTAC        207
2 gilthead_bream                --------------- --------------- --------------- --------------- ---------------------------------          0
3 grass_carp                    -------GTGTCATG GTTGGTATGGGACAG AAGGACAGCTACGTT GGTGATGAGGCTCAG AGCAAGAGAGGTATC CTGACCCTGAAGTAC        207
4 Chinese_minnow                TTACAGGGTGTCATG GTCGGTATGGGACAG AAGGACAGCTATGTT GGTGACGAGGCTCAG AGCAAGAGAGGTATC CTGACTCTGAAGTAC        332
5 Jap_Silver_carp               -------GTGTCATG GTTGGTATGGGACAG AAGGACAGCTACGTT GGTGATGAGGCTCAG AGCAAGAGAGGTATC CTGACCCTGAAGTAC        129
6 zebrafish2                    -------GAGTGATG GTTGGCATGGGACAG AAAGACTCCTATGTG GGAGATGAGGCCCAG AGCAAGAGAGGTATC CTGACCCTCAAATAC        283
7 zebrafish1                    -------GAGTGATG GTTGGCATGGGACAG AAAGACTCCTATGTG GGAGATGAGGCTCAG AGCAAGAGACGTATC CTGACCCTCAAATAC        325

                                                                                FHbact-f2                       FHbact-r1
                                451             465 466             480 481             495 496             510 511             525 526             540
1 yellow_perch                  CCCATTGAGCATGGT ATCGTCACCAACTGG GACGACATGGAGAAG ATCTGGCATCACACC TTCTACAACGAGCTG AGAGTTGCGCCCGAG        297
2 gilthead_bream                --------------- ----TCACCAACTGG GATGACATGGAGAAG ATCTGGCATCACACC TTCTACAACGAGCTG AGAGTTGCCCCTGAG         71
3 grass_carp                    CCCATCGAGCACGGT ATTGTCACCAACTGG GACGATATGGAGAAG ATCTGGCATCACACC TTCTACAACGAGCTG CGTGTTGCCCCAGAG        297
4 Chinese_minnow                CCCATTGAGCACGGT ATTGTCACCAACTGG GACGATATGGAGAAG ATCTGGCATCACACC TTCTACAATGAGCTG CGTGTTGCCCCAGAG        422
5 Jap_Silver_carp               CCCATCGAGCACGGT ATTGTCACCAACTGG GATGATATGGAGAAG ATCTGGCATCACACC TTCTACAACGAGCTG CGTGTTGCCCCAGAG        219
6 zebrafish2                    CCCATTGAGCACGGT ATTGTGACTAACTGG GATGACATGGAGAAG ATCTGGCATCACACC TTCTACAATGAGCTC CGTGTTGCCCCTGAG        373
7 zebrafish1                    CCCATTGAGCACGGT ATTGTGACCAACTGG GATGACATGGAGAAG ATCTGGCATCACACC TTCTACAATGAGCTC CGTGTTGCCCCTGAG        415

                                                                                                                FHbact-r2
                                541             555 556             570 571             585 586             600 601             615 616             630
1 yellow_perch                  GAGCACCCCGTCCTG CTCACAGAGGC-TCC CCTGAACCCCAAGGC CAACAGGGAAAAGAT GAC------------ ---------------        359
2 gilthead_bream                GAGCACCCTGTCCTG CTCACAGAGGC-CCC CCTGAACCCCAAAGC CAACAGGGAGAAGAT GAC------------ ---------------        133
3 grass_carp                    GAGCACCCCGTCCTG CTCACAGAGGC-CCC CCTGAACCCCAAGGC CAACAGGGAAAAGAT GAC------------ ---------------        359
4 Chinese_minnow                GAGCACCCCGTCCTG CTCACAGAGGC-CCC CCTGAACCCCAAGGC CAACAGGGAAAAGAT GACACAGGTTGGTTT TTGGCTAGAAAATGG        511
5 Jap_Silver_carp               GAGCACCCCGTCCTG CTCACAGAGGC-CCC CCTGAACCCCAAGGC CAACAGGGAAAAGAT GAC------------ ---------------        281
