Chapter 5: Potential effects of Clofibric acid on the adult fathead minnow (*Pimephales promelas*)

5.1 Introduction

The issue of pharmaceuticals in the environment is a growing topic. It was only with the development of more sensitive analytical techniques that it became evident that so many drugs and personal care products, covering a wide spectrum of therapeutic uses for humans and animals, have been inadvertently released into the environment. Simultaneously, there has been rapid progression of endocrine disruption research, involving the investigation of the effects occurring in different organisms from exposure to natural and synthetic oestrogens, including the pharmaceutical ethinyloestradiol. This has brought about interest in other drugs present in the environment. Although the pharmaceuticals present in the environment are generally present only at low concentrations, the effect of chronic exposure to these chemicals must be determined, because low concentrations can still cause effects (c.f. EE_2).

The aim of this thesis was to use different methods to determine the effect, if any, of clofibric acid on fish, both in the embryo and adult stages. The following chapter summarizes the results of a number of exposure studies designed to evaluate such effects in adult fathead minnow (*Pimephales promelas*) and to determine whether any of these effects are significant.

It has become increasing apparent from the literature that there are a multitude of mechanisms through which xenobiotics can interfere with physiological processes. Pharmaceuticals are designed to have a biological effect in a specific organism (usually a human), and may have similar or identical effects in fish. Alternatively, their effects on a non-target organism may be very different. Either way, the effects need to be investigated. This chapter investigates possible effects of clofibric acid on various biological parameters, ranging from physiological and biochemical through to effects at the molecular

level, to try and determine if there are any specific effects occurring in fish exposed to the drug.

For all the experiments reported within this chapter, the dosing set-up was as described in Section 2.1. Adult fathead minnow (*Pimephales promelas*) from breeding stocks held at Brunel University (parents were imported from the USA) were used. They were fed three times per day: once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated) and twice with flake food (King British Tropical flake fish food, Lillicos, Surrey). Water quality parameters were monitored daily.

5.2 Adult Experiment 2

As this is the second of the adult experiments (the first being the Bream experiment reported in Chapter 4), the experiments will be numbered from 2 onwards in this chapter.

A preliminary, short-term, pilot static exposure experiment was carried out to determine the protocol to be used in further experiments and also to identify potential endpoints. It included establishing and then validating some techniques, assessing the viability of protocols, and establishing the identity of those tissues in which transcription of certain genes occurred. For this first adult fathead minnow exposure experiment, female fish were used, although there was also one male fish per tank, to ensure the female fish remained reproductively active.

This static exposure study was carried out over a 7-day period and included both control and treated (nominal concentration of 1mg/l clofibric acid) groups of fish. Twenty-five adult fish were used, consisting of 4 males (two per treatment) and 21 females (11 control and 10 exposed) divided between 4 tanks

(two control and two dosed). Stock solutions were prepared as stated in Section 2.1.2 to produce a tank concentration of 1 mg/l, but instead of a flow through system, a static renewal system was employed. The tanks were set up a few days prior to addition of the fish, to ensure equilibrium of the chemical within the tanks. After the fish were put in, the water in the tanks was replaced twice during the exposure period. Temperature and dissolved oxygen levels were maintained and monitored daily throughout the exposure period.

After the seven-day dosing period, all fish were anaesthetised and terminated as described in Section 2.1.3. General somatic parameters were measured and blood, liver and gonad samples were collected as described in Section 2.1.4, and stored for analysis of lipoprotein (blood) and VTG and ZP3 mRNA levels (gonad and liver tissue).

5.2.1 Measurement of Cholesterol within Lipoproteins

Serum samples (previously stored at 4°C and used within 3 days) were analysed using the SEBIA HDL/LDL CHOL Direct method, which was modified for use with fish as described in Chapter 2, Section 2.2.2. Each gel contained six samples and one standard, which was run on all gels (in this experiment plasma from fish 25 was used as the standard). The resulting gels were scanned using an Alpha Imager 1220 and analysis system V5.5; an example is shown in Figure 5.1. Densitometry provided a way to quantify the area under the curve (Figure 5.2), and these numbers could then be analysed statistically.

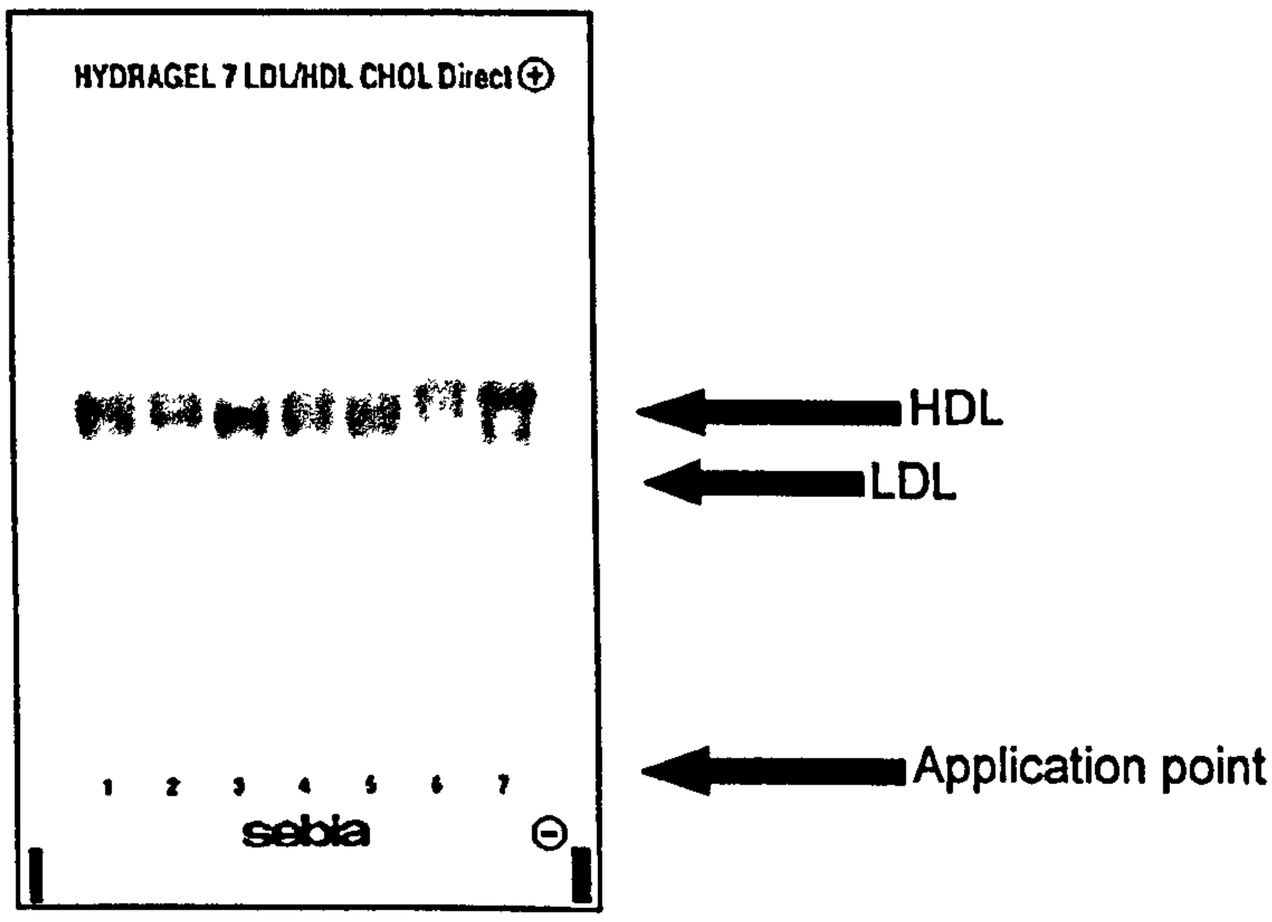
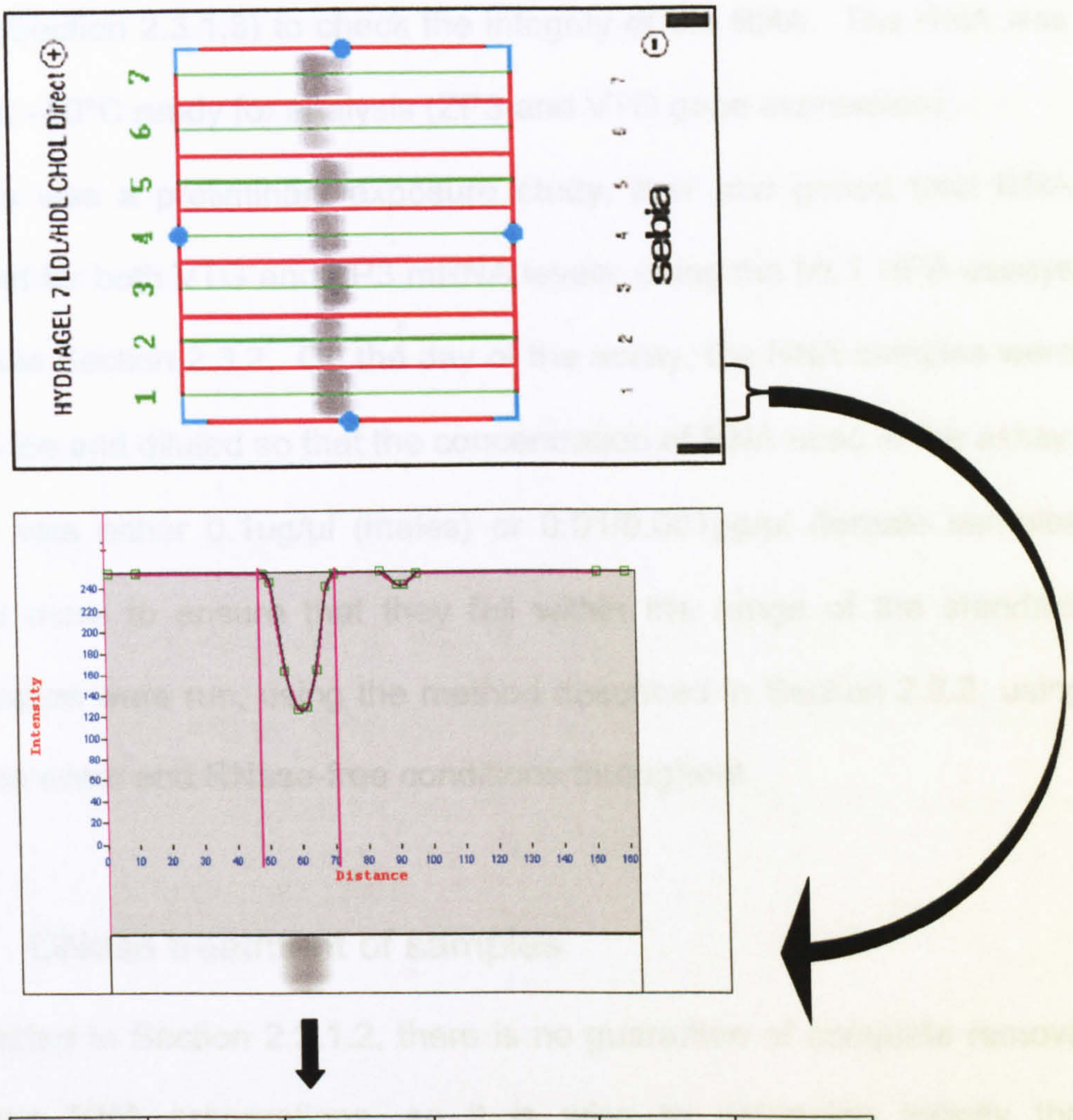


Figure 5.1: An example of the results obtained by using a SEBIA gel to separate and identify plasma lipoproteins. Six samples (lanes 1 to 6) and one standard – lane 7 in this case were run on each gel.



PEAK	DIST	WIDTH	HEIGHT	AREA	%
1	61	24	4	173	91.1
2	90	12	2	17	8.9

Figure 5.2: Calculation of band intensity using densitometry. The top panel shows a Sebia gel used to quantify cholesterol within lipoproteins. Using the Alpha Imager software, a grid is overlaid and densitometry is measured within this grid. The track for sample 1, with the corresponding densitometry analysis, is shown in the middle panel. Band intensity is calculated by measuring the area under the curve, and this is shown in the lower panel.

5.2.2 Molecular protocols

Total RNA was extracted from liver and gonad tissue using the method previously described in Section 2.3.1. After extraction, the precipitated RNA was re-suspended in ~200µl milliQ, double-autoclaved water (amount of water was dependant on the expected yield of RNA) and quantified spectrophotometrically using the Genequant (Section 2.3.1.1). A few micro-

litres of the total RNA from each extraction was then visually inspected on an agarose gel (Section 2.3.1.3) to check the integrity of the RNA. The RNA was then stored at –80°C ready for analysis (ZP3 and VTG gene expression).

As this was a preliminary exposure study, liver and gonad total RNA were analysed for both VTG and ZP3 mRNA levels, using the MLT HPA assays as described in Section 2.3.2. On the day of the assay, the RNA samples were defrosted on ice and diluted so that the concentration of RNA used in the assay, per sample, was either 0.1ug/µl (males) or 0.01/0.001µg/µl (female samples were diluted more to ensure that they fell within the range of the standard curve). Samples were run, using the method described in Section 2.3.2, using nuclease-free water and RNase-free conditions throughout.

5.2.2.1 DNase treatment of samples

As stated in Section 2.3.1.2, there is no guarantee of complete removal of DNA from RNA preparations, so it is wise to determine initially that contaminating genomic DNA is not present in the purified RNA. Some methods are more sensitive to DNA contamination than others, and so in this experiment I carried out DNase I treatment to determine if there was a need for this step prior to conducting the HPA assays. 5 random control samples were chosen to be treated with DNase I. A DNase digestion reaction was set up using a 120µl reaction volume containing:

- 10µg (100µl) RNA sample
- 8µl DNase I reaction buffer (200µM Tris-HCL (pH 8.4), 20mM $MgCl_2$, 500mM KCL)
- 12µl Buffer (20mM sodium acetate (pH 6.5), 5mM $CaCl_2$, 0.1mM PMSF, 50% (v/v) glycerol)

The reaction mixture was incubated for 15 minutes at room temperature. After incubation, the reaction volume was increased by the addition of 12µl EDTA. Samples were then heated for 10 minutes at 65°C to heat-inactivate the enzyme. These 5 samples were then run in the VTG HPA, along side untreated samples, to determine if there was any negative effect of contaminating genomic DNA in the assay.

5.2.3 Statistical analyses

Differences between 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 tests failed, non-parametric statistical tests were used.

5.2.4 Results

5.2.4.1 Morphometric data

There were no significant differences when comparing mean length or weight of control and dosed fish (Table 5.1). Female fish had mean weights of 3.6±0.2 and 3.8±0.2g and mean lengths of 64.5±0.9 and 64.4±0.9mm in the dosed and control groups, respectively. Male fish mean weights were 13.5±3.9 and 9.3±1.5g, for control and dosed fish respectively, and the mean length of both groups of fish was 87.0mm. The mean HSI for female fish was 2.4±0.3 and 2.8±0.3 and the males 2.8±0.7 and 1.6±0.2 in control and exposed fish respectively. The mean GSIs for females were 11.9±1.2 and 9.9±1.3 and males 1.6±0.1 and 1.2±0.4.

Table 5.1: Weight, length, HSI and GSI of fish within each treatment group (control and 1mg/l Clofibric acid) at termination of the experiment (day 7). Data are expressed as means and SEM.

	Sex	N numbers	Weight (g)	Length (mm)	HSI	GSI
Control	**F**	**11**	**3.8±0.2**	**64.4±0.9**	**2.4±0.3**	**11.9±1.2**
CA - exposed	**F**	**10**	**3.6±0.2**	**64.5±0.9**	**2.8±0.3**	**9.9±1.3**
Control	**M**	**2**	**13.5±3.9**	**87.0±8.5**	**2.8±0.7**	**1.6±0.1**
CA - exposed	**M**	**2**	**9.3±1.5**	**87.0±4**	**1.6±0.2**	**1.2±0.4**

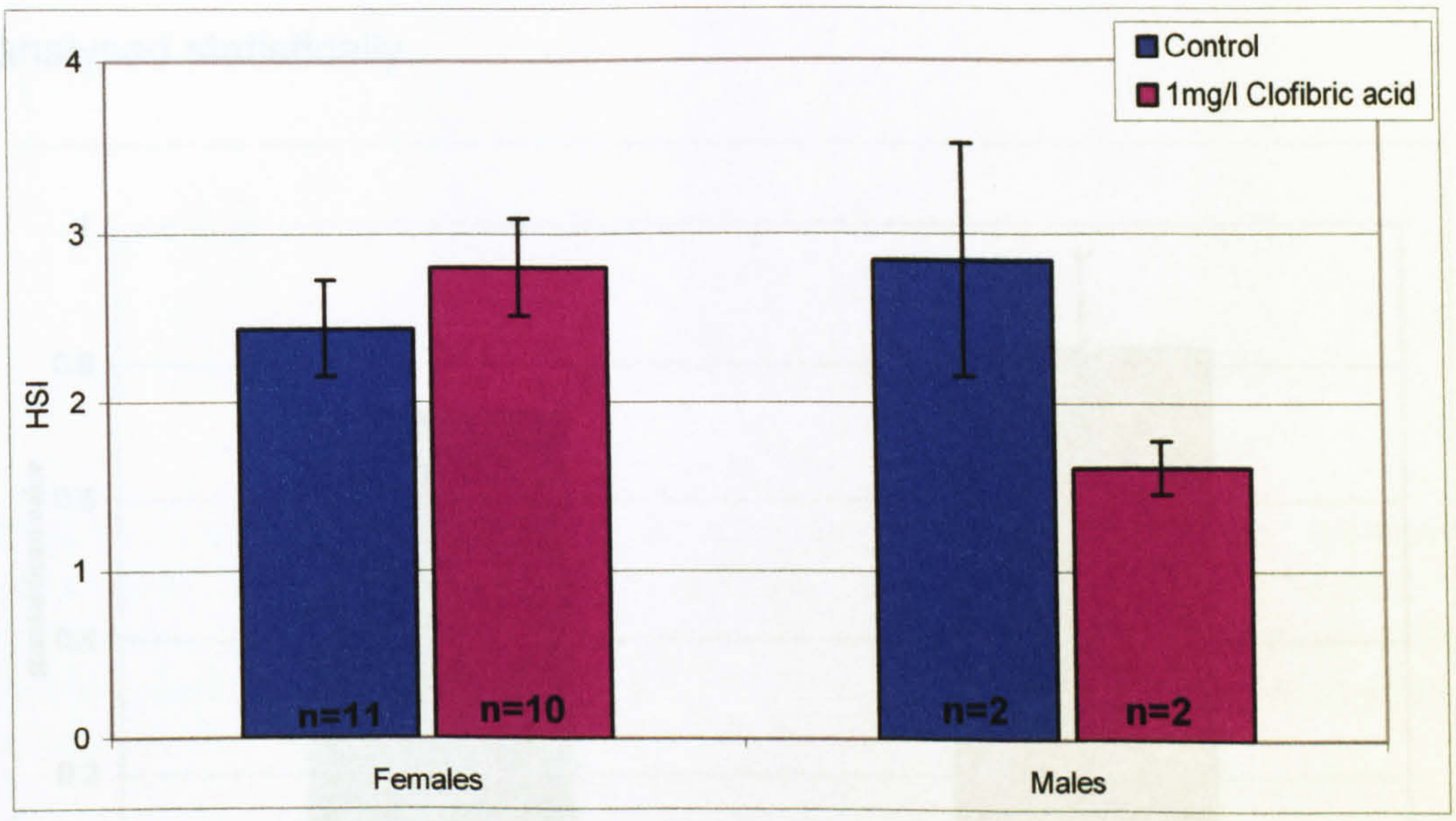


Figure 5.3: The HSI of adult fathead minnows exposed to a nominal concentration of 1mg/l Clofibric acid in Experiment 2. Data are expressed as mean ± SEM. There were no significant effects of Clofibric acid.

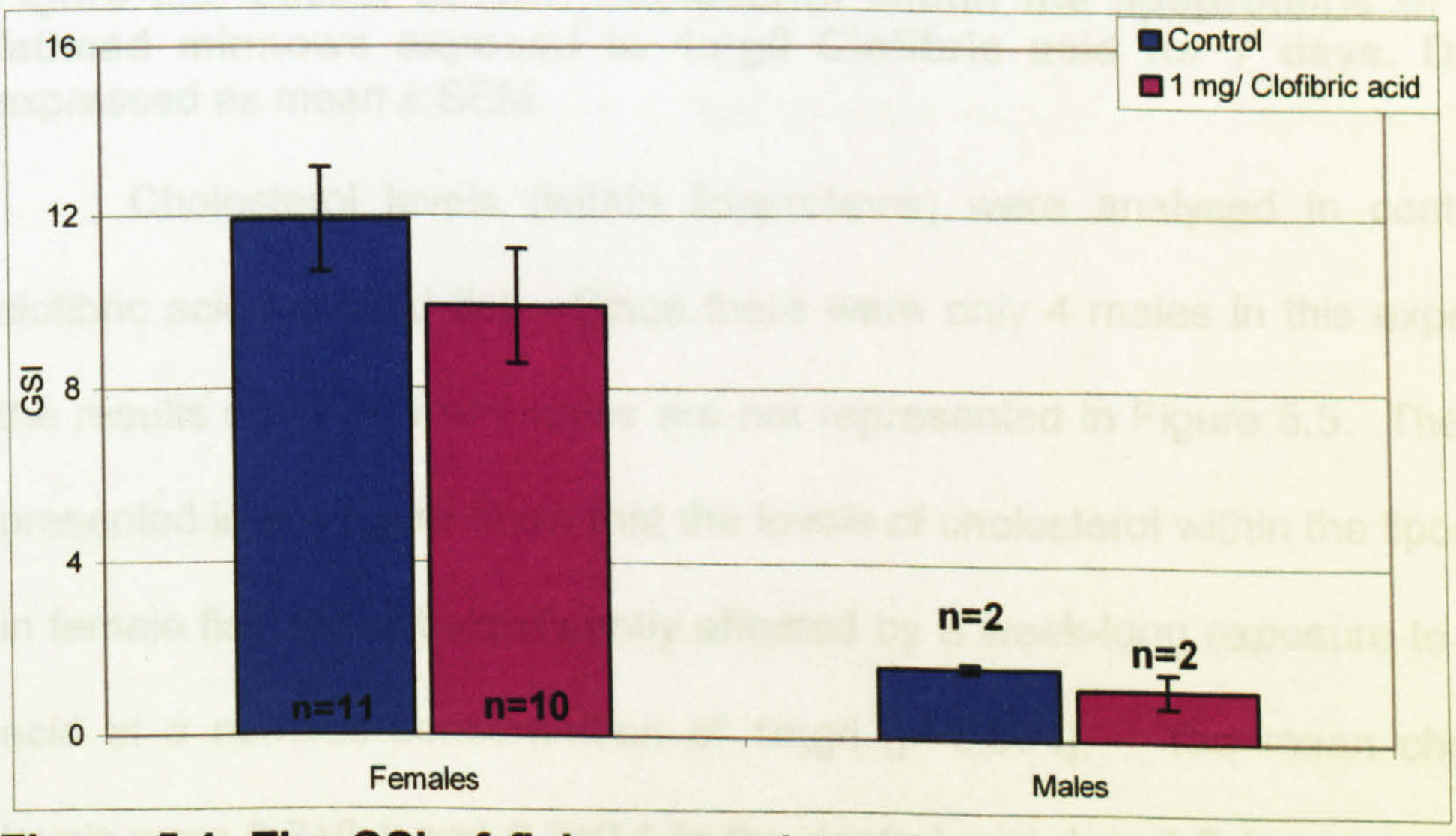


Figure 5.4: The GSI of fish exposed to a nominal concentration of 1mg/l Clofibric acid in Experiment 2. Data are expressed as mean ± SEM. There were no significant effects of clofibric acid.

The results for the HSI and GSI (Figures 5.3 and 5.4) were both found to be statistically non-significant (p=0.38 and p=0.28, respectively) when comparing control and dosed female fish. The results, however, do indicate possible trends - HSI is increased and GSI decreased in response to exposure to clofibric acid. Conclusions could not be made from the results from the male fish, as the 'n' number (n=2) was too low and the data could therefore not be analysed statistically.

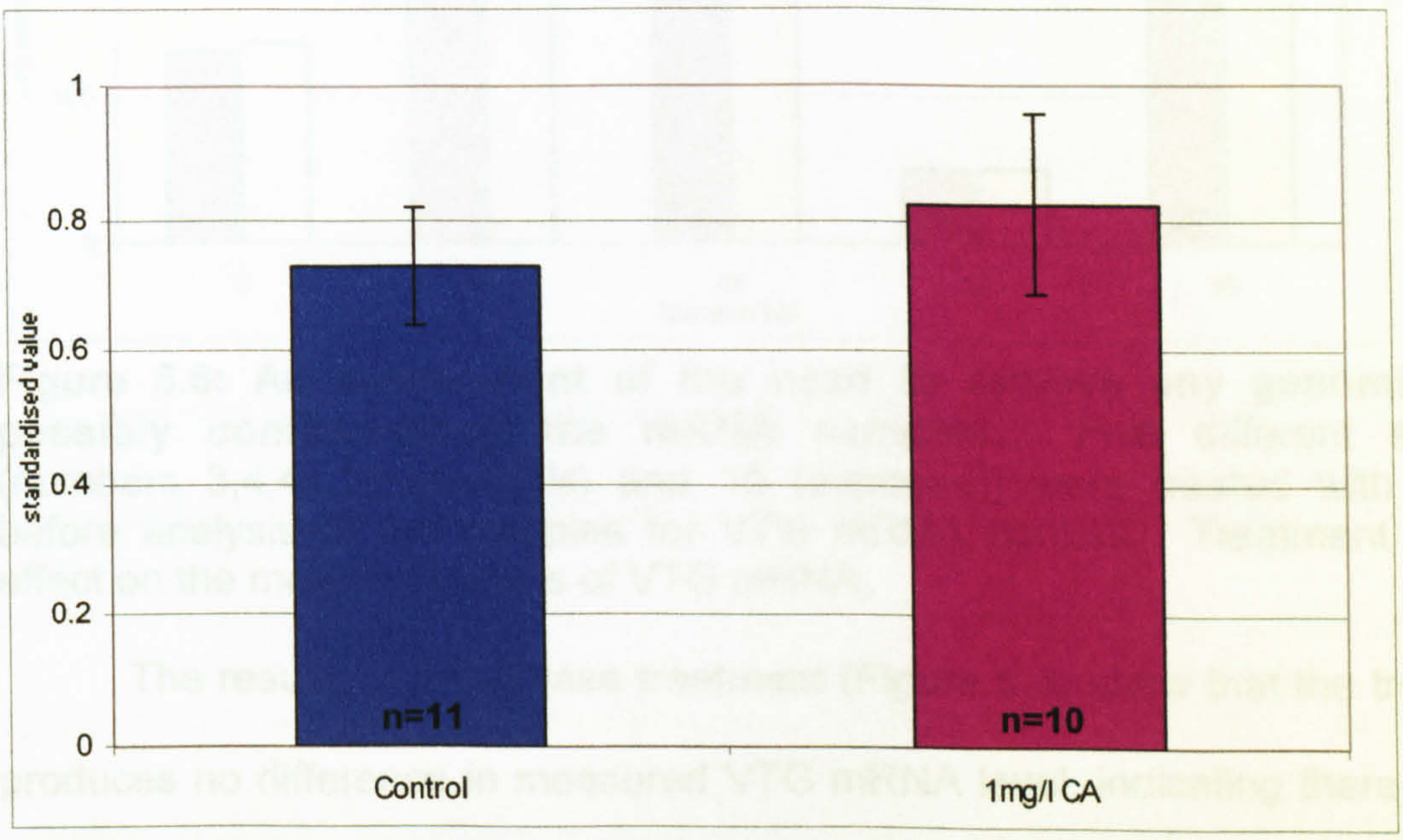


Figure 5.5: Levels of HDL Cholesterol within the lipoproteins of female fathead minnows exposed to 1mg/l Clofibric acid for 7 days. Data are expressed as mean ± SEM.

Cholesterol levels (within lipoproteins) were analysed in control and clofibric acid-exposed fish. Since there were only 4 males in this experiment, the results obtained from males are not represented in Figure 5.5. The results presented in this figure show that the levels of cholesterol within the lipoproteins in female fish are not significantly affected by a week-long exposure to clofibric acid at a nominal concentration of 1mg/l (p=0.504). The mean cholesterol levels were 0.7±0.1 and 0.8±0.1 in the control and dosed fish, respectively. In the males the levels were 0.98±0.18 and 0.77±0.23 in the control and dosed fish, respectively (n=2 in each case).

5.2.4.2 Molecular endpoints

Assessment of the need to remove any genomic DNA contaminating the mRNA samples

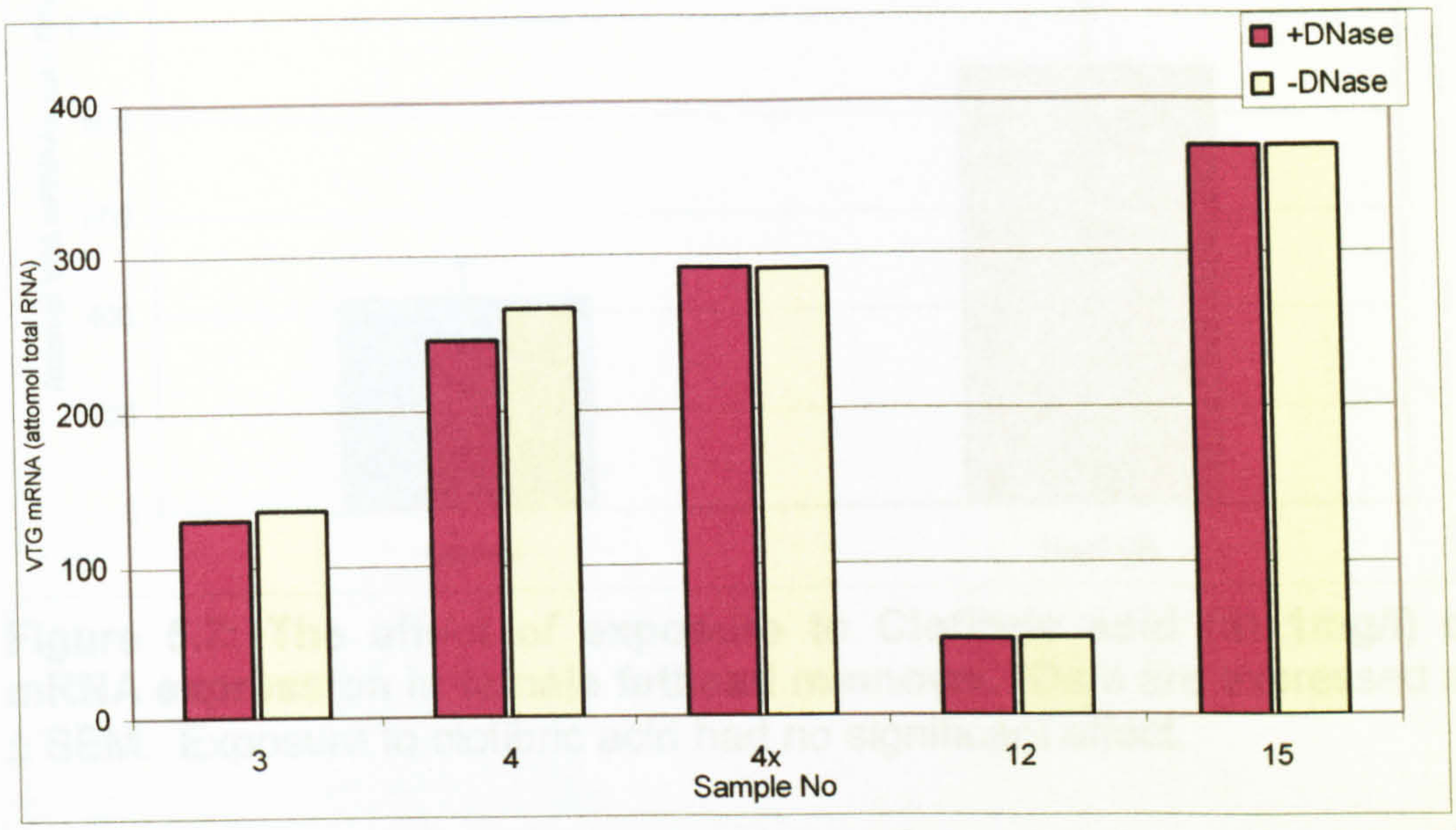


Figure 5.6: An assessment of the need to remove any genomic DNA possibly contaminating the mRNA samples. Five different samples (numbers 3,4,4x,12 (controls) and 15 (exposed)) were treated with DNase before analysis of the samples for VTG mRNA content. Treatment had no effect on the measured levels of VTG mRNA.

The results of the DNase treatment (Figure 5.6) show that the treatment produces no difference in measured VTG mRNA level, indicating there was no detectable DNA contamination of the RNA preparations. Consequently, DNase treatment was not carried out in subsequent exposures. Additionally, these results highlight the reproducibility of this assay: replicates of each sample produced very similar mRNA levels.

VTG mRNA levels

The VTG mRNA HPA assay was carried out on both liver and gonad samples from this experiment, using the method described in Section 2.3.2. It was confirmed that, as predicted, VTG mRNA is synthesised in the livers of female fish, but it is not produced in the gonad of female fish and is very low (close to the detectable levels) in male fish (data not shown).

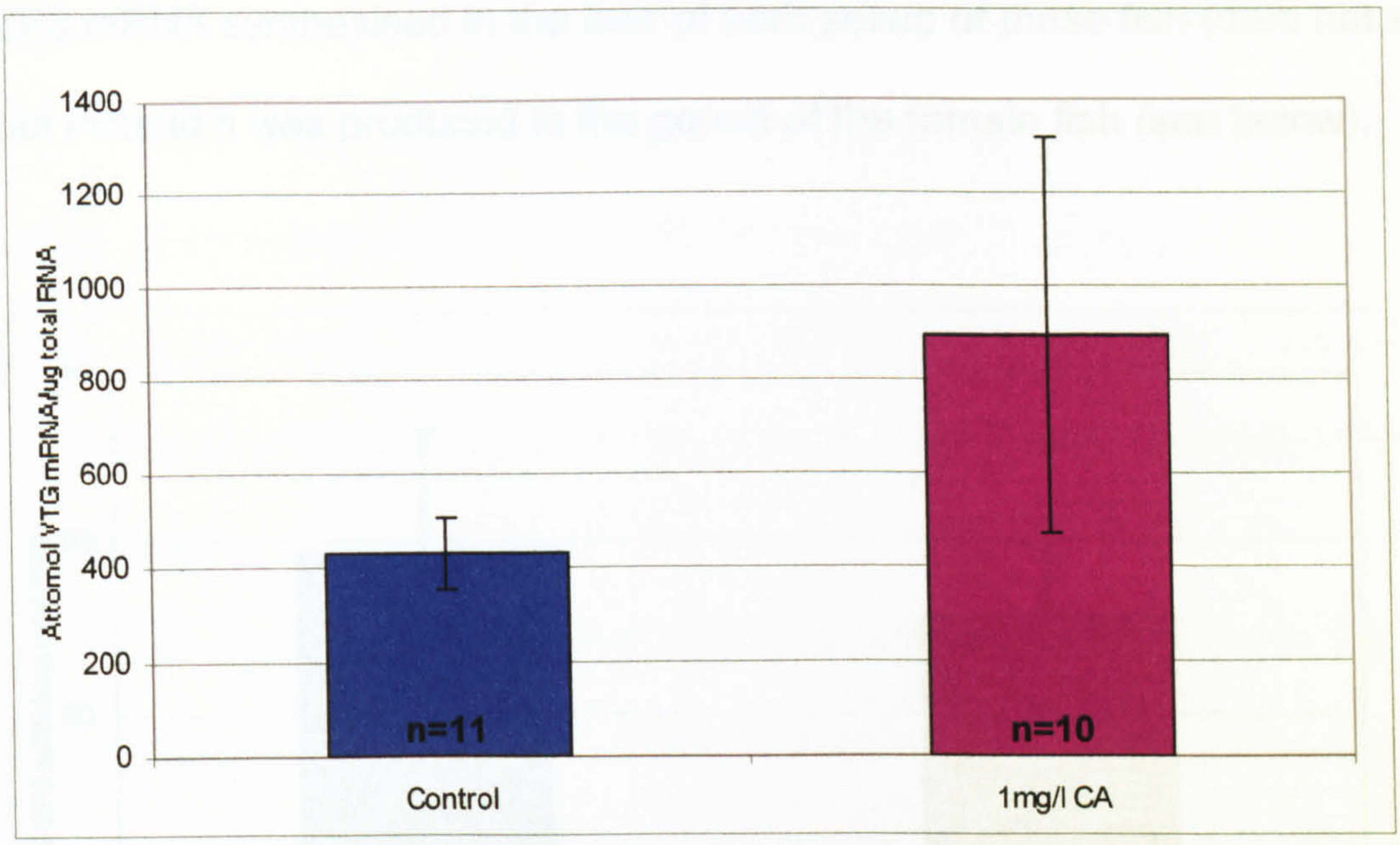


Figure 5.7: The effect of exposure to Clofibric acid (at 1mg/l) on VTG mRNA expression in female fathead minnows. Data are expressed as mean ± SEM. Exposure to clofibric acid had no significant effect.

Figure 5.7 shows the results of the HPA assay (Section 2.3.2) carried out on livers from control and exposed female fish. The mean levels of VTG mRNA were 431.8±76.4 attomol VTG mRNA/μg total RNA, in the control fish and 895.7±423.1 attomol VTG mRNA/μg total RNA in the dosed fish. These results suggest a slight increase in VTG mRNA due to exposure to clofibric acid, although this was not significant when analysed statistically ($p=0.6$). The levels in the males were very low (control levels were 0.0 and 0.6 and dosed were 0.0 and 13 attomol VTG mRNA/μg total RNA), and as there were only 4 males used in the study, the data are not shown in this figure and are not considered further.

ZP3 mRNA levels

Using the method previously described in Section 2.3.2, the ZP3 mRNA HPA assay was used to measure ZP3 mRNA levels in both liver and gonad samples collected from this experiment. It was concluded that there was no

ZP3 mRNA synthesised in the liver of both sexes of these fish (data not shown), but instead it was produced in the gonad of the female fish (see below).

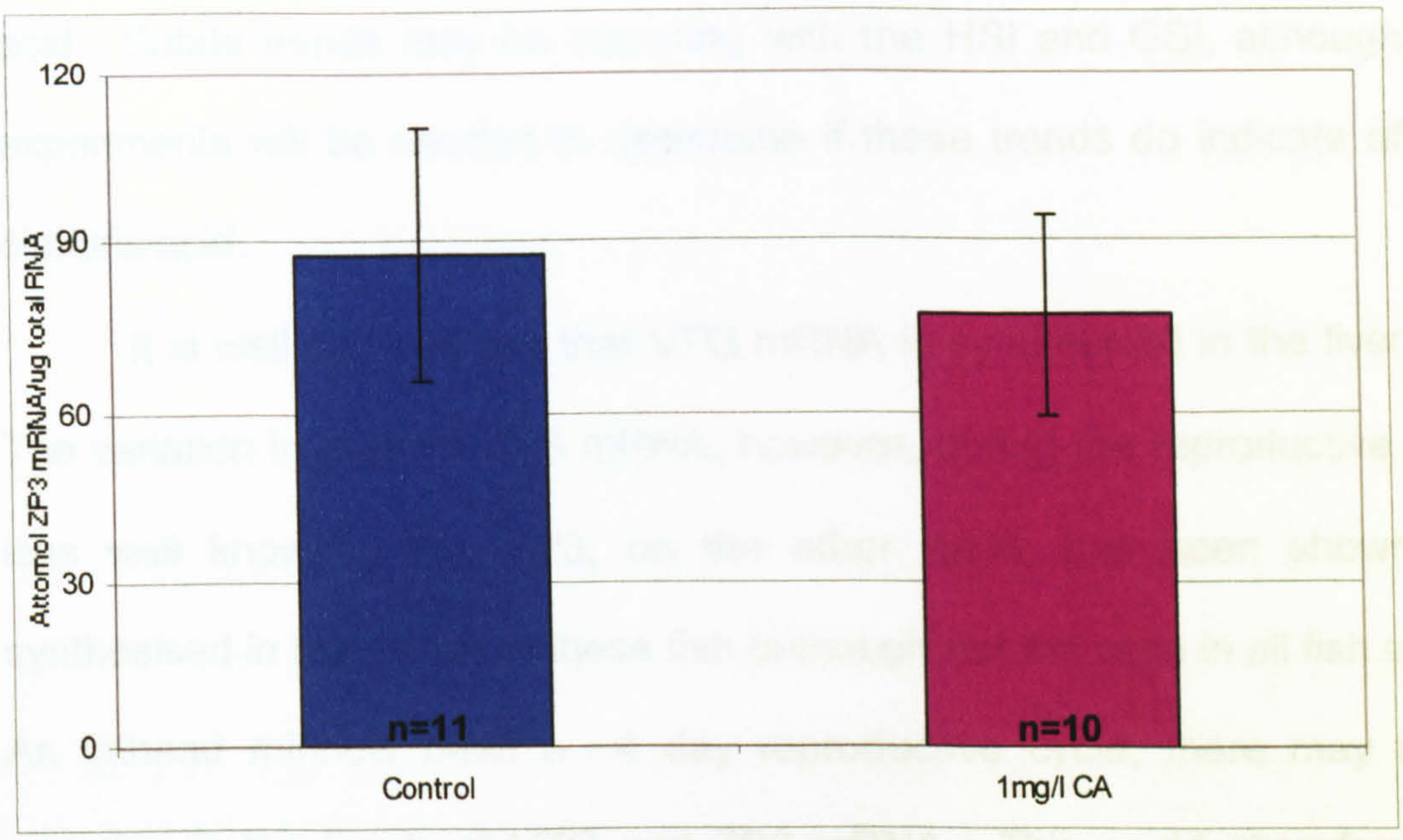


Figure 5.8: The effect of exposure to Clofibric acid (at 1mg/l) on ZP3 mRNA expression in female fathead minnows. Data are expressed as mean ± SEM. Exposure to clofibric acid had no significant effect.