6 zebrafish2                    GAGCACCCTGTGCTG CTCACTGAGGCTCCC CCTGAATCCCAAAGC CAACAGAGAGAAGAT GACACAGATCATGTT CGAGACCTTCAAC--        461
7 zebrafish1                    GAGCACCCTGTGCT- --------------- --------------- ---------------------------- ---------------        429

                                631             645 646             660 661             675 676             690 691             705 706             720
1 yellow_perch                  --------------- --------------- --------------- --------------- --------------- ---------------        359
2 gilthead_bream                --------------- --------------- --------------- --------------- --------------- ---------------        133
3 grass_carp                    --------------- --------------- --------------- --------------- --------------- ---------------        359
4 Chinese_minnow                TGCATTGAAGTCTCT TGTCTGTCTTCTGTC TTTTAAGTTCTCCTT TTCATTCGTTCACTT CCTCCAGGCTTTGTT TCCTCTGAGCTCCTG        601
5 Jap_Silver_carp               --------------- --------------- --------------- --------------- --------------- ---------------        281
6 zebrafish2                    --------------- --------------- --------------- --------------- --------------- ---------------        461
7 zebrafish1                    --------------- --------------- --------------- --------------- --------------- ---------------        429

                                721             735 736             750 751             765 766             780 781             795 796             810
1 yellow_perch                  --------------- --------------- --------------- --------------- --------------- ---------------        359
2 gilthead_bream                --------------- --------------- --------------- --------------- --------------- ---------------        133
3 grass_carp                    --------------- --------------- --------------- --------------- --------------- ---------------        359
4 Chinese_minnow                AGTTTCTCATCTTTT GCTGGAAGCAGCAGG TTATCTATACTTTTG CCTGCCTGTTTTGCA GTCTCCTGCACTCTT ATTCTTTGTGCACTT        691
5 Jap_Silver_carp               --------------- --------------- --------------- --------------- --------------- ---------------        281
6 zebrafish2                    --------------- --------------- --------------- --------------- --------------- ---------------        461
7 zebrafish1                    --------------- --------------- --------------- --------------- --------------- ---------------        429
```

```
                  811         825 826         840 841         855 856         870 871         885 886         900
1 yellow_perch    --------------- --------------- --------------- --------------- --------------- ---------------  359
2 gilthead_bream  --------------- --------------- --------------- --------------- --------------- ---------------  133
3 grass_carp      --------------- --------------- --------------- --------------- --------------- ---------------  359