Figure 5.8 shows the results of the ZP3 HPA assay (Section 2.3.2) carried out on gonads from control and exposed female fish. The mean levels were 87.79±22.3 attomol ZP3 mRNA/μg total RNA in the control fish and 76.9±17.5 attomol ZP3 mRNA/μg total RNA in the dosed fish, and were not significantly different when analysed statistically ($p=0.7$). The male fish levels were found to be 0 ($n=2$ in control and dosed fish), and were therefore not considered further.

As can be seen, there was considerable variability with the levels of ZP3 and VTG mRNA in females, even in the control fish. This could be due to the levels varying during the spawning cycle.

5.2.5 Summary of the results from adult Experiment 2

There were no obvious effects of clofibric acid on either weight, length or levels of cholesterol within the HDL of fish after 7 days exposure to clofibric acid. Subtle trends may be occurring with the HSI and GSI, although further experiments will be needed to determine if these trends do indicate effects of clofibric acid.

It is well documented that VTG mRNA is synthesised in the liver of fish. The variation in level of VTG mRNA, however, during the reproductive cycle is less well known. The ZP3, on the other hand, has been shown to be synthesised in the gonad of these fish (although not the case in all fish species). As fathead minnow have a ~4 day reproductive cycle, there may be daily variation in the levels of VTG and ZP3 mRNA. This could, therefore, be an uncontrollable variable within the data. Subtle effects of clofibric acid may well be masked by these reproductive fluctuations in levels of VTG and ZP3 mRNA. Greater numbers of fish used in the experiment would be needed to overcome this problem.

As no known previous studies have been carried out on the effects of clofibric acid on fish, it is hard to determine if what I observed was a 'normal' result. In the absence of statistical significance, no real conclusions can be drawn from the results of this exposure. This may be in part due to the short length of the exposure, the static nature of the system, and/or the low numbers of fish used. It may well be, however, that clofibric acid does not affect the parameters I investigated. Trends may, however, be visible, and consequently the following experiments build on the results obtained from this preliminary study.

5.3 Adult Experiment 3

Two subsequent studies were carried out, with clofibric acid present at a nominal concentration of 1 mg/l for periods of three weeks each, and then a further study, using two concentrations of clofibric acid (10µg/l and 1mg/l), was carried out, again for a three week period (Sections 5.3, 5.4 and 5.5 cover the results from these three experiments).

A 21-day flow-through exposure study (Experiment 3) was carried out as described in Section 2.1, using 79 fathead minnows - five control tanks containing a total of 23 females and 21 males and five dosed tanks containing 1mg/l clofibric acid and a total of 16 females and 19 males. Stock solution of the drug (0.83g/l clofibric acid added to double distilled water in 4 litre amber Winchester bottles) was dosed into the tanks as described previously in Section 2.1.2, to obtain a nominal concentration of 1mg/litre. The clofibric acid was dosed continuously for the 21 days throughout the experiment as well as for a week prior to this, in an attempt to ensure chemical equilibrium within the tanks before the fish were added.

At termination of the exposure, all fish were anaesthetised as described in Section 2.1.3. Basic parameters (length, weight, GSI and HSI) were taken and liver and gonad were carefully collected as described in Section 2.1.4, ready for analysis of VTG and ZP3 mRNA levels, respectively. Blood was also collected from this experiment, for lipoprotein analysis. However, technical problems occurred, and no reliable lipoprotein data were obtained, and hence results are not shown.

As with the previous experiment, all data were analysed statistically using SigmaStat (Section 2.4.2). In cases of normality, a t-test was used, and in cases where normality failed, specified non-parametric tests were used.

Total RNA was extracted using the method previously described in Chapter 2, Section 2.3. As before, precipitated RNA was re-suspended in ~200µl milliQ, double autoclaved water (amount of water was dependant on expected yield of RNA) and quantified using the Genequant (2.3.1.1). It was visually inspected for integrity on agarose gel (Section 2.3.1.3) and stored at –80°C ready for analysis using HPA kits supplied by MLT (Cardiff). For this particular experiment, liver samples were used for the VTG mRNA analysis and gonads for the ZP3 mRNA analysis,.

5.3.1 Results

5.3.1.1 Morphometric analysis

Table 5.2: Weight, length, HSI and GSI of fish within each treatment group (control and 1mg/l Clofibric acid) from adult Experiment 3. Data are expressed as means ± SEM.

	N numbers	Sex	Weight (g)	Length (mm)	HSI	GSI
Control	23	F	1.47±0.07	48.48±0.77	3.88±0.39	5.30±0.90
CA - exposed	21	F	1.53±0.08	48.05±0.87	4.31±0.42	8.94±1.18
Control	16	M	2.71±0.13	59.31±0.74	2.80±0.30	0.83±0.17
CA - exposed	19	M	2.49±0.11	57.16±0.80	2.76±0.34	1.78±0.22

Female fish weighed a mean of 1.47±0.07 and 1.53±0.08g and were between 48.48±0.77 and 48.05±0.87mm in length in the control and dosed groups, respectively. Male fish had mean weights of 2.71±0.13 and 2.49±0.11g and their mean lengths were 59.31±0.74 and 57.16±0.80mm in the control and dosed groups. As with Experiment 2, there were no significant differences in either length or weight between control and dosed female fish. It seems however that the clofibric acid exposed male fish were slightly smaller than the control males. They weighed less and were slightly shorter, although in both cases these differences (compared to the control fish) were not significant.

The fish used in Experiment 3 and all subsequent experiments were from a younger batch of fish (however, still all sexually mature) and consequently weighed very much less that those used in the previous experiment - the males were less than 20% the weight of previous fish, for example.

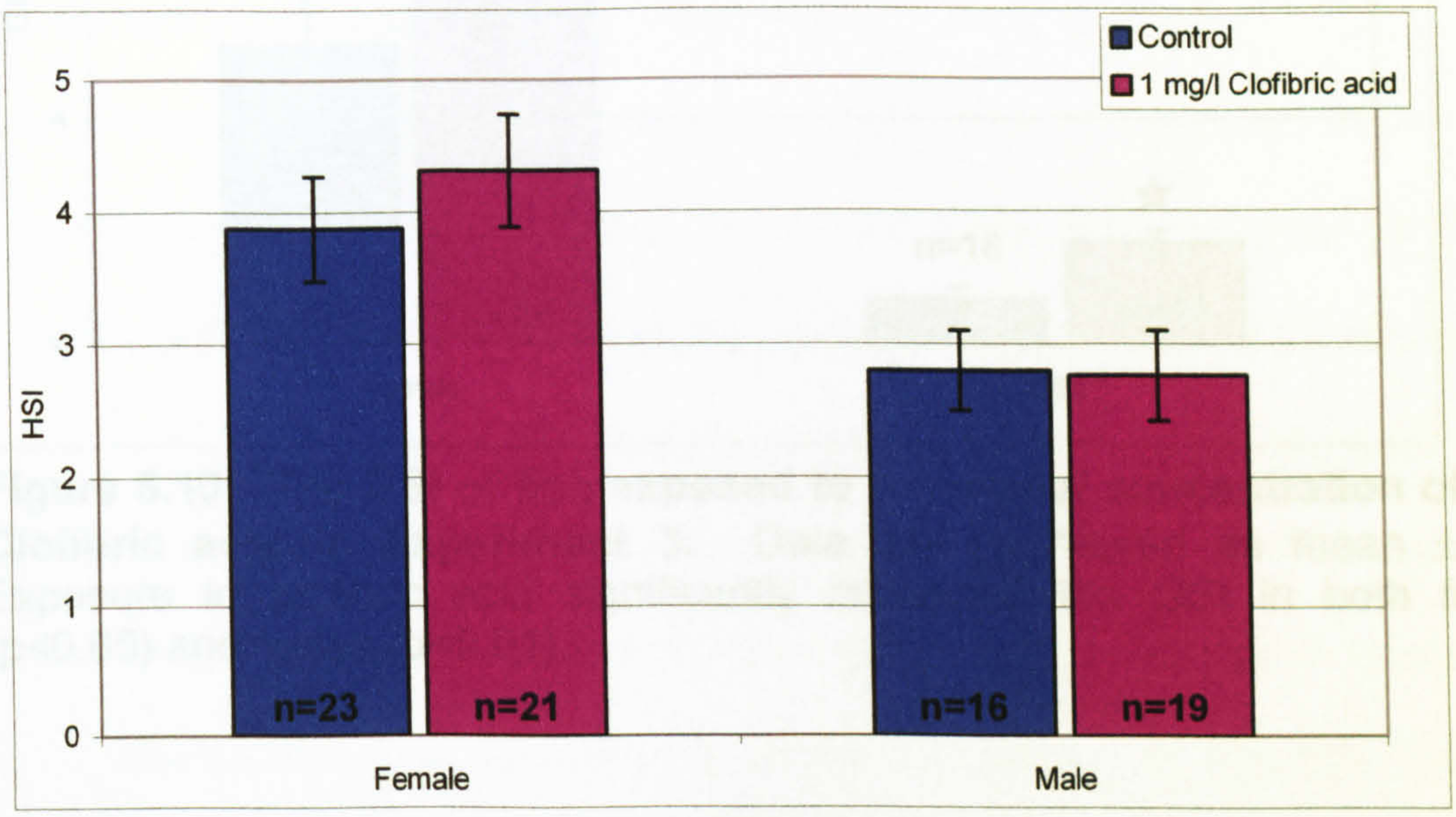


Figure 5.9: The HSI of adult fathead minnows exposed to a nominal concentration of 1mg/l Clofibric acid in Experiment 3. Data are expressed as mean ± SEM.

The mean HSI for control and dosed female fish was 3.8±0.39 and 4.3±0.42, and for males in both cases it was 2.8. The mean GSI for females (control and dosed) was 5.30±0.9 and 8.94±1.18, and for males 0.83±0.17 and 1.78±0.22. It can be seen (Figure 5.9) that the HSI in females was slightly higher in the dosed fish, although this difference was not significantly statistically (p=0.4), and in the males it remained the same irrespective of treatment (p=0.9). This result agrees with the trend observed in the previous experiment, even though the HSI measurements were slightly lower.

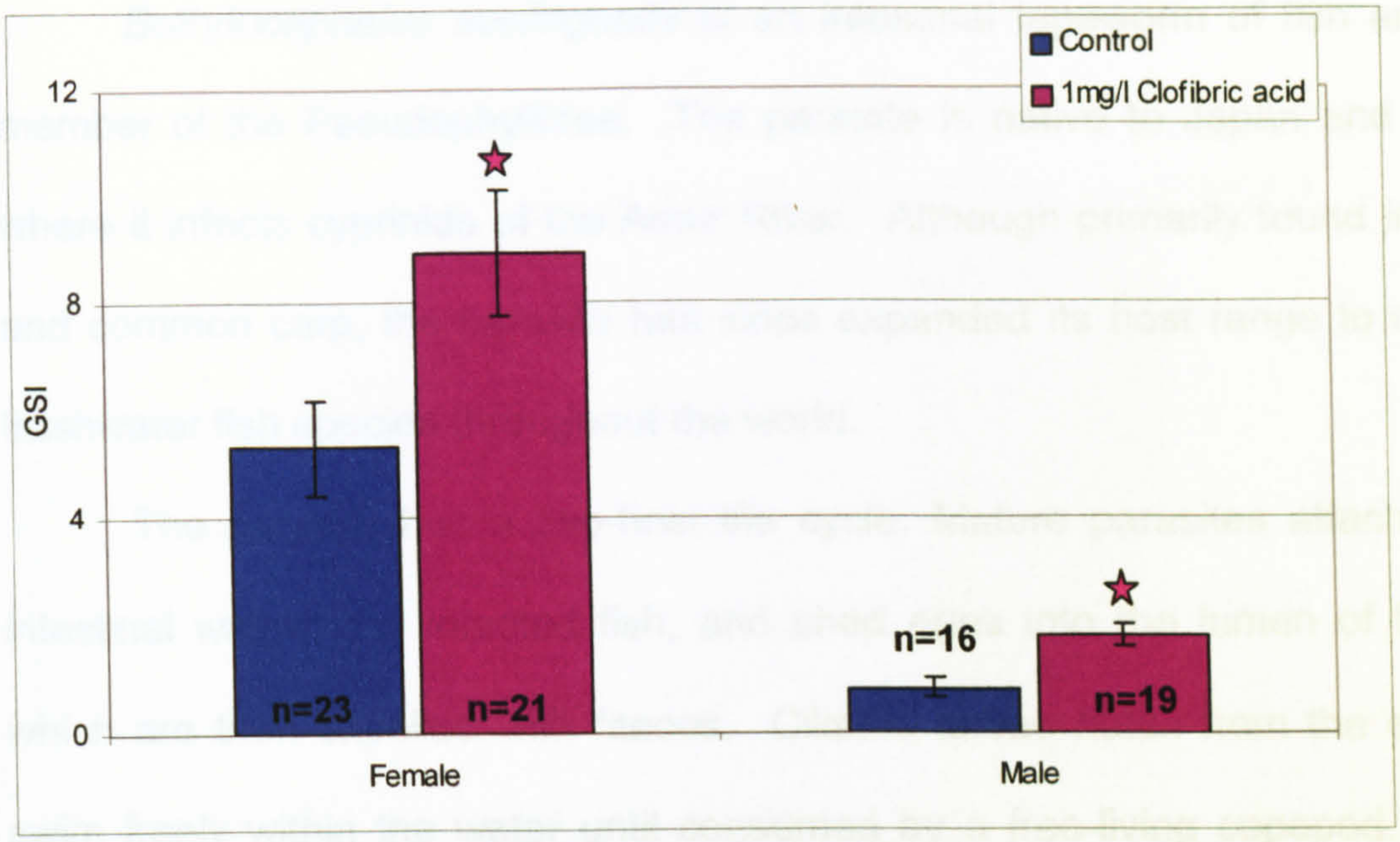


Figure 5.10: The GSI of fish exposed to a nominal concentration of 1mg/l Clofibric acid in Experiment 3. Data are expressed as mean ± SEM. Exposure to clofibric acid significantly increased the GSI in both females ($p<0.05$) and males ($p<0.01$).

The GSI results, however, do not agree with those of the previous study. Both the female and male dosed fish had GSIs which were significantly higher (females $p=0.017$ and males $p=0.002$) compared to those of the control groups (Figure 5.10). However, during dissection it was found that there were worms present in many of these fish (see following section). Furthermore, these parasites were found in more of the control fish than the dosed, and may have been responsible for the lower mean GSI in the control groups.

5.3.1.2 The presence of worms

At termination of this experiment it was discovered that there were worms present in some of the fish. The worm was identified (by myself, and later confirmed by Chris Williams, Fish Health laboratory, Environment Agency) as the Asian Tape worm (*Bothriocephalus aceilognathi*), which was inadvertently imported with the fish from the USA.

Bothriocephalus aceilognathi is an intestinal tapeworm of fish and is a member of the Pseudophyllidea. The parasite is native to Japan and China, where it infects cyprinids of the Amur River. Although primarily found in grass and common carp, the parasite has since expanded its host range to over 90 freshwater fish species throughout the world.

The parasite has a two-host life cycle. Mature parasites attach to the intestinal wall of the infected fish, and shed eggs into the lumen of the gut, which are then expelled with faeces. Ciliated larvae hatch from the egg and swim freely within the water until consumed by a free-living copepod. Larval development takes place within this intermediary host. Fish serve as the definitive host, obtaining infection by the ingestion of the infected copepods.

Bothriocephalus aceilognathi cause blockages of the intestinal tract, intestinal perforation, destruction of the intestinal mucosa, reduced growth and even death of the host fish. Histopathological studies have shown that heavy parasite burdens can result in severe catarrhal-haemorrhagic enteritis, intestinal inflammation and considerable damage to gut structure and function. Physiological investigations have revealed disruption to the blood composition and digestive processes of infected hosts. When more than 4 worms are present in a single fish, they have been found to affect carbohydrate and protein metabolism and cause reduced nutritional status.

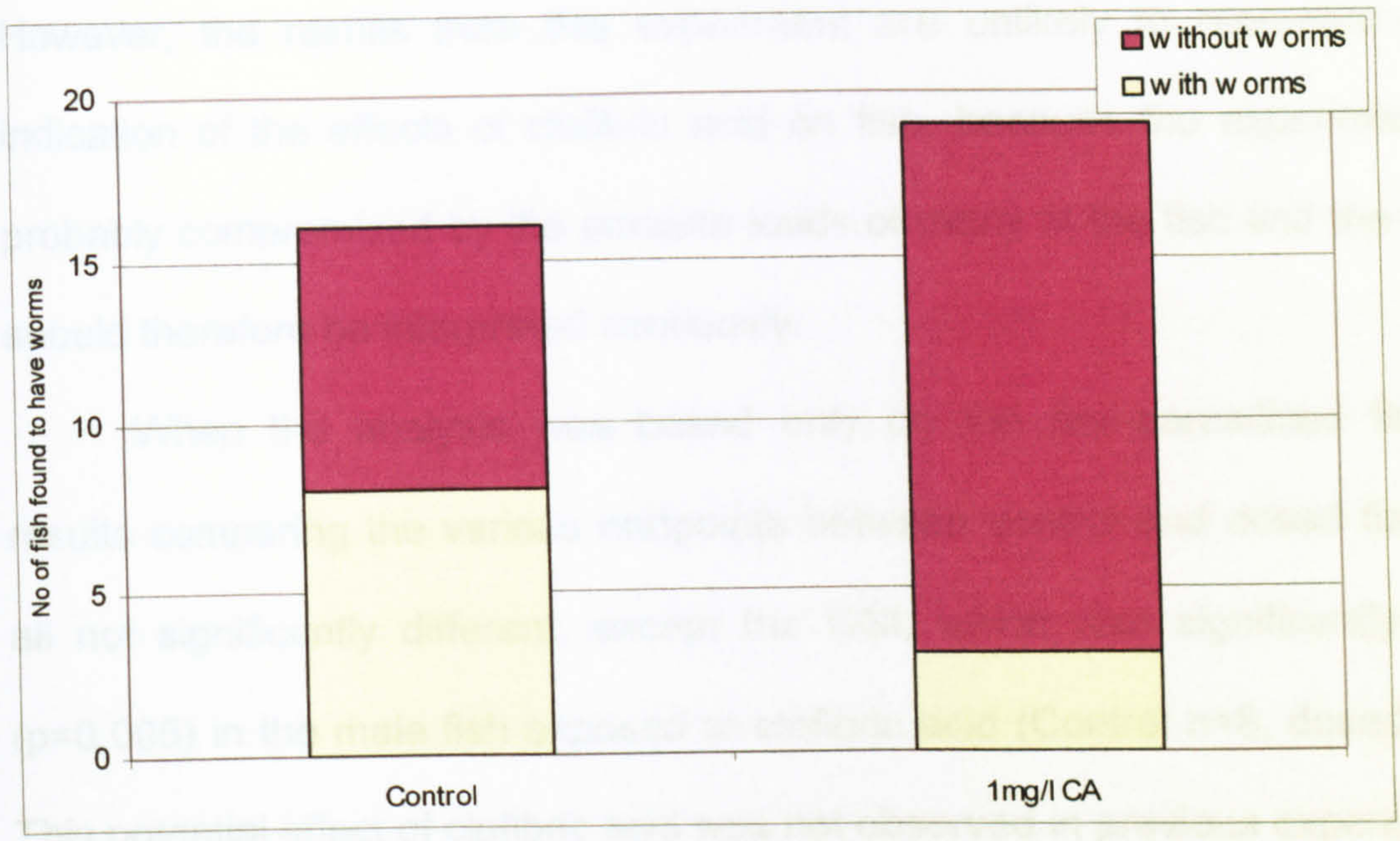


Figure 5.11: The proportion of fish containing worms in Experiment 3. As can be seen, approximately half of control fish (both sexes) contained worms, whereas only about 30% of the fish exposed to Clofibric acid did.

As can be seen in Figure 5.11, in this experiment there were 18 control fish with worms and 21 without, and in the dosed fish there were 32 without worms and 8 with worms. Worms were present in 10 control females and 5 dosed females, and in the males 8 of the control fish and 3 of the dosed fish were infected. Statistical analysis of data could therefore still be carried out on

the fish with no worms present (because there were still enough fish). However, the results from this experiment are unlikely to represent a true indication of the effects of clofibric acid on fish, because the experiment was probably compromised by the parasite loads of many of the fish and the results should therefore be interpreted cautiously.

When the analysis was based only on the non-parasitized fish, the results comparing the various endpoints between control and dosed fish were all not significantly different, except the GSI, which was significantly higher ($p=0.005$) in the male fish exposed to clofibric acid (Control n=8, dosed n=16). This potential effect of clofibric acid was not observed in previous experiments.

5.3.1.3 Molecular endpoints

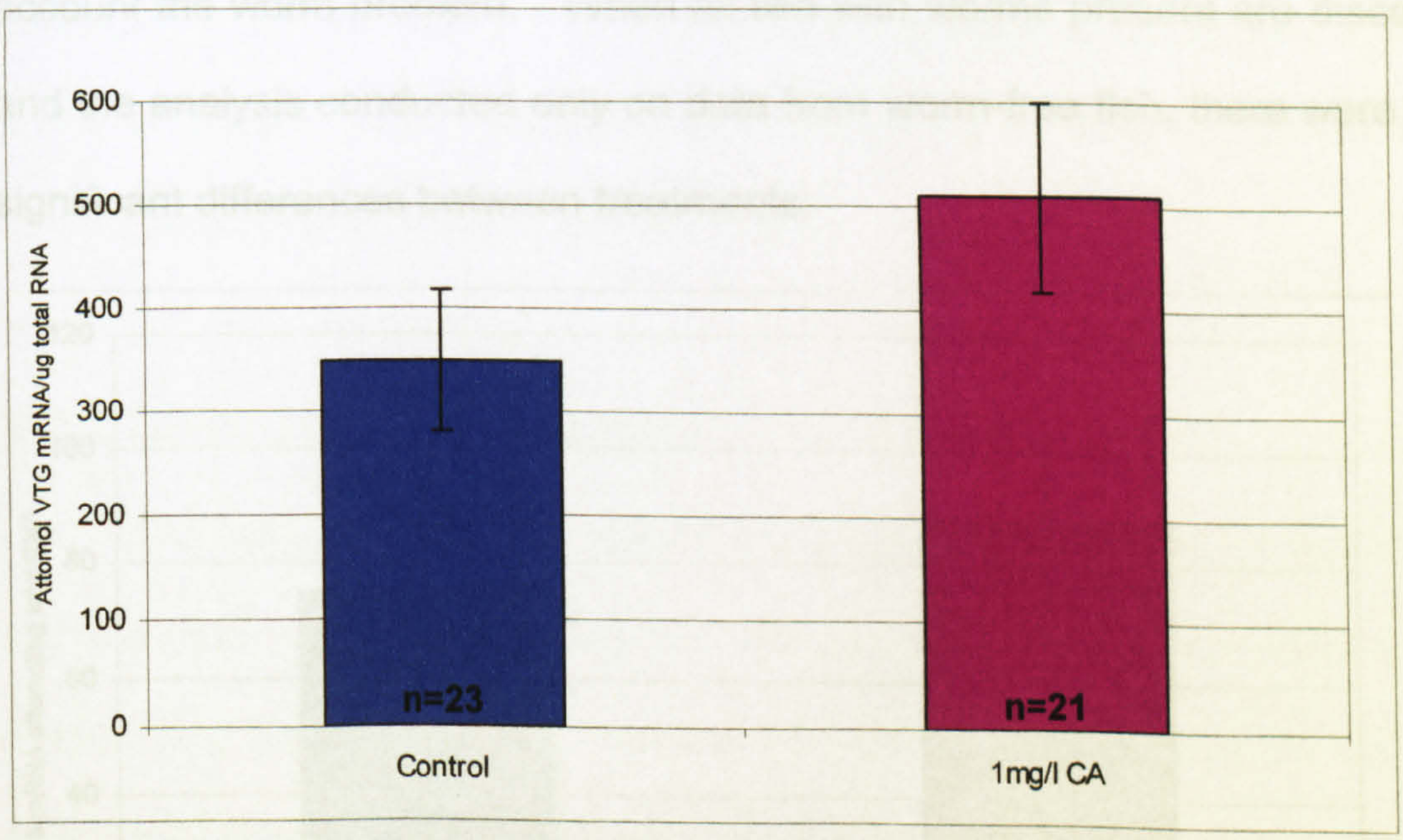


Figure 5.12: The effect of exposure to Clofibric acid (at 1mg/l) on the level of VTG mRNA in female fathead minnows from Experiment 3. Data are expressed as mean ± SEM. Exposure to clofibric acid had no significant effect.

Figure 5.12 shows the results from the HPA assay used to measure VTG mRNA levels in exposed and control female fish. Control female fish had a mean level of VTG mRNA of 348.4±69.3 attomol VTG/µg total RNA, whereas

dosed female fish had a mean level of 508.9±91.2. The males had a level in the controls at 0.2±0.12 and in the dosed 0.3±0.17 attomol VTG/µg total RNA (results not shown graphically).

The results from the females indicate that there was no significant difference (p=0.1) between the control and dosed groups, although as can been seen in Figure 5.12, there may be a trend. Data from male fish were also analysed and the levels of VTG mRNA found were, as expected, very low compared to the females, and when control and dosed male fish were compared their VTG mRNA levels were shown to be statistically not significantly different (p=0.1).

The VTG mRNA results from this experiment do show the same indications as the previous exposure. These results do not, however, take into account the worm problem. When all fish with worms present are discounted, and the analysis conducted only on data from worm-free fish, there were still no significant differences between treatments.

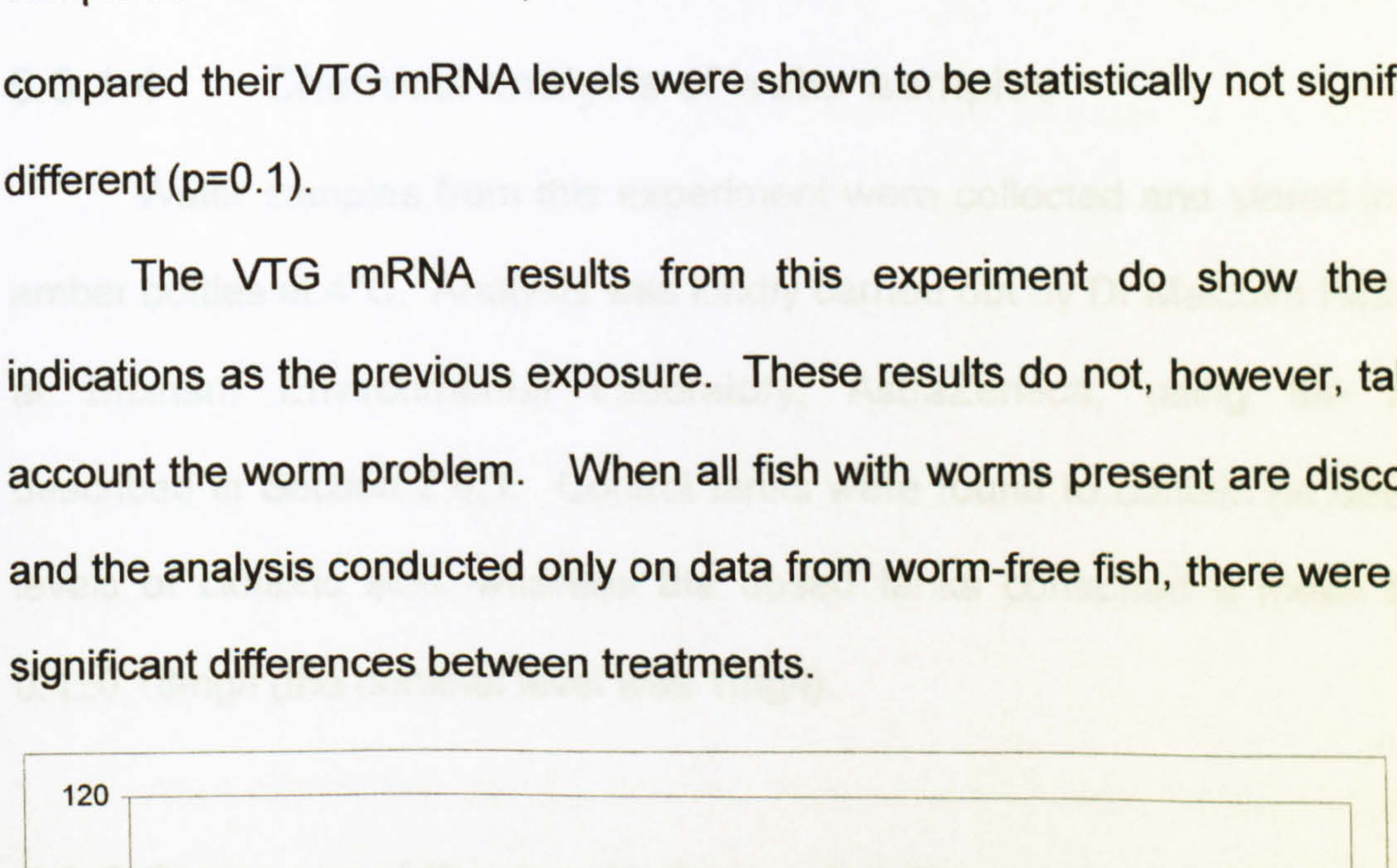


Figure 5.13: The effect of exposure to Clofibric acid (1mg/l) on ZP3 mRNA expression in female fish from Experiment 3. Data are expressed as mean ± SEM. There was no significant effect of clofibric acid.

The results of the ZP3 HPA assay show (Figure 5.13) the control females with a mean level of ZP3 at 74.9± 17.1 attomol ZP3/µg total RNA, and dosed females with 87.4±14.9 attomol ZP3/µg total RNA. Male fish were also analysed and the levels found were 0 in the control fish and 0.19±0.13 in the dosed fish (the results are not shown graphically). These results were tested for significance and none was found in either sex; p=0.5 for females and p=0.1 for males.

5.3.1.4 Chemical analysis of water samples

Water samples from this experiment were collected and stored in 250ml amber bottles at 4°C. Analysis was kindly carried out by Dr Malcolm Hetheridge at Brixham Environmental Laboratory, AstraZeneca, using the method described in Section 2.4.1. Control tanks were found to contain no detectable levels of clofibric acid, whereas the dosed tanks contained a mean level of 0.4±0.18mg/l (the nominal level was 1mg/l).

5.3.2 Summary of the results from adult Experiment 3

The results from this experiment again gave some indications that clofibric acid might be affecting the fish, although nothing concrete could be determined, as worms were present and the effect of these on the fish is unknown. It was noted, however, that the numbers of worms present in the exposed fish were significantly less than in the control fish (Figures 5.11), and probably as a consequence the mean GSI in the exposed fish was higher. The worm factor unfortunately added another unexpected variable, and so the experiment had to be repeated, taking this factor out.

5.4 Adult Experiment 4

A subsequent study was carried out using more fish, hopefully ones that did not have worms. A 21-day flow through exposure study was once more carried out, as a repeat of adult Experiment 3. A total of 130 fathead minnows (*Pimephales promelas*) were distributed within 5 control and 5 dosed tanks (1mg/l clofibric acid). The control tanks contained 57 female and 8 male fish and the dosed tanks contained 53 female and 12 male fish. Stock solutions were prepared as stated in Section 2.1.2, and so for the nominal tank concentration to be 1 mg/l, 0.83g/l clofibric acid was dissolved in double distilled water (3.332g in 4L) in amber stock bottles as previously specified. Dosing of the tanks was started a week before fish were placed in the tanks, to ensure equilibrium of the chemical within the tanks. In contrast to previous experiments, levels of total triglycerides and total cholesterol were analysed in all fish, and sperm counts of male fish were also determined, using the protocol described in Section 2.2.1.

After the dosing period, the fish were anaesthetised, length, weight, GSI and HSI were measured, and serum was collected for lipoprotein analysis, as previously described (Section 2.2.2), and stored at 4°C for a maximum of 3 days. Any serum available after lipoprotein analysis was frozen (-20°C) as soon as the lipoprotein assay was completed, so it could be used for total triglyceride and cholesterol analysis (see Section 2.2.3). Liver, ovary and testis (apart from the tip) were collected using RNase-free conditions (Section 2.1.4) and snap-frozen ready for VTG and ZP3 mRNA analysis. The tip of the one of the testis was kept for the sperm count (Section 5.4.4.1), which was determined as follows:

The tip of one testis from each male fish was collected, weighed and placed in 100µl catfish extender solution (Section 2.2.1) and stored at 4°C until analysed (within 1 week of collection). The testis tip was then homogenised thoroughly using a clean pipette tip for each sample. 1µl of this homogenate was then added to 90µl of trypan blue (filtered) and left in a 'damp box' for 10 minutes. Counting of mature sperm was carried as described in Section 2.2.1. These results were then analysed (taking into account the actual weight of the tip of the gonad used) and the number of sperm per mg gonad was calculated. Statistical comparisons were then carried out to determine if there was an effect of clofibric acid on sperm count in male fathead minnows.

5.4.1 Measurement of Cholesterol within lipoproteins

Serum samples were again analysed using the SEBIA HDL/LDL CHOL Direct method (Section 2.2.2). Each gel contained six samples and one standard, which in this experiment was human plasma (see Figure 5.14).

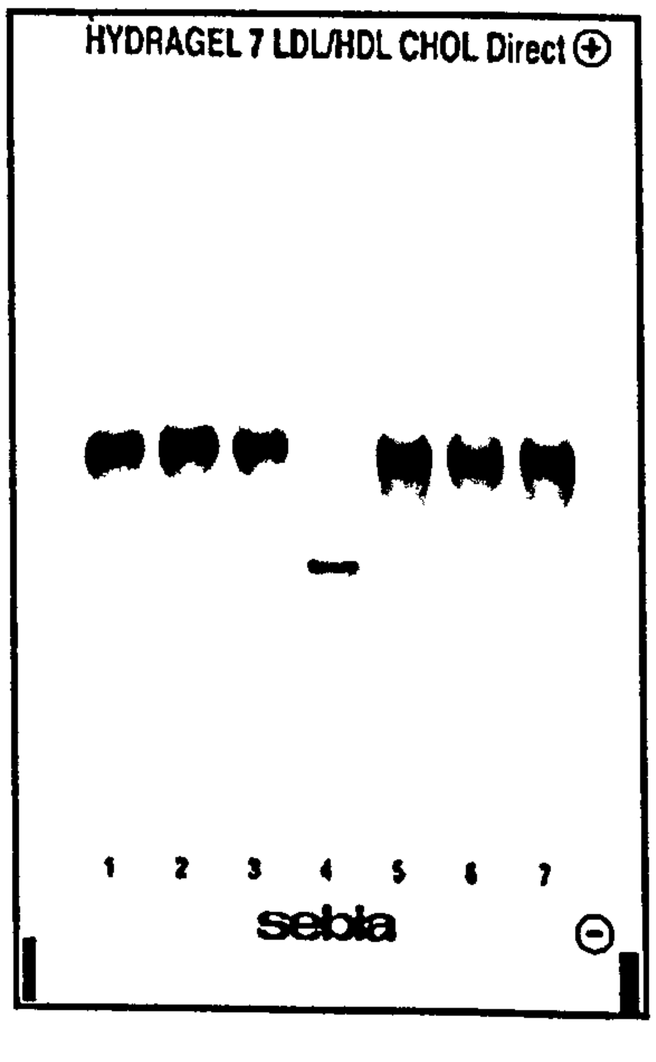


Figure 5.14: An example of the results of a SEBIA gel used to separate and quantify (after densitometry). Six different serum samples from fathead minnows were run in lanes 1, 2, 3, 5, 6, and 7, whereas an internal control (a human plasma sample) was run in lane 4.

5.4.2 Manual Cholesterol and Triglyceride Assays

Any spare serum available after the lipoprotein gels had been run was used to quantify total cholesterol and triglyceride. Total cholesterol was measured using kits supplied by ThermoTrace (Victoria, Australia), which are based on the methodologies of Allain *et al*, (1974) and Roeschlau *et al*, (1974). Total triglyceride was measured using a kit also supplied by ThermoTrace (developed by Waco Pure Chemicals Industry Ltd, with modifications from McGowan *et al*, 1983, Fossati, *et al*, 1982). Both assays also had modifications making them appropriate for use with fish serum (Section 2.2.3) (Jensen and Taylor, 2002; Jensen, 2003, personal communication).

5.4.3 Molecular methods

Total RNA, extracted as described before from livers of fish, was re-suspended in nuclease-free water, quantified and stored at –80°C until needed in the HPA assays. The total RNA samples were diluted to concentrations of 0.01μg/μl for females and 0.1μg/μl for males. As with adult Experiments 2 and 3, samples were run as described in Section 2.3.2 and concentrations of mRNA (both ZP3 and VTG) were then determined using a luminometer.

5.4.4 Results

5.4.4.1 Morphometric analysis

Table 5.3: Weight, length, HSI and GSI within each treatment group (control and 1mg/l Clofibric acid) at termination of the experiment (day 21). Data are expressed as means ± SEM.

	N numbers	Sex	Weight (g)	Length (mm)	HSI	GSI
Control	**57**	**F**	**1.83±0.05**	**53.91±0.44**	**2.31±0.06**	**9.0±0.01**
CA - exposed	**53**	**F**	**1.98±0.07**	**54.19±0.53**	**2.95±0.09**	**9.9±0.63**
Control	**8**	**M**	**3.62±0.4**	**65.75±1.5**	**2.3±0.1**	**1.0±0.1**
CA - exposed	**12**	**M**	**3.1±0.3**	**62.9±1.3**	**2.7±0.1**	**1.0±0.1**

There were no significant differences in either mean length ($p=0.6$ and 0.1 for females and males, respectively) or mean weight ($p=0.07$ for females and $p=0.2$ for males) of fish exposed to clofibric acid at 1mg/l, when compared to the control fish (Table 5.3). Female fish weighed 1.83±0.05g and were 53.91±0.44mm in length in the control group, and weighed 1.98±0.07g and were 54.19±0.53mm in length in the dosed group. Male control fish weighed 3.62±0.4g and were 65.75±1.5mm in length, and dosed fish weighed 3.1±0.3g and were 62.9±1.3mm in length.