4 Chinese_minnow  TTGTTTCTTTACTCT GGATTTCCAACTAAC CCTTGCATGGATGTG TGGATTGTCTGCTGT AAATATTTGAGCATC AGTTAACTTCTCCAC  781
5 Jap_Silver_carp --------------- --------------- --------------- --------------- --------------- ---------------  281
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  901         915 916         930 931         945 946         960 961         975 976         990
1 yellow_perch    ---------CCAGAT CATGTTCGAGACCTT CAACACCCCTGCCAT GTACGTTGCCATCCA GGCCGTGCTGTCCCT GTATGCCTCTGGTCG  440
2 gilthead_bream  ---------CCAGAT CATGTTCGAGACCTT CAACACCCCCGCCAT GTATGTTGCCATCCA GGCTGTGCTGTCCCT GTATGCCTCTGGTCG  214
3 grass_carp      ---------ACAGAT CATGTTCGAGACCTT CAACACCCCCGCCAT GTACGTTGCCATCCA GGCTGTGCTGTCCCT GTATGCCTCTGGTCG  440
4 Chinese_minnow  TCTCTCTTTCCAGAT CATGTTCGAGACCTT CAACACCCCAGCCAT GTACGTTGCCATCCA GGCTGTGTTGTCCCT GTATGCCTCTGGTCG  871
5 Jap_Silver_carp ---------ACAGAT CATGTTCGAGACCTT CAACACCCCTGCCAT GTACGTTGCCATCCA AGCTGTGCTGTCCCT GTATGCCTCTGGTCG  362
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  991        1005 1006        1020 1021        1035 1036        1050 1051        1065 1066        1080
1 yellow_perch    TACCACTGGTATCGT CATGGACTCCGGTGA TGGTGTGACCCACAC AGTGCCCATCTATGA GGGCTATGCCCTGCC CCACGCCATCCTGCG  530
2 gilthead_bream  TACCACTGGTATTGT CATGGATTCCGGTGA TGGTGTGACCCACAC AGTGCCCATCTATGA GGGCTATGCCCTGCC CCACGCCATCCTGCG  304
3 grass_carp      TACCACTGGTATCGT GATGGACTCTGGTGA TGGTGTCACCCACAC TGTGCCCATCTACGA GGGTTACGCCCTGCC CCACGCCATCCTCCG  530
4 Chinese_minnow  TACCACTGGTATCGT GATGGACTCTGGTGA TGGTGTCACCCACAC CGTGCCCATCTACGA GGGGTACGCCCTGCC CCATGCCATCCTCCG  961
5 Jap_Silver_carp TACCACTGGTATTGT GATGGACTCTGGTGA TGGTGTCACCCACAC TGTGCCCATCTACGA GGGTTACGCCCTGCC CCATGCCATCCTCCG  452
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1081       1095 1096       1110 1111       1125 1126       1140 1141       1155 1156       1170
1 yellow_perch    TCTGGACTTGGCTGG CCGTGACCTCACAGA CTACCTCATGAAGAT CCTGACAGAGCGTGG GTACTCATTCACCAC CACAGCTGAGAGGGA  620
2 gilthead_bream  TCTGGACTTGGCCGG CCGCGACCTCACAGA CTACCTCATGAAGAT CCTGACAGAGCGTGG CTACTCCTTCACCAC CACAGCCGAGAGGGA  394
3 grass_carp      TCTGGACTTGGCTGG CCGTGACCTGACTGA CTACCTCATGAAGAT CCTGACCGAGAGAGG CTACAGCTTCACCAC CACAGCCGAGAGGGA  620
4 Chinese_minnow  TCTGGACTTGGCTGG CCGTGACTTGACCGA CTACCTCATGAAGAT CCTGACCGAGAGAGG TTACAGCTTCACCAC CACAGCTGAGAGGGA 1051
5 Jap_Silver_carp TCTGGACTTGGCTGG CCGTGACCTGACAGA CTACCTCATGAAGAT CCTGACCGAGAGAGG CTACAGCTTCACCAC CACAGCCGAGAGGGA  542
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1171       1185 1186       1200 1201       1215 1216       1230 1231       1245 1246       1260
1 yellow_perch    AATCGTGCGTGACAT CAAGGAGAAGCTGTG CTATGTCGCCCTGGA CTTCGAGCAGGAGAT GGGCACTGCTGCCTC CTCTTCCTCCCTGGA  710
2 gilthead_bream  AATCGTGCGTGACAT CAAGGAGAAGCTGTG CTACGTCGCCCTGGA CTTCGAGCAGGAGAT GGGTACCGCTGCCTC CTCCTCCTCCCTGGA  484
3 grass_carp      AATTGTCCGTGACAT CAAGGAGAAGCTCTG CTATGTGGCTCTTGA CTTCGAGCAGGAGAT GGGCACTGCTGCTTC CTCCTCCTCCCTGGA  710
4 Chinese_minnow  AATTGTCCGTGACAT CAAGGAGAAGCTCTG CTATGTGGCTCTTGA CTTCGAGCAGGAGAT GGGCACCGCTGCTTC CTCCTCCTCCCTGGA 1141