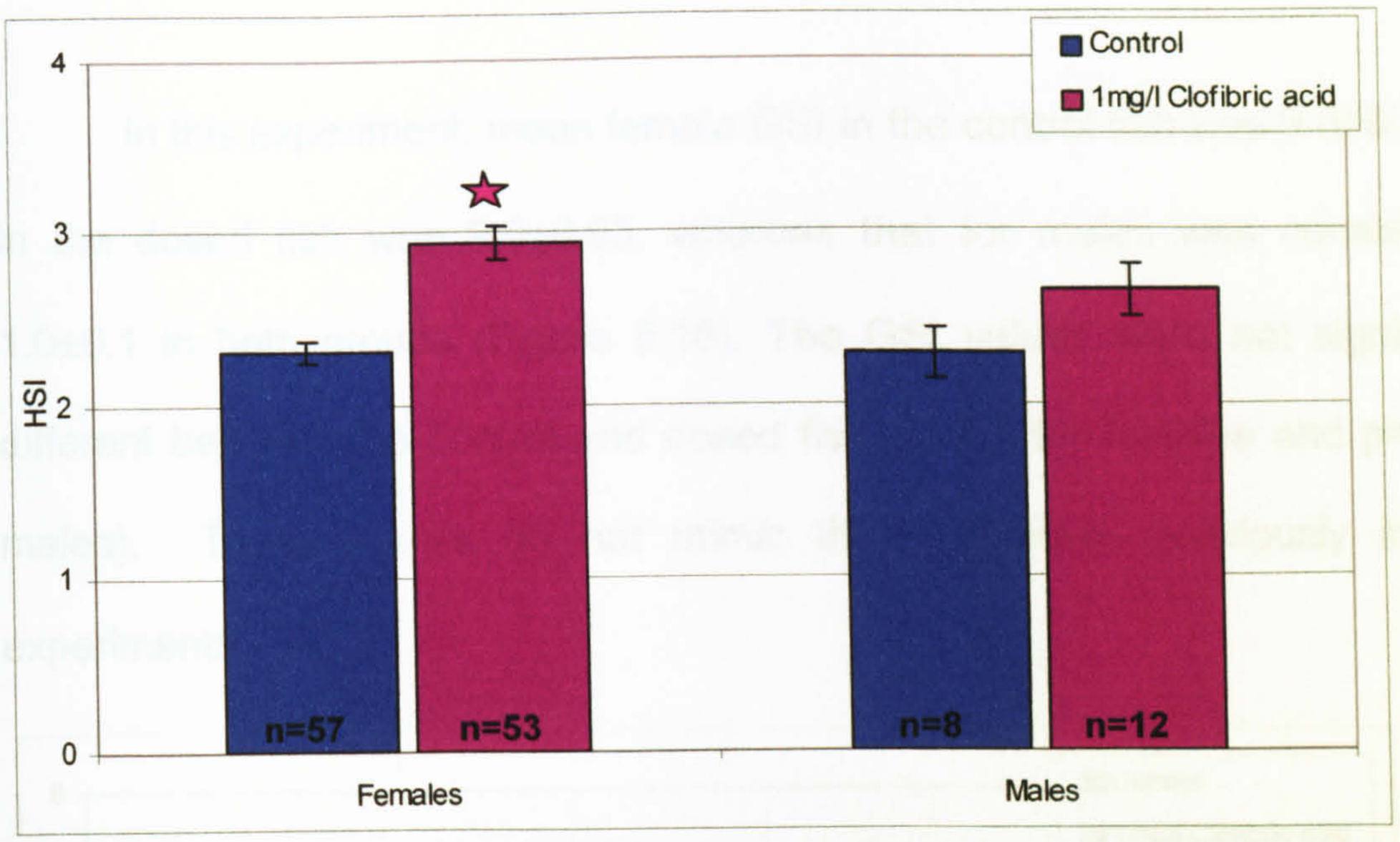


Figure 5.15: HSI of fish exposed to a nominal concentration of 1mg/l of Clofibric acid in adult Experiment 4. Data are expressed as mean ± SEM, (* p <0.001).

The mean HSI for female fish was significantly higher (p=0.001) in the dosed fish compared to the controls (Figure 5.15). The HSI in the males was not significantly different (p=0.1) between the two groups of fish; however, the results do mimic those obtained from the females. These results support those obtained from the previous two adult exposure studies.

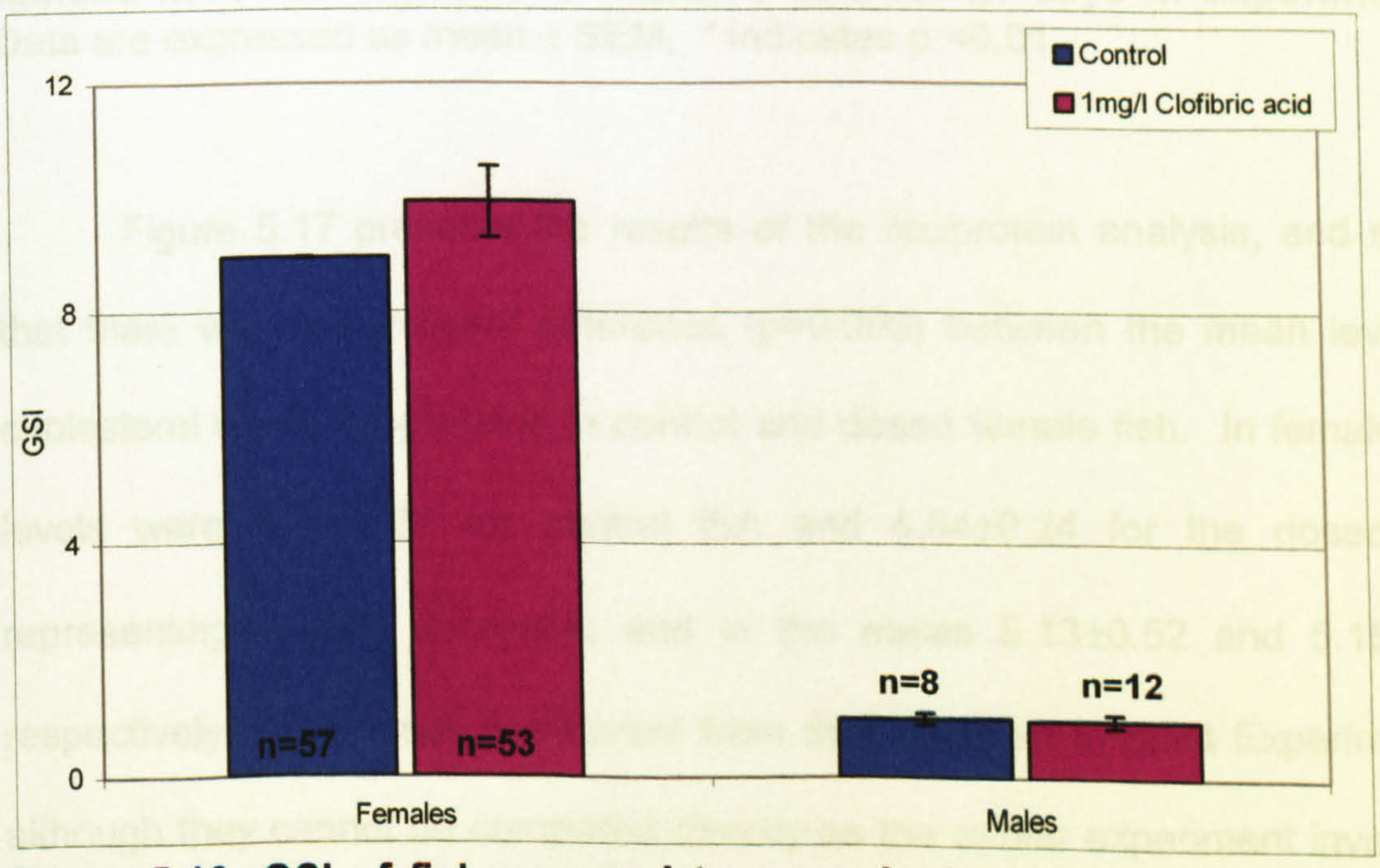


Figure 5.16: GSI of fish exposed to a nominal concentration of 1mg/l of Clofibric acid in adult Experiment 4. Data are expressed as mean ± SEM. There was no significant effect of clofibric acid on either sex.

In this experiment, mean female GSI in the control fish was 9.0±0.01 and in the dosed fish was 9.9±0.63, whereas that for males was consistent at 1.0±0.1 in both groups (Figure 5.16). The GSI values were not significantly different between the control and dosed fish (p=0.2 for females and p=0.7 for males). These results do not mimic those obtained previously in other experiments.

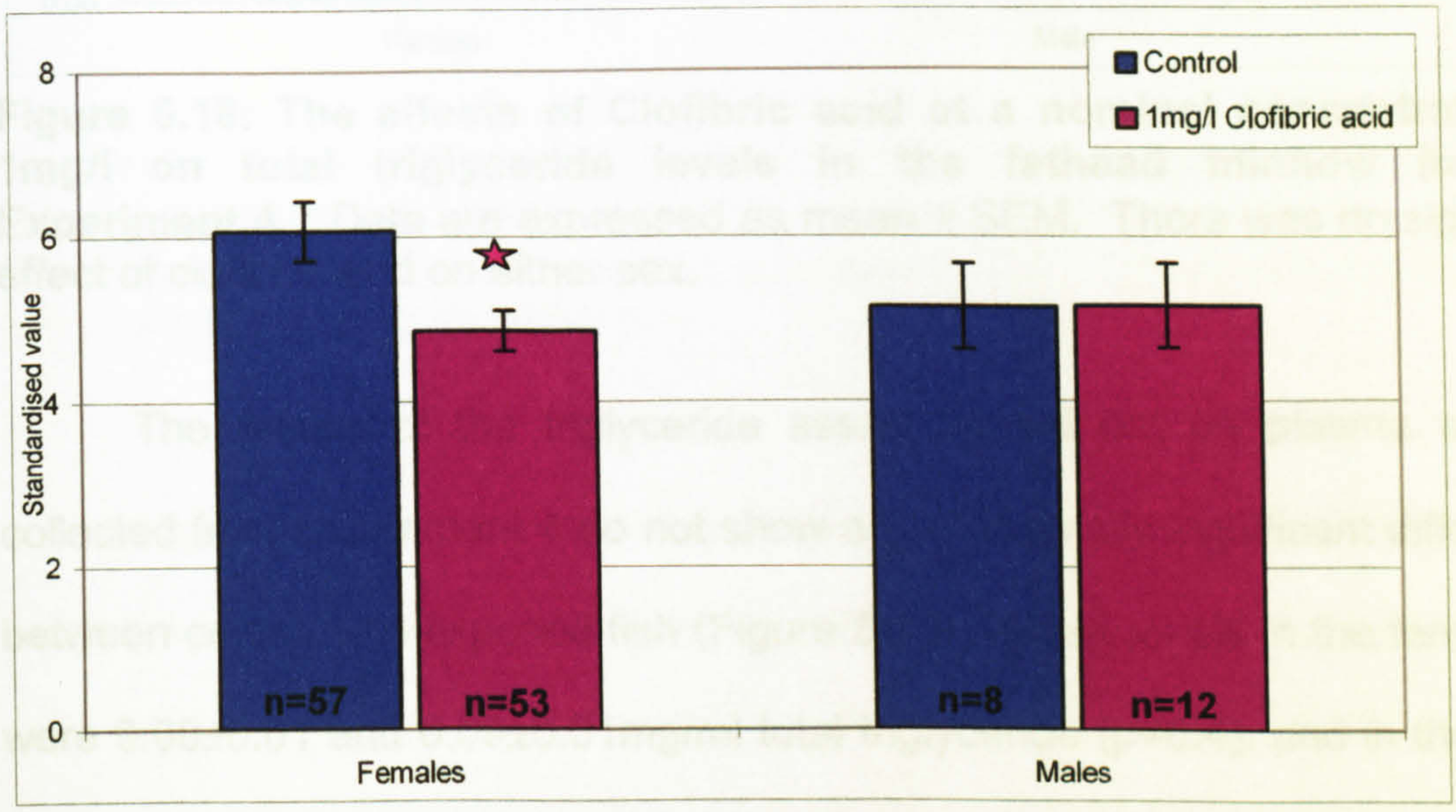


Figure 5.17: Levels of Cholesterol within the lipoproteins of female fathead minnows exposed to Clofibric acid for 21 days in Experiment 4. Data are expressed as mean ± SEM, * indicates p <0.01.

Figure 5.17 presents the results of the lipoprotein analysis, and shows that there was a significant difference (p=0.006) between the mean levels of cholesterol within lipoproteins in control and dosed female fish. In females the levels were 6.07±0.37 for control fish and 4.84±0.24 for the dosed fish, representing a 20% decrease, and in the males 5.13±0.52 and 5.15±0.51 respectively. This result is different from that obtained in adult Experiment 2, although they cannot be compared directly as the earlier experiment involved a 7-day exposure, and not 3 weeks, as this one did.

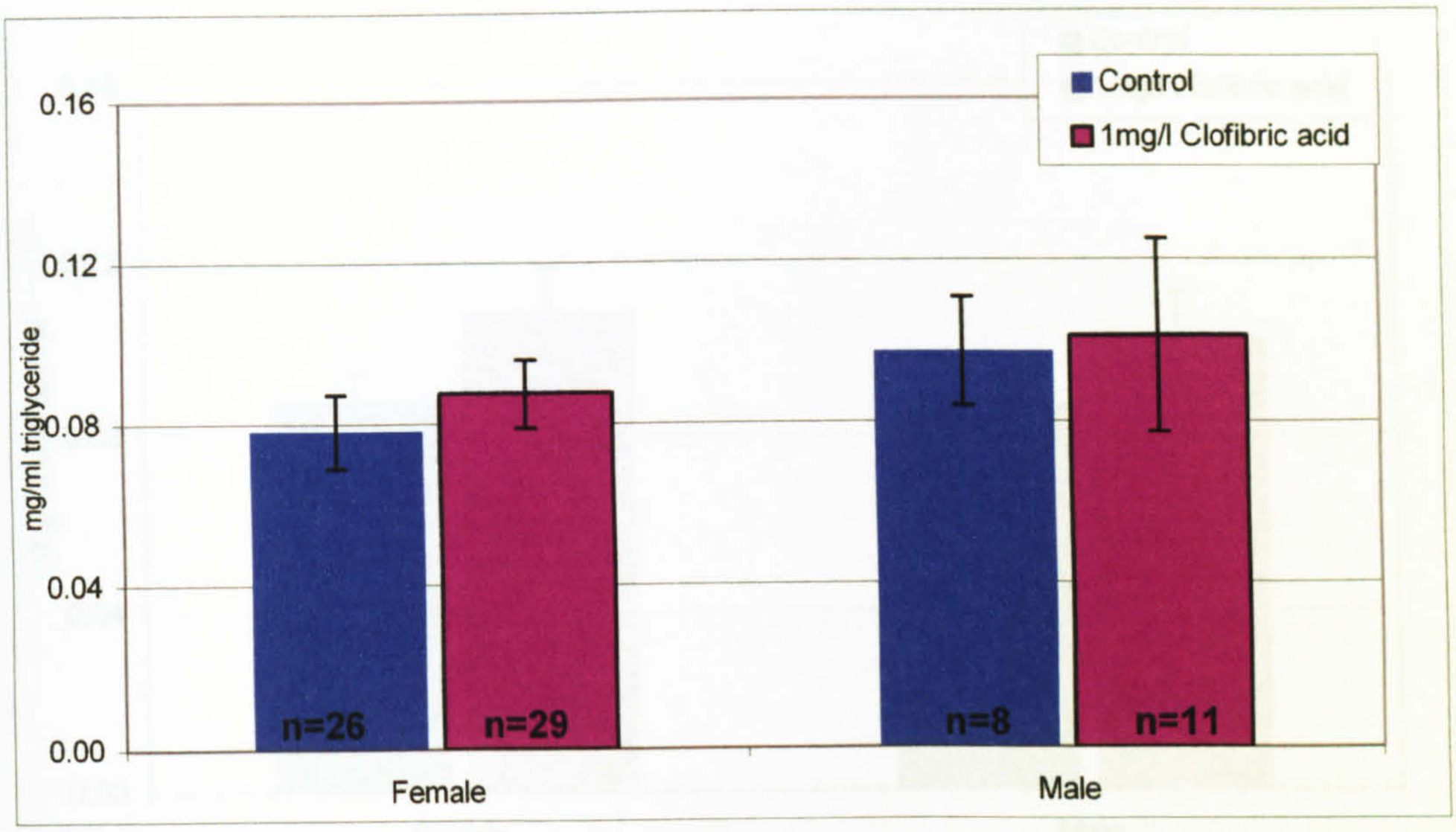


Figure 5.18: The effects of Clofibric acid at a nominal concentration of 1mg/l on total triglyceride levels in the fathead minnow in adult Experiment 4. Data are expressed as mean ± SEM. There was no significant effect of clofibric acid on either sex.

The results of the triglyceride assay carried out on plasma samples collected from Experiment 4 do not show any statistically significant differences between control and exposed fish (Figure 5.18). Mean levels in the female fish were 0.08±0.01 and 0.09±0.01mg/ml total triglyceride (p=0.4), and in the males triglyceride levels were 0.1±0.02mg/ml in both control and dosed fish (p=0.9).

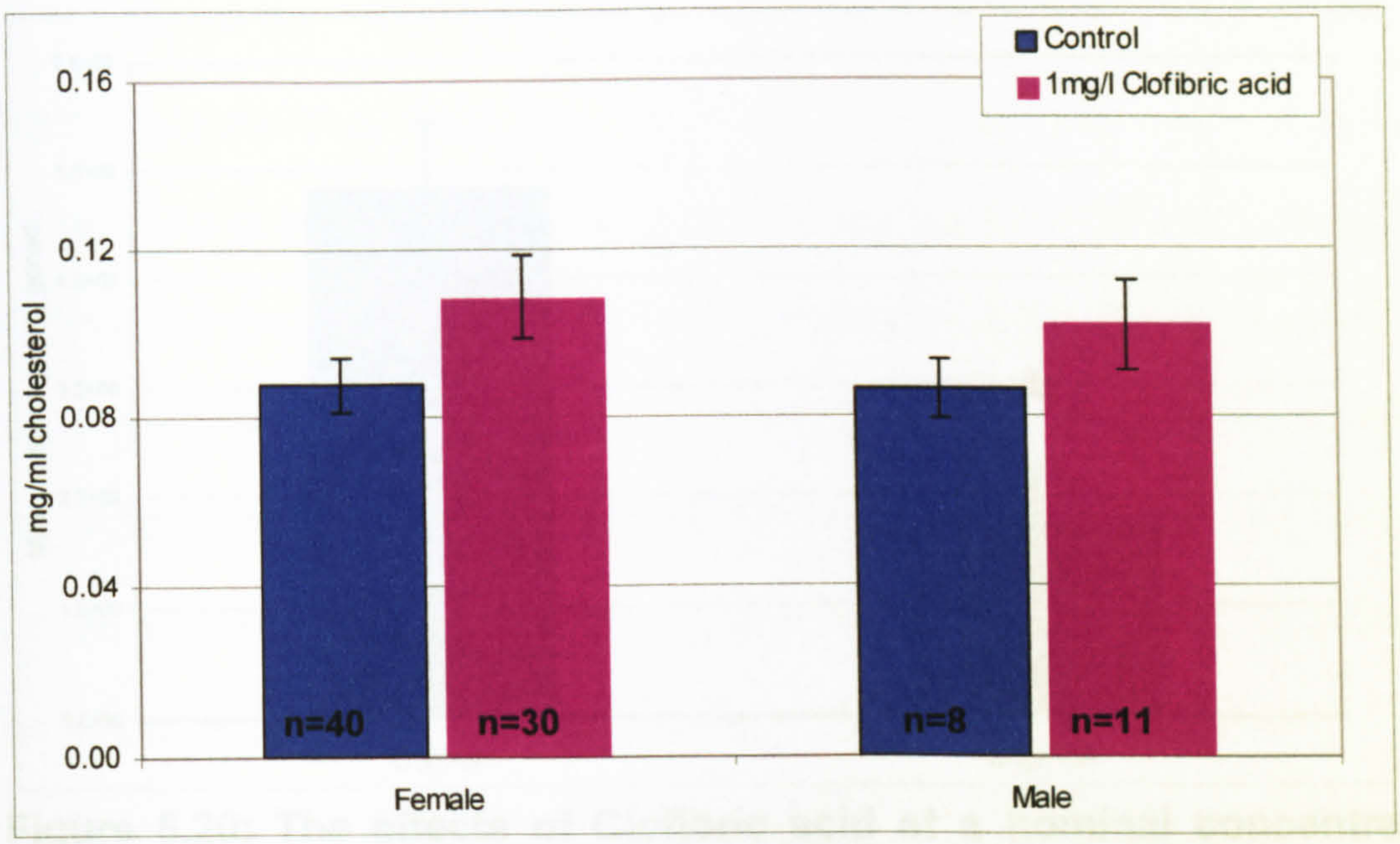


Figure 5.19: The effects of Clofibric acid at a nominal concentration of 1mg/l on total cholesterol levels in the fathead minnow in adult Experiment 4. Data are expressed as mean ± SEM. There was no significant effect of clofibric acid on either sex.

Figure 5.19 shows the results of the manual total cholesterol assay carried out on fish exposed to clofibric acid at 1mg/l. Mean levels in the females were found to be 0.09±0.01 and 0.11±0.01mg/ml in the control and dosed, respectively, whereas in the males they were 0.09±0.01 and 0.10±0.01, respectively. When analysed, there were found to be no statistically significant differences due to treatment ($p=0.1$ for females and 0.3 for males), although a trend for higher cholesterol levels in clofibric acid exposed fish occurred in both sexes.

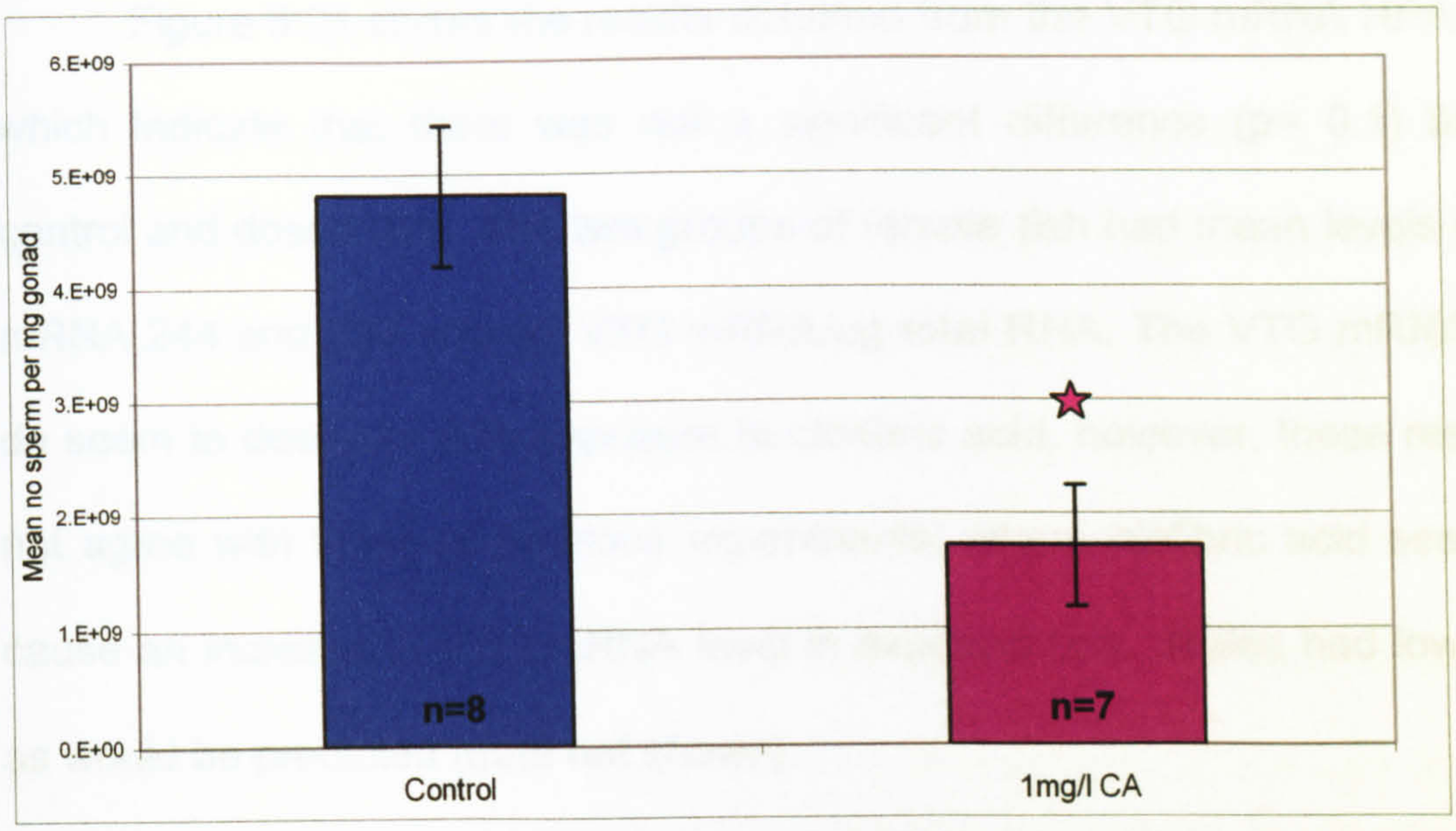


Figure 5.20: The effects of Clofibric acid at a nominal concentration of 1mg/l on sperm counts of the fathead minnow in adult Experiment 4. Data are expressed as mean ± SEM, (* indicates $p < 0.01$).

Figure 5.20 shows the results of the sperm analysis. It shows that clofibric acid exposure at 1mg/l for 21 days can significantly decrease mean sperm count in adult male fish ($p=0.002$).

5.4.4.2 Molecular endpoints

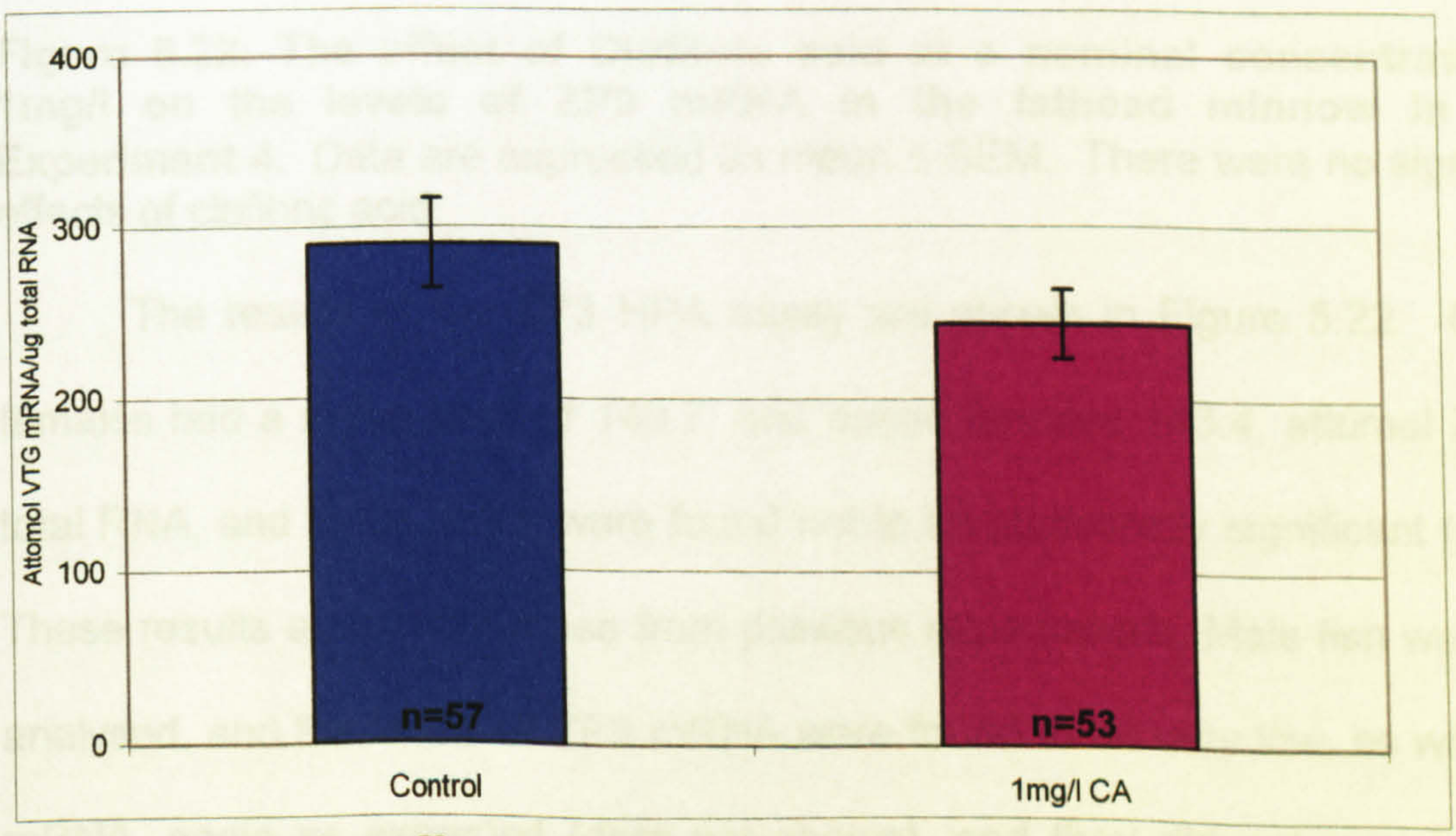


Figure 5.21: The effect of Clofibric acid at a nominal concentration of 1mg/l on the levels of VTG mRNA in the fathead minnow in adult females in Experiment 4. Data are expressed as mean ± SEM. There was no significant effect of exposure to clofibric acid.

Figure 5.21 shows the results obtained from the VTG mRNA HPA assay, which indicate that there was not a significant difference (p= 0.1) between control and dosed fish. The two groups of female fish had mean levels of VTG mRNA 244 and 290 attomol VTG mRNA/µg total RNA. The VTG mRNA levels do seem to decrease with exposure to clofibric acid, however, these results do not agree with those of previous experiments, where clofibric acid seemed to cause an increase in VTG mRNA level in exposed fish. Males had low levels, as would be predicted (data not shown).

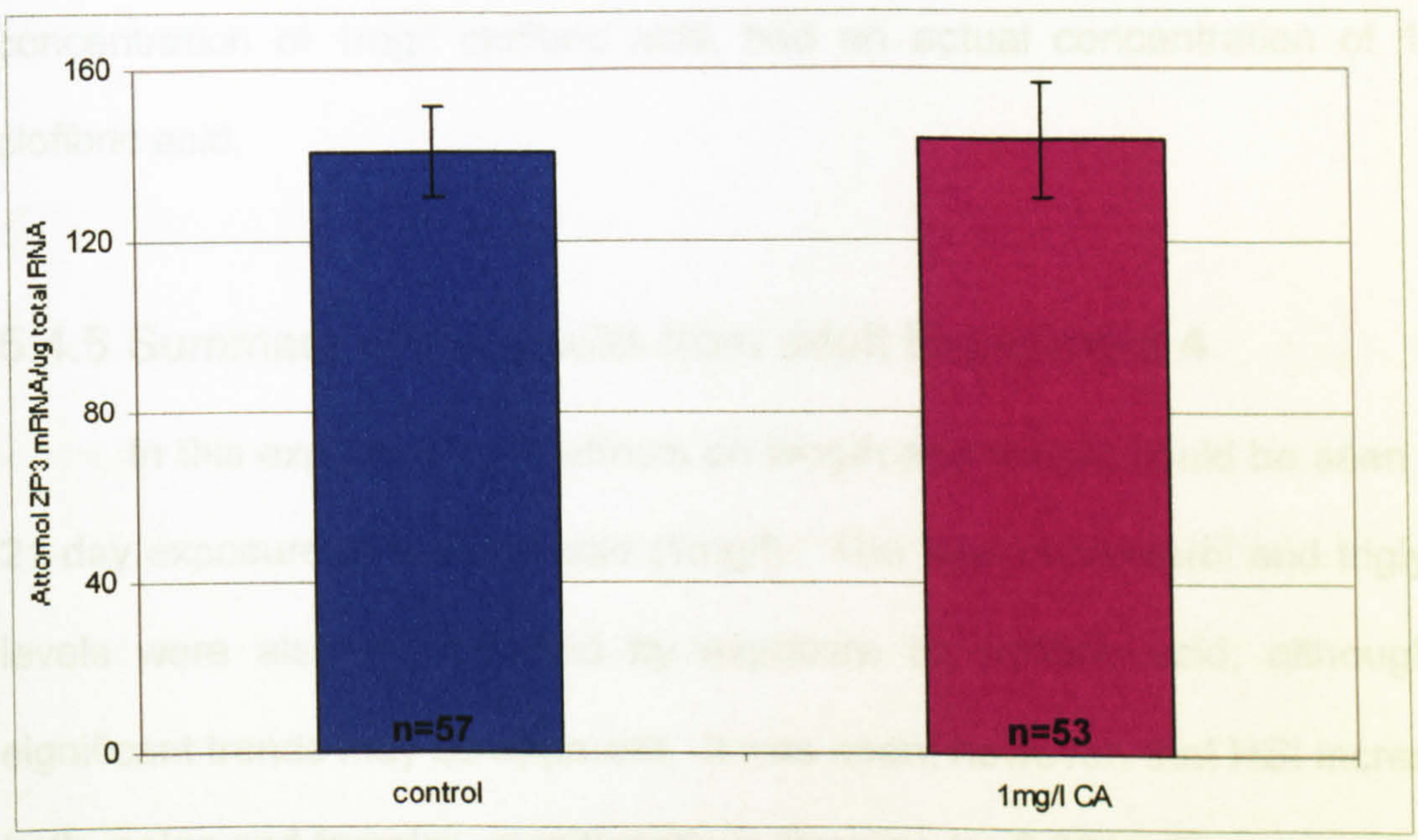


Figure 5.22: The effect of Clofibric acid at a nominal concentration of 1mg/l on the levels of ZP3 mRNA in the fathead minnow in adult Experiment 4. Data are expressed as mean ± SEM. There were no significant effects of clofibric acid.

The results of the ZP3 HPA assay are shown in Figure 5.22. Control females had a mean level of 140.7, and dosed females 143.4, attomol ZP3/ug total RNA, and these levels were found not to be statistically significant (p=0.6). These results agree with those from previous experiments. Male fish were also analysed, and the levels of ZP3 mRNA were found to be very low, as with VTG mRNA, again as expected (data not shown), and they did not vary between control and dosed fish (p=0.9).

5.4.4.3 Water samples

Water samples from this experiment were collected and stored in 250ml amber bottles at 4°C. 1% formaldehyde was added to preserve the samples until the analysis could be done. Analysis was kindly carried out by Dr Malcolm Hetheridge at Brixham Environmental Laboratory, AstraZeneca, using the method described in Section 2.4.1.

The samples of tank water collected from the control tank contained no detectable clofibric acid, as expected. The dosed tank, which had a nominal concentration of 1mg/l clofibric acid, had an actual concentration of 1.3mg/l clofibric acid.

5.4.5 Summary of the results from adult Experiment 4

In this experiment, no effects on length and weight could be seen after a 21-day exposure to clofibric acid (1mg/l). The total cholesterol and triglyceride levels were also not effected by exposure to clofibric acid, although non-significant trends may be apparent. It was seen, however, that HSI increased in both males and females, significantly in females ($p=0.001$). These results agree with the results found in both previous experiments.

One of the most interesting results obtained from this experiment was perhaps the 20% decrease in the mean levels of cholesterol within HDL of female fish exposed to clofibric acid ($p=0.006$). This result does not agree with that of Experiment 2 (which showed no significant effect), however, the numbers of fish used in this experiment was five times that used before and perhaps consequently a more sensitive and indicative result. Another very interesting result was the highly significant decrease ($p=0.002$) in sperm number when comparing control and exposed male fish:- Although only a

preliminary finding, the results led to further investigation (and another significant decrease in sperm count) in the following experiment.

The results from the ZP3 mRNA assays agreed with the results of previous experiments, namely that there were no significant differences between exposed and control fish. The levels of VTG mRNA, however, show different results from previous experiments (although the presence of worms in the previous exposure may have effected the previous results). Overall, the results suggest that exposure to clofibric acid at 1mg/l for 21 days has little, if any, effects on VTG and ZP3 mRNA levels.

5.5 Adult Experiment 5

A further three-week experiment was then carried out, in which fathead minnows were exposed to 2 different concentrations of clofibric acid (10µg/l and 1mg/l). Nine tanks were set up as described in Section 2.1: three control, three at a nominal concentration of 10µg clofibric acid per litre and three at a nominal concentration of 1mg clofibric acid per litre. Each of the three treatments had 18 female and 18 male fish (six per sex per tank). Stock solutions were prepared as described in Section 2.1.2 to give nominal tank concentrations of 1mg/l and 10µg/l clofibric acid. For 1 mg/l, 0.83g clofibric acid was added per litre, to double distilled water (3.332g in 4L), and for 10µg/l, 0.0083g, clofibric acid was added per litre, to double distilled water (0.033g in 4L), and these stock solutions were then stirred overnight at ~40°C. Dosing of the tanks was carried out using the method described in Section 2.1.2, and this was started one week prior to entry of the fish, to ensure equilibrium of the chemical within the tanks.

After 21-days exposure the experiment was terminated, all fish were anaesthetised, killed and sampled as previously described (Section 2.1.3). Morphometric parameters were recorded and blood was collected using heparin and aprotinin, and plasma was separated (see Section 2.1.3) ready for steroid analysis. Livers were carefully dissected and immediately snap-frozen using RNase free conditions, to allow the measurement of the mRNA levels of lipoprotein lipase and glucose-6-phosphate-dehydrogenase, as described in Section 2.1.4 (see Chapter 6 for results). Testes were also carefully dissected, and placed into catfish extender (Section 2.2.1) ready for sperm counting.

5.5.1 Steroid Radioimmunoassays

Plasma samples collected from this experiment were analysed using radioimmunoassays to determine and compare the levels of testosterone and 11-keto-testosterone in control and dosed fish. The general principal and method of the steroid RIA has previously been described in Section 2.2.5.

5.5.2 Sperm count

In contrast to Experiment 4, in this experiment whole testes were carefully dissected, weighed and placed into 1.5ml eppendorf tubes containing catfish extender (Section 2.2.1). These were then stored until needed for analysis (within 1 week). The testes were homogenised thoroughly using a clean pipette tip and 1µl of the homogenate was then added to 99µl of trypan blue and left in a 'damp box' for 10 minutes. 20µl of this sample was then applied to the haemocytometer and cell counting rules then applied (Section 2.2.1). Mature sperm were counted and analysis was carried out on number of sperm per mg gonad to determine any exposure effects.

5.5.3 Results

5.5.4 Morphometric analysis

Table 5.4: Weight, length, HSI and GSI for each treatment group (control, 10µg/l and 1mg/l Clofibric acid) at termination of the experiment. Data are expressed as means ± SEM.

	N numbers	Sex	Weight (g)	Length (mm)	HSI	GSI
Control	**18**	**F**	**1.9±0.1**	**50.4±0.9**	**2.9±0.2**	**8.9±0.6**
10µg/l CA	**18**	**F**	**1.7±0.1**	**50.9±0.9**	**3.1±0.1**	**10.3±1.0**
1mg/l CA	**18**	**F**	**1.7±0.1**	**49.7±0.9**	**3.1±0.2**	**9.5±0.8**
Control	**18**	**M**	**3.3±0.2**	**63.2±0.8**	**2.2±0.1**	**0.9±0.1**
10µg/l CA	**18**	**M**	**2.8±0.2**	**60.9±1.0**	**2.2±0.1**	**0.8±0.1**
1mg/l CA	**18**	**M**	**3.0±0.2**	**61.5±1.1**	**2.3±0.1**	**0.8±0.1**

The results in Table 5.4 show that there were no significant differences in either length or weight between control and exposed fish. Mean weights of females were 1.9±0.1g in the controls and 1.7±0.1g in both groups exposed to clofibric acid, and the weights of males were 3.3±0.2g in the controls, and in the two doses of clofibric acid 2.8±0.2g and 3.0±0.2g, respectively. Mean lengths for females were 50.4±0.9mm, 50.9±0.9mm and 49.7±0.9mm and males were 63.2±0.8mm, 60.9±1.0mm and 61.5±1.1mm.

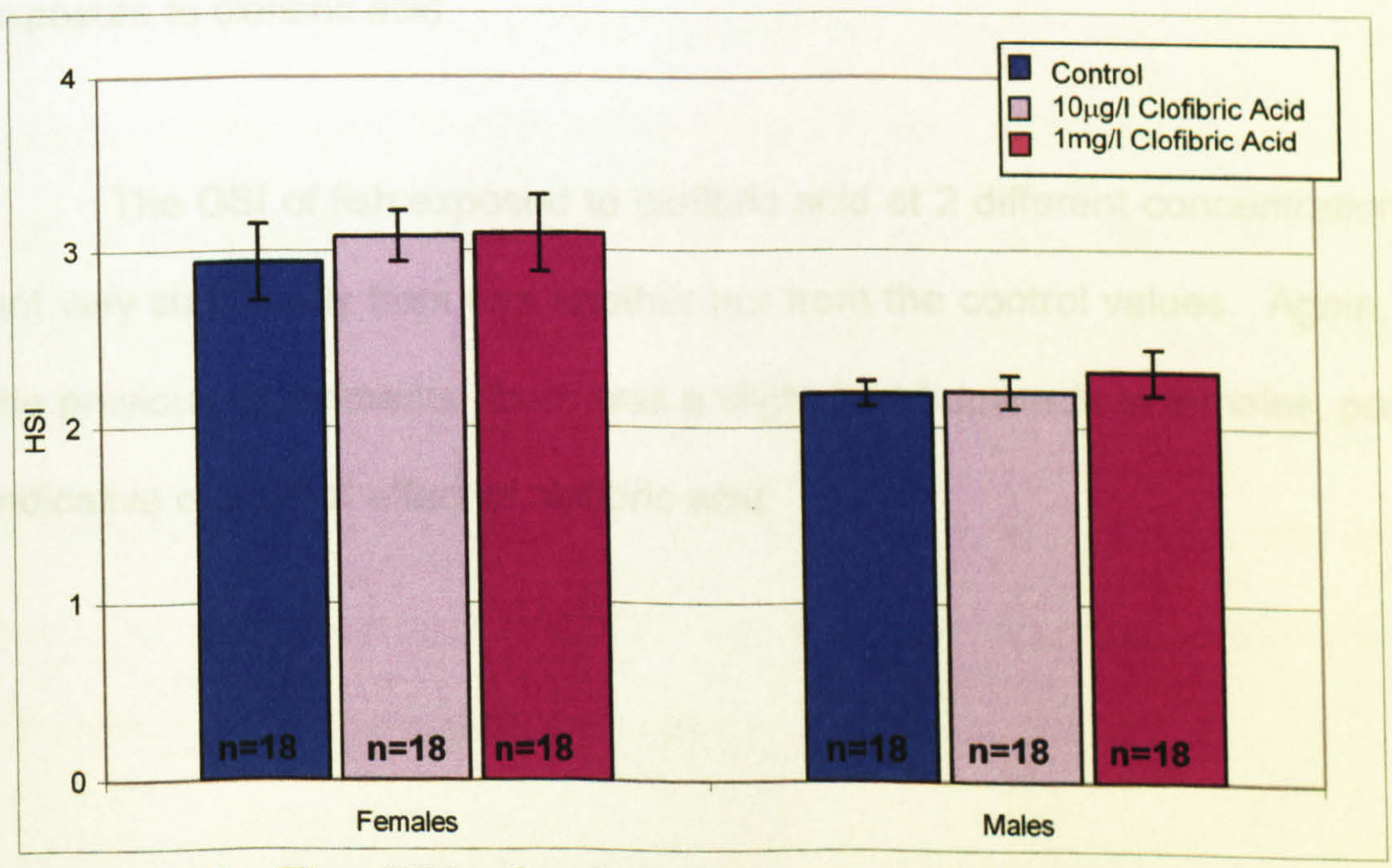


Figure 5.23: The HSI of adult fathead minnow exposed to nominal concentrations of 10µg/l and 1mg/l Clofibric acid in Experiment 5. The data are expressed as means ± SEM. No significant differences were found.