5 Jap_Silver_carp AATTGTCCGTGACAT CAAGGAGAAGCTCTG CTATGTGGCTCTTGA CTTTGAGCAGGAGAT GGGCACTGCTGCTTC CTCCTCCTCCTGGGA  632
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1261       1275 1276       1290 1291       1305 1306       1320 1321       1335 1336       1350
1 yellow_perch    GAAGAGCTACGAGCT GCCCGACGGACAGGT CATCACCATCGGCAA TGAGAGGTTCCGTTG CCCAGAGGCCCTCTT CCAGCCTTCCTTCCT  800
2 gilthead_bream  GAAGAGCTATGAGCT GCCCGACGGACAGGT CATCACCATCGGCAA TGAGAGGTTCCGTTG CCCAGAGGCCCTCTT CCAGCCATCCTTCCT  574
3 grass_carp      GAAGAGCTACGAGCT GCCTGACGGACAGGT CATCACCATTGGCAA TGAGAGGTTCAGGTG CCCAGAGGCCCTGTT CCAGCCATCCTTCTT  800
4 Chinese_minnow  GAAGAGCTATGAGCT GCCTGACGGACAGGT CATCACCATTGGCAA TGAGAGGTTCAGGTG CCCAGAGGCCCTGTT CCAGCCATCCTTCTT 1231
5 Jap_Silver_carp AAAGAGCTATGAGCT G-------------- --------------- --------------- --------------- --------------- ---------------  648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1351       1365 1366       1380 1381       1395 1396       1410 1411       1425 1426       1440
1 yellow_perch    CGGTA---------- --------------- --------------- --------------- --------------- ---------------  805
2 gilthead_bream  CGGTA---------- --------------- --------------- --------------- --------------- ---------------  579
3 grass_carp      GGGTA---------- --------------- --------------- --------------- --------------- ---------------  805
4 Chinese_minnow  GGGTAGGTTTCCTTA CAAACATTCCCTGGT GTGTGTATGTACACT AGATTTAATTTGAAG GCGAGTACAAGAACA CTGGCTAATCATTTT 1321
5 Jap_Silver_carp --------------- --------------- --------------- --------------- --------------- ---------------  648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1441       1455 1456       1470 1471       1485 1486       1500 1501       1515 1516       1530
1 yellow_perch    --------------- ---TGGAGTCCTGCG GAATCCATGAGACCA CCTACAACAGCATTA TGAAGTGTGATGTCG ACATCCGTAAGGACC  877
2 gilthead_bream  --------------- ---TGGAGTCCTGTG GAATCCATGAGACCA CCTACAACAGCATCA TGAAGTGCGACGTCG ACATCCGTAAGGACC  651
3 grass_carp      --------------- ---TGGAGTCTTGCG GTATCCATGAGACCA CCTTCAACTCCATCA TGAAGTGTGACGTCG ACATCCGTAAGGACC  877
4 Chinese_minnow  TGTCTTGCTCTGCAG GTATGGATTCTTGCG GTATCCATGAGACCA CCTTCAACTCCATCA TGAAGTGTGACGTCG ACATCCGTAAGGACC 1411
5 Jap_Silver_carp --------------- --------------- --------------- --------------- --------------- ---------------  648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1531       1545 1546       1560 1561       1575 1576       1590 1591       1605 1606       1620
1 yellow_perch    TGTACGCCAACACCG TGCTGTCTGGAGGTA CCACCATGTACCCCG GCATTGCCGACAGGA TGCAGAAGGAGATCA CAGCCCTGGCCCCAT  967
2 gilthead_bream  TGTATGCCAACACTG TGCTGTCTGGAGGTA CCACCATGTACCCCG GCATCGCCGACAGGA TGCAGAAGGAAATCA CAGCCCTGGCCCCAT  741
3 grass_carp      TGTATGCCAACACTG TATTGTCTGGTGGTA CCACCATGTACCCTG GCATTGCTGACAGGA TGCAGAAGGAGATCA CATCCCTGGCCCCCA  967
4 Chinese_minnow  TGTATGCCAACACTG TATTGTCTGGTGGTA CCACCATGTACCCTG GCATTGCTGACAGGA TGCAGAAGGAGATCA CATCCCTGGCCCCCA 1501