The mean HSI values (Figure 5.23) for both males and females were not significantly different when comparing each of the different doses, both to each other and to the controls. There was, however, as with previous experiments, a slight upward trend, with the HSI of both males and females exposed to clofibric acid being slightly higher than the same sex controls.

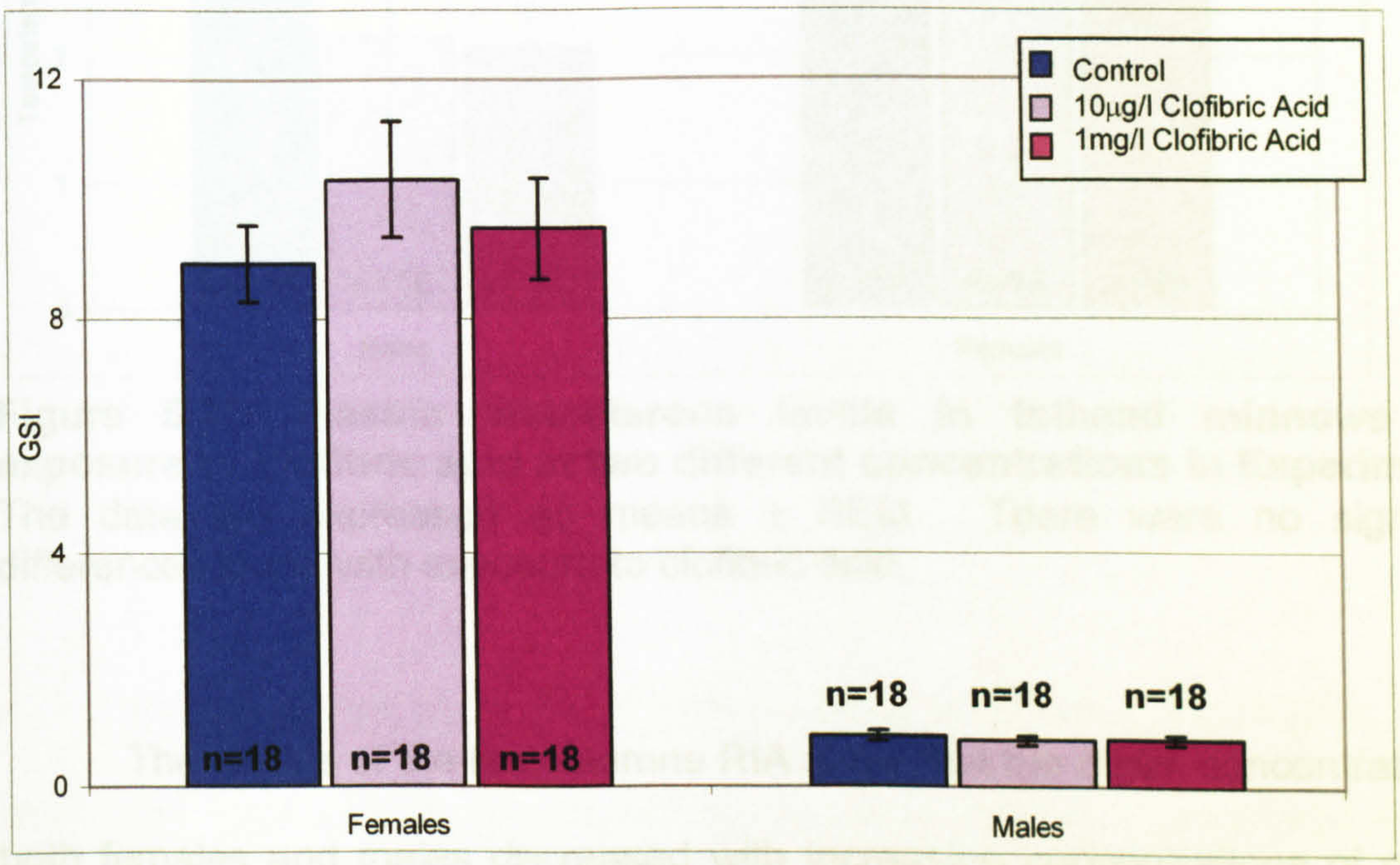


Figure 5.24: The GSI of adult fathead minnow exposed to nominal concentration of 10µg/l and 1mg/l Clofibric acid in Experiment 5. The data are expressed as means ± SEM. No significant differences were found with exposure to clofibric acid.

The GSI of fish exposed to clofibric acid at 2 different concentrations did not vary statistically from one another nor from the control values. Again, as in the previous experiments, there was a slight trend upwards in females, possible indicative of a small effect of clofibric acid.

5.5.4.1 Results of the Steroid Assays

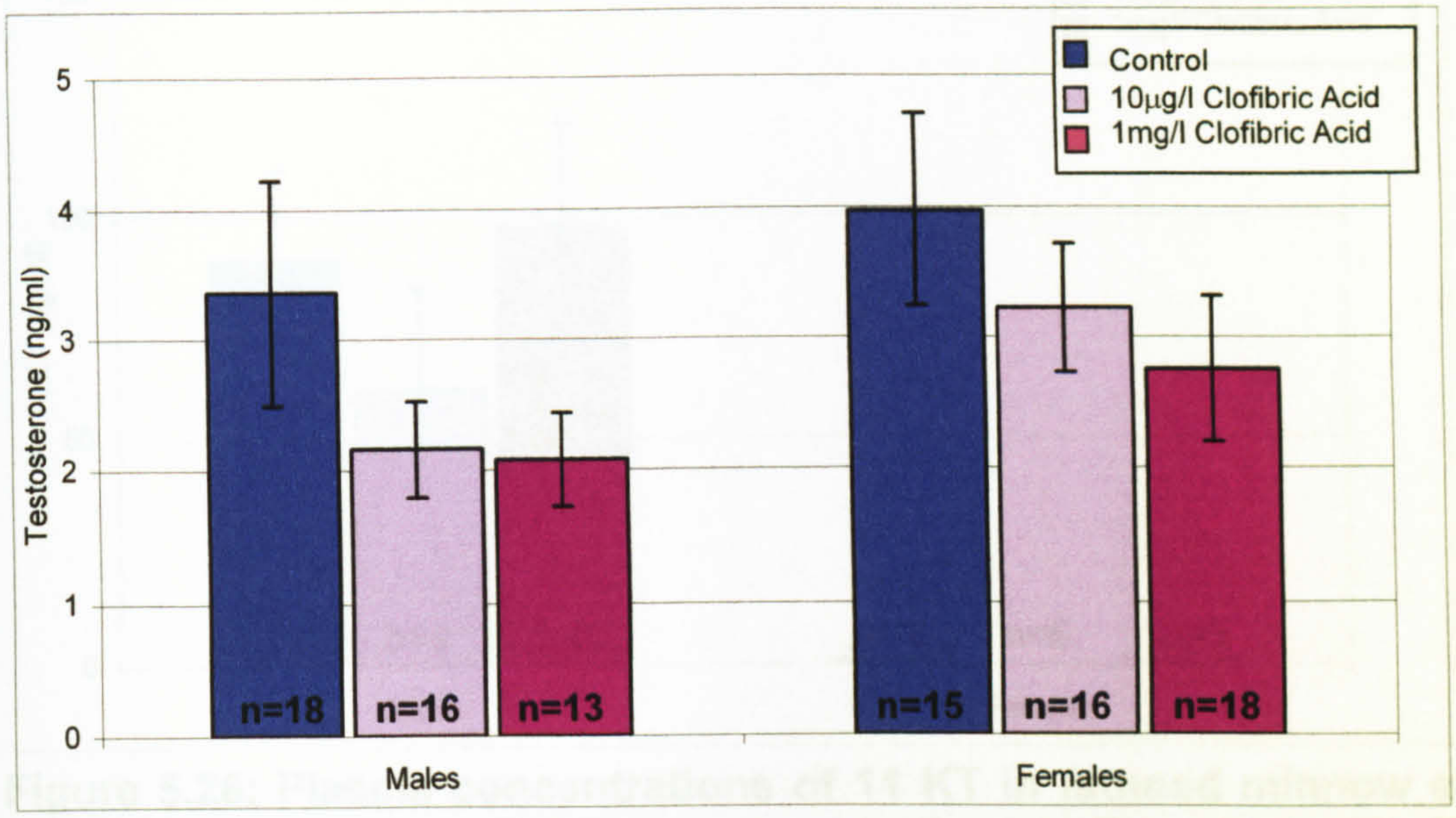


Figure 5.25: Plasma testosterone levels in fathead minnows after exposure to Clofibric acid at two different concentrations in Experiment 5. The data are expressed as means ± SEM. There were no significant differences found with exposure to clofibric acid.

The results of the testosterone RIA show that the mean concentrations in both females and males decreased with increasing concentrations of clofibric acid. Males had levels of 3.36±0.87ng/ml, 2.16±0.36ng/ml and 2.08±0.36ng/ml in the control and the dosed fish, respectively, and females 3.97±0.74ng/ml, 3.22±0.49ng/ml and 2.76±0.56ng/ml (Figure 5.25). Although the mean levels are not significantly different statistically within each sex, the results from both sexes suggest a definite trend to a lower testosterone level upon exposure to clofibric acid. Statistical analyses (ANOVA and paired t-tests) were subsequently carried out comparing the data within each sex and also between sexes for each individual treatment and the data as a whole. No significant differences were determined in any case.

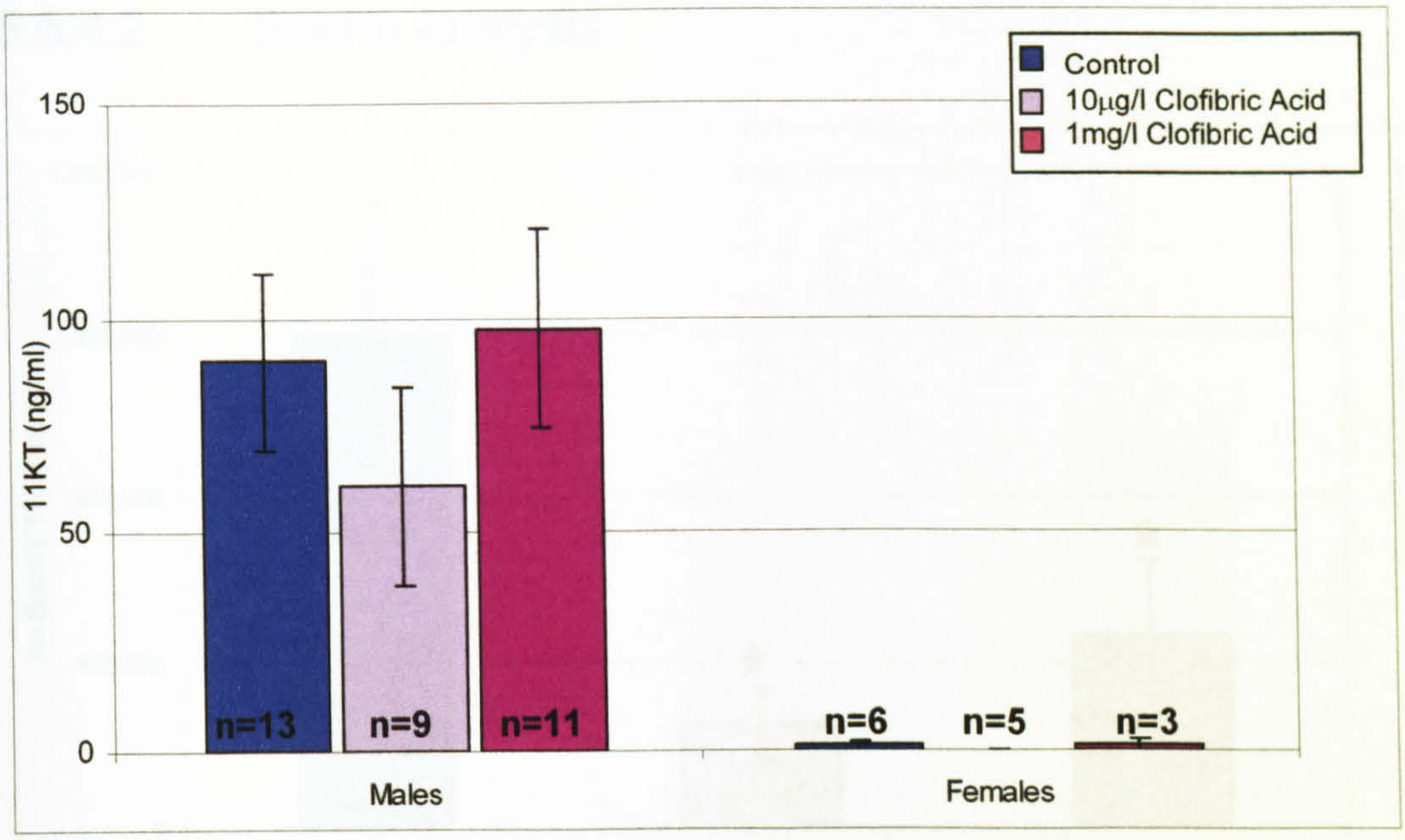


Figure 5.26: Plasma concentrations of 11 KT in fathead minnow exposed to Clofibric acid at two different concentrations in Experiment 5. The data are expressed as means ± SEM. There were no significant differences found with exposure to clofibric acid.

Plasma levels of 11 KT in females were very low – 1.0±1ng/ml, 0ng/ml and 1.3±1.3ng/ml in the control and two exposed groups, respectively, whereas the males had levels of 90±20.8ng/ml, 61.0±23.1ng/ml and 97.36±23.1ng/ml, respectively (Figure 5.26). There was no obvious effect of clofibric acid on plasma 11-KT concentrations, nor even a suggestion of clofibric acid affecting 11-KT levels. The large SEMs indicated a high level of variability in plasma 11-KT levels in male fish, even without treatment.

5.5.4.2 Sperm Analysis

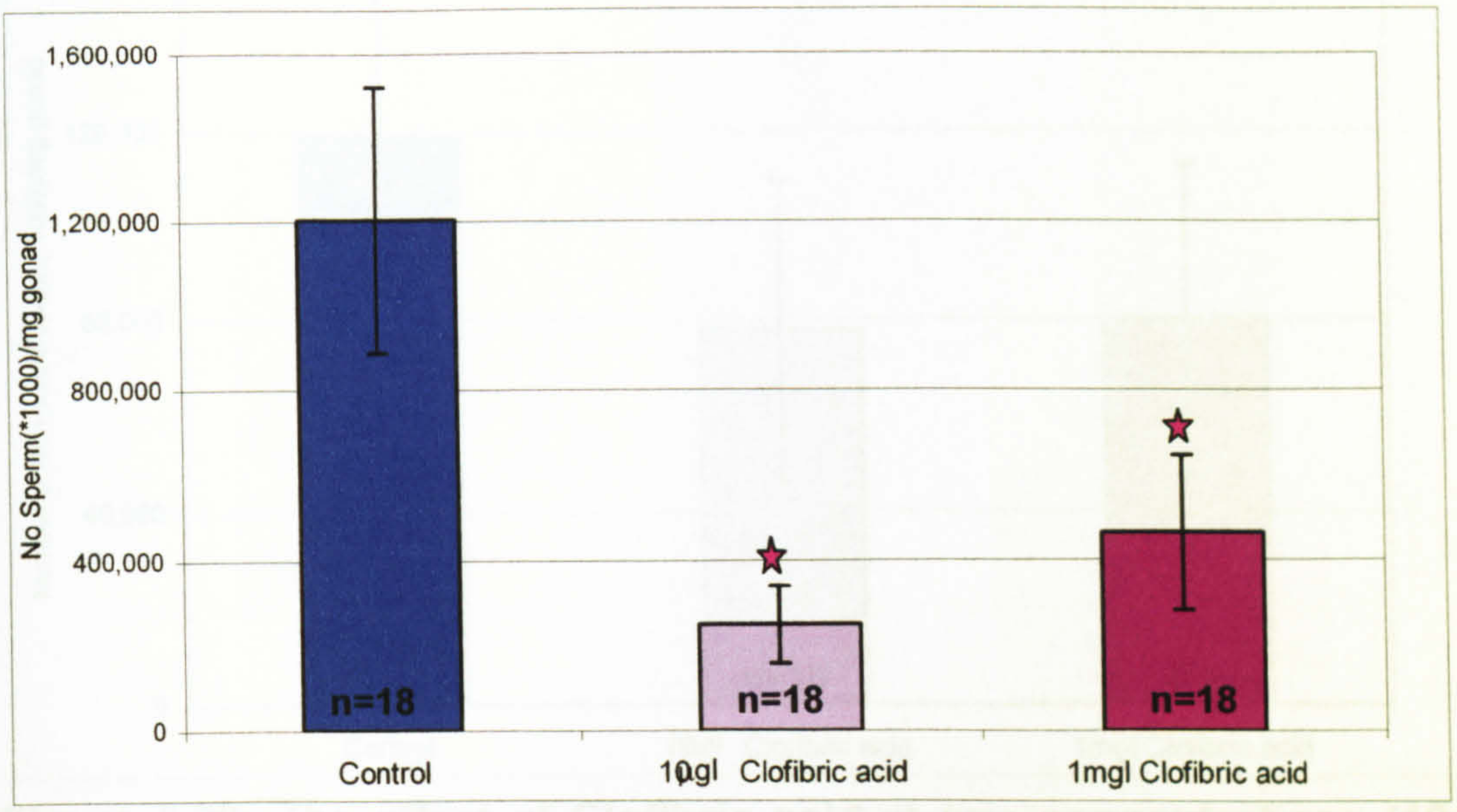


Figure 5.27: The effect of Clofibric acid at two concentrations (10µg and 1mg per litre) on the numbers of viable sperm in the testes of adult fathead minnows. Data are presented as means ± SEM. * indicates $p<0.01$ at 10µg/l and $p<0.05$ at 1mg/l concentrations, when compared to the control.

Figure 5.27 shows the results of the sperm count data from Experiment 5. The results show a highly significant effect of clofibric acid when compared to the controls. These results indicate that clofibric acid at both 10µg/l and 1mg/l had a deleterious effect on the production of viable sperm from adult male fish. They strongly agree with the results from the previous experiment, and confirm that clofibric has an effect on the number of viable mature sperm in the testes.

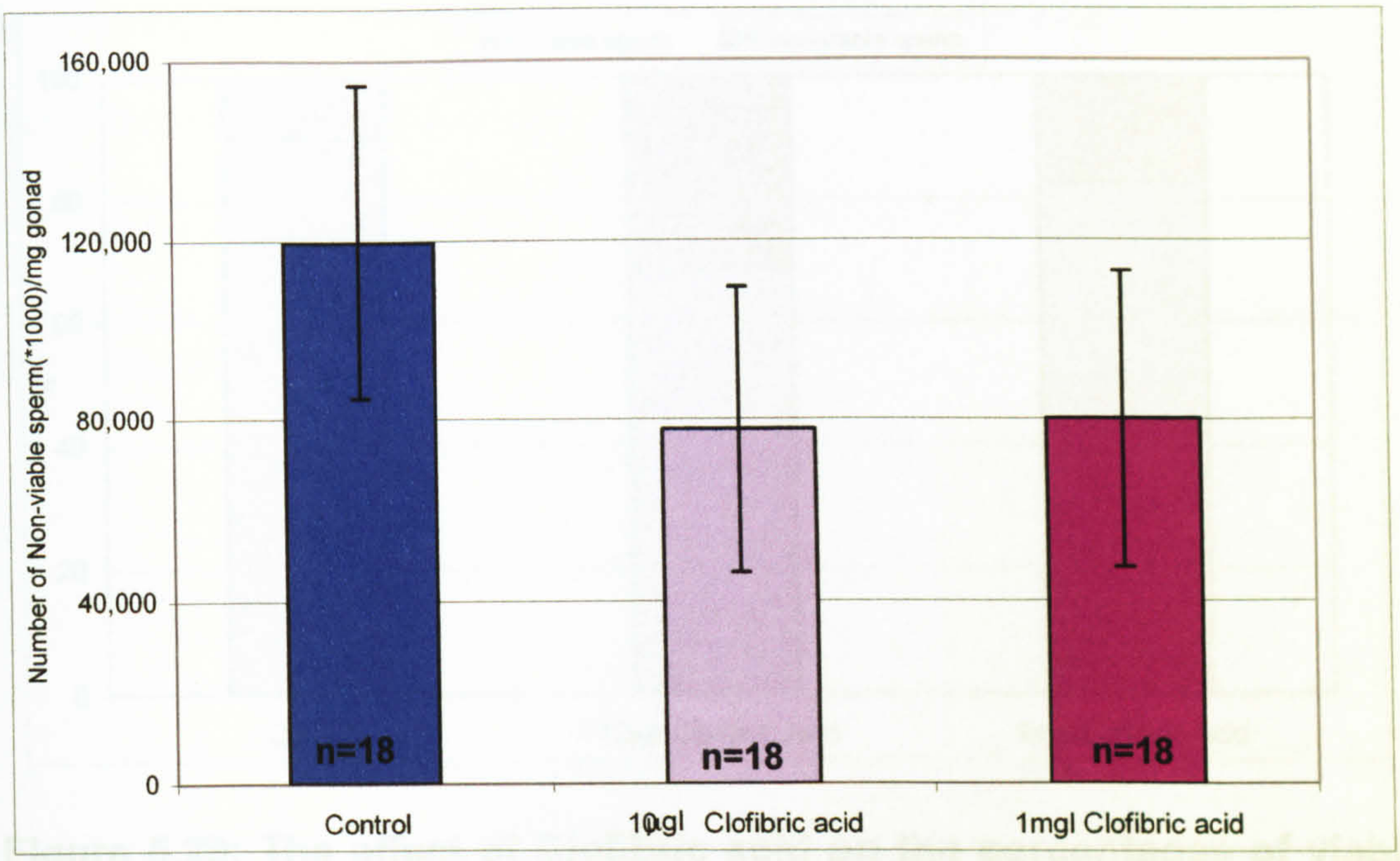


Figure 5.28: The effect of Clofibric acid at two concentrations (10µg and 1mg per litre) on the number of non-viable sperm of fathead minnows. The data are expressed as means ± SEM. There were no significant differences found with exposure to clofibric acid.

Figures 5.28 shows the numbers of non-viable sperm present in the samples from Experiment 5. When compared to Figure 5.27, it can be seen that there were about ten times more viable than non-viable sperm in these fish. There were no significant differences found when both doses were compared to the control, however there were less non-viable sperm present in the testes of both of the dosed groups of fish.

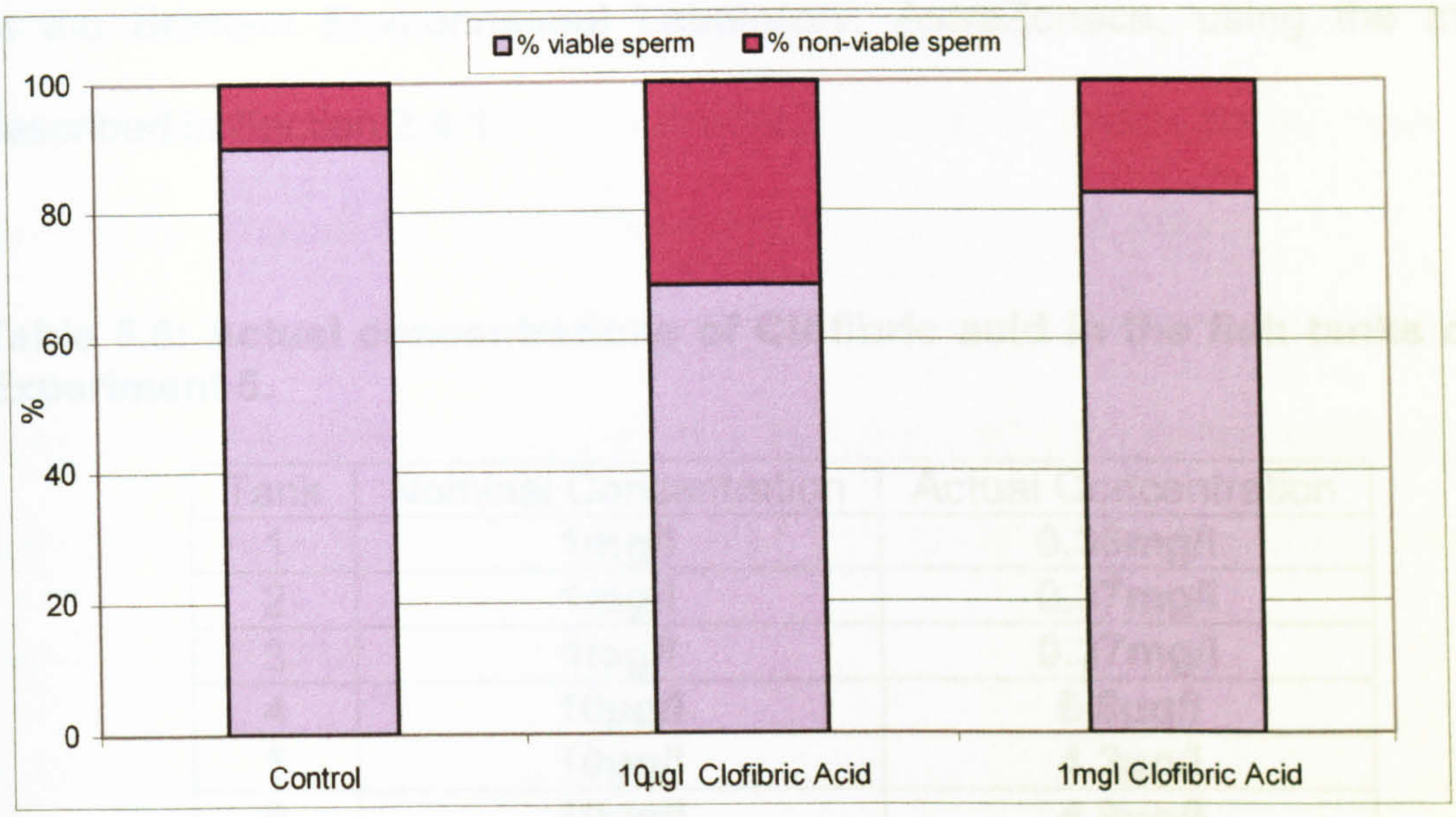


Figure 5.29: The effect of Clofibric acid on the percentages of viable and non-viable sperm from Experiment 5. The data are expressed as means ± SEM. No significant differences found with exposure to clofibric acid.

Figure 5.29 shows the proportions of viable and non-viable sperm present in testicular samples from Experiment 5. As shown in the Figure, the proportion of sperm in 'normal', undosed fish was found to be 9.9% of the total count, whereas at the 10µg/l dose this figure more than trebled (31.2%), and at the highest dose (1mg/l clofibric acid) this percentage was double that of the control (17.4%). This result shows that exposure of fish to clofibric acid not only reduces sperm number but may also affect sperm quality (not only are less sperm produced in exposed fish, but more of these are non-viable). However, the non-viable sperm numbers were not significantly different, so this apparent decrease in sperm quality may not be true.

5.5.4.3 Analysis of Water samples

Water samples from this exposure were collected on the final day of the experiment and were stored at 4°C in 250ml amber bottles until analysis could be carried out. Analysis was again kindly carried out by Dr Malcolm Hetheridge

at the Brixham Environmental Laboratory, AstraZeneca, using the method described in Section 2.4.1.

Table 5.5: Actual concentrations of Clofibric acid in the fish tanks during Experiment 5.

Tank	Nominal Concentration	Actual Concentration
1	**1mg/l**	**0.36mg/l**
2	**1mg/l**	**0.37mg/l**
3	**1mg/l**	**0.37mg/l**
4	**10µg/l**	**5.6µg/l**
5	**10µg/l**	**4.2µg/l**
6	**10µg/l**	**4.9µg/l**
7	**Control**	**<1µg/l**
8	**Control**	**<1µg/l**
9	**Control**	**<1µg/l**

Table 5.5 above shows the results of the water analysis. Concentrations in the control tanks were all below the 1µg/l detection level, whereas the 10µg/l nominal concentration tanks contained a mean value of 4.9µg/l (approximately half of the nominal concentration) and the tanks with a nominal concentration of 1mg/l contained an actual concentration of 0.36mg/l (approximately a third of the nominal concentration).

5.5.5 Summary of the results from adult Experiment 5

The results obtained from this experiment are probably the most interesting of all those obtained. There were again, as with previous experiments, no significant effects on somatic parameters. Subtle, albeit not statistically significant, effects were seen on steroid levels, particularly so on testosterone levels in both males and females. However, the most pronounced effects of clofibric acid were on sperm counts; both test concentrations significantly reduced the sperm count. Moreover, because there is a significant

effect on viable sperm and not non-viable sperm, this indicates that the effect of clofibric acid is on the actual production of viable sperm rather than sperm viability itself (as would be seen with a non-specific toxic effect).

The lack of a dose-response relationship for the effect of clofibric acid on sperm count is unexplained, but may indicate that both concentrations tested caused the maximum effect, and hence that lower doses would be needed to obtain a dose-response curve.

5.6 Overall summary of the effects of Clofibric acid on the adult fathead minnow.

As stated previously, prior to this research there had been no reports of experiments carried out to determine the effects of clofibric acid on adult fish. At present the only human pharmaceutical with a reasonable amount of data on effects in fish is EE_2. With all other pharmaceuticals there is very little published material concerning possible effects on any wildlife, including fish.

The experiments reported in this particular chapter were therefore aimed at identifying effects of clofibric acid exposure, if any, on the fathead minnow, using a series of flow through experiments.

In most cases the results at first glance seem generally insignificant; there were not any pronounced changes in most of the parameters monitored. However, when looked at more closely, there may be indications or trends suggesting effects of clofibric acid exposure which were reproduced in some or all experiments. In order to be able to more readily visualise these possible changes, and to allow comparisons between experiments, the results from all four experiments have been summarised in Table 5.6, which follows.

Table 5.6: A summary of the effects of Clofibric acid on a variety of endpoints in the adult fathead minnow. (Where: * indicates a significant effect, ⇑ = Higher in exposed fish, ⇓ = Lower in exposed fish, ⇔ = No response/stayed the same).

a) Females

EXPT	HSI	GSI	HDL CHOL	TOTAL CHOL	TOTAL TRI	VTG	ZP3	TESTO
2	⇑	⇓	⇔			⇑	⇔	
3	⇑	⇑*				⇑	⇔	
4	⇑*	⇑	⇓*	⇑	⇑	⇓	⇔	
5	⇑	⇑						⇓

b) Males

EXPT	HSI	GSI	HDL CHOL	TOTAL CHOL	TOTAL TRI	TESTO	SPERM
2	⇓	⇓					
3	⇔	⇑*					
4	⇑	⇔	⇔	⇑	⇑		⇓*
5	⇔	⇔				⇓	⇓*

The results from the four experiments presented in this chapter indicate that length and weight were unaffected by exposure to clofibric acid. However, there were indications of a general upward trend in HSI after exposure of females to clofibric acid (in one of the experiments it was significantly higher). Similar upward trends in HSI have been determined in studies looking at Clofibrate (Baker *et al*, 2004) and bezafibrate (Krause *et al*, 1996) in rats. There were found to be variations in the levels of biological response of the liver, as with my study, where liver weight increase varied from 3-15% within one dose, and 15-31% at a higher dose (Baker *et al*, 2004).

In the case of HSI for the males, on the other hand, the results were a bit more varied between experiments, and consequently no general trend could be determined. This apparent sexual difference may be attributed to the fact that the 'n' numbers were lower for the males, and consequently slight changes may not have been detected so easily.

As occurred with the HSI, most of the results from experiments reported in this chapter indicated a possible upward trend in GSI in the female fish exposed to clofibric acid. This was the case in all except one of the experiments (the static, short term Experiment 2). In Experiment 3 there was a statistically significant increase in the GSI with exposure to clofibric acid, although this result could have been influenced by the presence of worms, and consequently these results must be interpreted cautiously. The GSI results from the male fish again gave no significant effects or even a trend. However, in Experiment 3 (with worms present) there was a significant increase in male GSI, although again these results should be interpreted cautiously. The results from the two longer-term Experiments (4 and 5) both indicate that there were no trends, either upward or downward, with regard to the effect of clofibric acid on

the GSI. Thus it seems most likely that clofibric acid, at least at the concentration of 1mg per litre, does not have any significant effect on the size of the gonads in relatively short-term experiments. There have not been any previous reports of the effects of Clofibric acid on GSI in fish or mammals, therefore it is impossible to ascertain whether any of these results are usual.

When considering cholesterol within high-density lipoproteins, a significant decrease was observed in the females from Experiment 4 ($p<0.01$), whereas in the males it remained constant, as was also the case in the females from the week-long static Experiment 2. The significant difference seen in Experiment 4 where the numbers of fish within each group were over 50 indicate that clofibric acid is definitely having some effect on HDL cholesterol levels of female fish. These results are contrary to those found in humans and rodents, where HDL cholesterol is found to increase with treatment of fibrates (Staels *et al,* 1998), indicating possible differences in the way that fibrates may act in fish.

The total cholesterol and triglyceride results from Experiment 4 indicated an upward trend in both male and female fish. However, as these two parameters were not measured in the other experiments reported within this chapter, it was impossible to determine if this was a reproducible effect. These effects are discordant to those reported previously, which state that fibrates decrease both plasma cholesterol and triglyceride levels in humans (Blane, 1987) and rodents (Baker *et al*, 2004; Toda *et al*, 2003). However, for these results, perhaps the most accurate summary one can provide is to conclude that, at the concentration tested, clofibric acid did not have any pronounced (significant) effect on total triglyceride and cholesterol levels in these fish.

In the experiments where VTG mRNA expression was measured in female fish, in two cases there was an upward trend and one case a downward trend. Again these were not significant effects when analysed statistically, so from that we can deduce that there are probably not any effects of clofibric acid on the level of expression of this particular gene. This was also the case with Zp3 mRNA expression. It was completely unaffected in the female fish exposed to clofibric acid from all the experiments. There are no previous reports from the literature on the effects of fibrates on either of these particular genes to compare these results to.

Plasma sex steroid levels were measured in only one experiment, Experiment 5, in response to the apparent effect of clofibric acid on spermatogenesis, which was first observed in Experiment 4. The plasma 11-keto-testosterone levels in both male and females from Experiment 5 were unaffected by clofibric acid. In contrast, testosterone levels in both the males and females exposed to clofibric acid at two concentrations in Experiment 5 suggested a definite effect, although unfortunately this was not statistically significant. There were also indications of a dose-related effect in this particular case. This tentative effect of clofibric acid is perhaps linked with the most significant of all the results from this chapter, which was the finding from the sperm counts from Experiments 4 and 5. In both experiments there were statistically significant decreases in numbers of mature sperm in the testes of males exposed to clofibric acid. Although there have been no previous studies looking at the effects of fibrates on steroidogenesis in fish, there is data in the literature showing suppression of expression of genes involved in steroid synthesis by exposure to another fibrate (fenofibrate) on mouse ovary (Toda *et al*, 2003), which is concordant with the present data. Fan *et al*, (2004) also

states that exposure (of rodents) to some peroxisome proliferators (acting through the PPARs) can result in alterations of steroid levels which may be mechanistically linked to adverse effects in reproductive organs.

The results from this chapter suggest that clofibric acid does not seem to have any strikingly significant effects on lipid metabolism in fish. They do, however seem to indicate some sex-specific effects on somewhat unexpected endpoints, for example on sperm counts and steroid levels; perhaps clofibric acid is interacting with steroidogenesis via the cholesterol synthesis pathway.

Some of the observed 'negative' results reported in this chapter, in terms of there being no significant effects of clofibric acid on fish, may not be because there is not an effect, but may instead be a consequence of the methodology used being inappropriate to demonstrate subtle effects on, for example, lipid metabolism. The use of the HDL/LDL gels may not have been sensitive or accurate enough to measure small differences, and consequently it might be that a more quantitative method should have been used. Other results do, however, show definitive effects and appear to be reliable endpoints for monitoring the effect of clofibric acid on adult fish.

The significant effects found in this chapter include an increase in the HSI of females, increases in GSI (although it was in fish with worms present), a decrease in HDL cholesterol in females and decreases in sperm numbers with two different concentrations of clofibric acid. Consistent with this, in the channel catfish, the HSI values have been shown to display a dose-responsive increase (although not significantly so) after clofibric acid exposure (Perkins and Schlenk, 1998). Yang *et al*, 1990 also found an increase in the HSI in response to an intra-peritoneal injection of rainbow trout with a fibrate (ciprofibrate). Scholz (2000) also states that liver weight (which would lead to an increased HSI) is

often increased in fish exposed to xenobiotics, for example, Clofibrate. In mammalian studies, Toda *et al*, 2003, showed that mice exposure to fenofibrate had an increased HSI. This was, however, inconsistent with previous findings.

One of the most interesting findings from this thesis was the significant decrease in sperm number due to exposure to clofibric acid at two different concentrations. Although this result was a surprise, it has previously been reported that arrest of spermatogenesis has been seen in both dogs and monkeys dosed with two times the maximum human dose of Clofibrate (based on surface area) (www.RxList.com). The data indicated that there was a decrease in the number of viable sperm in dosed fish. Also shown was that there was no significant difference in the number of non-viable sperm - implying that the proportion of non-viable sperm within the total sperm count is higher in the dosed fish, consequently affecting sperm quality. Moreover, Warner (1997) suggests that most male fish only release the minimum amount of sperm that is required for fertilisation, so that even a minor decrease in sperm quality and/or quantity will probably result in a decreased fertilisation rate. This could have a major impact on fish fecundity, causing markedly less fertile fish.

The results obtained also indicated that there was a dose-dependant effect on the levels of plasma testosterone with exposure to clofibric acid. In fish, HDL cholesterol is the main exogenous source of cholesterol for steroidogenesis. This cholesterol is transformed to pregnenolone, which is then metabolised to form testosterone. In males it then acts with FSH to promote spermatogenesis and also to stimulate spermiation. An alteration in Leydig cell function and testosterone formation could lead to developmental defects directly affecting androgen dependant tissues of the male reproductive system. In this

way, the lowering of testosterone by clofibric acid may well be the mechanism by which it also lowers sperm count and quality.

In the experiments where the sperm count decreased in males, there was also a significant decrease in the plasma HDL cholesterol concentration in the female fish (in male fish the n numbers were lower, so effects may not have been seen) and a non-statistically different decrease in the VHDL, vitellogenin, gene expression. It could therefore be hypothesised that the decrease in the levels of HDL cholesterol in the female fish may be also mimicked in the males (although not seen due to low 'n' numbers) and that this in turn may be linked to the decrease in levels of testosterone (via cholesterol biosynthesis) and, in turn, to the decrease in numbers of sperm seen. The female effects may have been more identifiable as it has been shown that they are more vulnerable to toxic exposure than males (Winzer *et al*, 2002).

In an experiment carried out in mice exposed to Bezafibrate, Gazouli *et al* (2002) found evidence to support the direct action of activated PPARα on steroidogenic cells such as the Leydig cells, where PPARα inhibits steroidogenesis through suppression of the transport of cholesterol into the mitochondria. Alterations in Leydig cell function and testosterone synthesis will in turn directly affect sperm production. More recently, Fan *et al* (2004) reported that as well as the known effects of fibrates on lipid metabolism genes, exposure also leads to an alteration in the expression of the genes involved in steroid metabolism.

One very important concern highlighted from the results shown here is that if fibrates are having effects on spermatogenesis and steroidogenesis in fish exposed to quite low levels, what is happening to the fertility of men taking them at prescribed levels (2g/day)? As heart disease is usually a late onset

disease, it is generally older people who are prescribed these drugs and therefore changes in fertility in this group of men, who have probably already had children, may go unnoticed. This work does highlight a real concern for the proportion of younger men who are taking fibrates for heart disease, and also young diabetic men who are now also being prescribed fibrates, (Anna-Maria Andersson, personal communication), who are yet to have a family.