5 Jap_Silver_carp ---------------- --------------- --------------- --------------- --------------- ---------------  648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1621       1635 1636       1650 1651       1665 1666       1680 1681       1695 1696       1710
1 yellow_perch    CCACCATGAAAATCA AG---------- --------------- --------------- --------------- ---------------  984
2 gilthead_bream  CCACCATGAAGATCA AG---------- --------------- --------------- --------------- ---------------  758
3 grass_carp      GCACAATGAAAATCA AG---------- --------------- --------------- --------------- ---------------  984
4 Chinese_minnow  GCACAATGAAAATCA AGGTGAGCTTTGACC CTTGCCGCTTATATC AGTATATATCTAAAC GCACTCTGCTGTGTT TAGCAACTCTGCATC 1591
5 Jap_Silver_carp --------------- --------------- --------------- --------------- --------------- ---------------  648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429

                  1711       1725 1726       1740 1741       1755 1756       1770 1771       1785 1786       1800
1 yellow_perch    --------------- ----------ATTAT TGCCCCACCAGAGCG TAAATACTCTGTCTG GATCGGAGGCTCCAT CCTGGCCTCTCTGTC 1049
2 gilthead_bream  --------------- ----------ATCAT TGCCCCACCTGAGCG TAAATACTCTGTCTG GATCGGAGGCTCCAT CCTGGCCTCCCTGTC  823
3 grass_carp      --------------- ----------ATCAT TGCCCCACCTGAGCG TAAATACTCTGTCTG GATCGGAGGCTCCAT CCTGGCCTCCCTGTC 1049
4 Chinese_minnow  GTGCTAATTGTCTGT TTCTCCTCAGATCAT TGCCCCACCTGAGCG TAAATACTCTGTCTG GATCGGAGGCTCCAT CCTTGCCTCCCTGTC 1681
5 Jap_Silver_carp --------------- --------------- --------------- --------------- --------------- ---------------  648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ---------------  461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ---------------  429
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                  1801        1815 1816        1830 1831        1845 1846        1860 1861        1875 1876        1890
1 yellow_perch    CACCTTCCAGCAGAT GTGGATCAGCAAGCA GGAGTACGATGAGTC CGGCCCCTCCATCGT CCACCGTAAATGCTT CTAA    1128
2 gilthead_bream  CACCTTCCAGCAGAT GTGGATTA------- --------------- --------------- --------------- ----     846
3 grass_carp      CACCTTCCAGCAGAT GTGGATTAGCAAGCA GGAGTACGATGAGTC TGGACCATCCATCGT CCACCGCGAATGCTT CTAA    1128
4 Chinese_minnow  CACCTTCCAGCAGAT GTGGATTAGCAAGCA GGAGTATGATGAGTC TGGACCATCCATTGC CCACCGCAAATGCTT CTAA    1760
5 Jap_Silver_carp --------------- --------------- --------------- --------------- --------------- ----     648
6 zebrafish2      --------------- --------------- --------------- --------------- --------------- ----     461
7 zebrafish1      --------------- --------------- --------------- --------------- --------------- ----     429
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Where: reverse primers; forward primers; over lap between primers, and parts of the Chinese minnow sequence identified as introns are shown in light grey.

Accession numbers for different sequences used: Yellow Perch: AY332493; Gilthead bream: F384096; Grass Carp: AF393832; Chinese minnow: AF200957; Sliver Carp: AB020852; zebrafish 1: CB366679; zebrafish 2: CB366658.