

# Chapter 3: Potential effects of Clofibric acid on early development of fathead minnow embryos

### 3.1 Introduction

The aim of this thesis was to utilize different approaches to determine the effects, if any, of clofibric acid exposure on different stages of fish development. The following chapter considers a single exposure experiment designed to determine the effect of different concentrations of clofibric acid on the development of fathead minnow embryos.

Egg quality is the most important factor for the production of healthy and high quality fish larvae. Factors affecting this quality are primarily determined by the intrinsic properties of the egg itself and also by the environment in which it is fertilised and subsequently incubated. Fish embryos are particularly sensitive to environmental pollutants by virtue of the fact that they are surrounded by them during the crucial stages of their development. Many chemicals have the potential to have effects at different levels – from the molecular to the whole organism and even the whole population. They can also cause adverse effects at different stages of development. Despite their increasing environmental significance, investigations concerning the effects of pharmaceutical drugs on early developmental stages are lacking. Having said that, however, in the field of ecotoxicology, the early life stage test using fish embryos is one of the most widely used tools for investigating the detrimental effects of aquatic pollution. Several authors consider the early life stage to be the most sensitive (including Kristensen, 1995; Hallare *et al*, 2004), although this may not always be the case with all compounds and all species.

The fathead minnow is a small, freshwater species that adapts well to aquaria, as previously discussed in Chapter 2. It is easy to grow and maintain in different environments, it has a short generation time and breeds all year

round, and produces large numbers of eggs. These factors therefore make it an excellent model for studying embryo effects induced by pollutants.

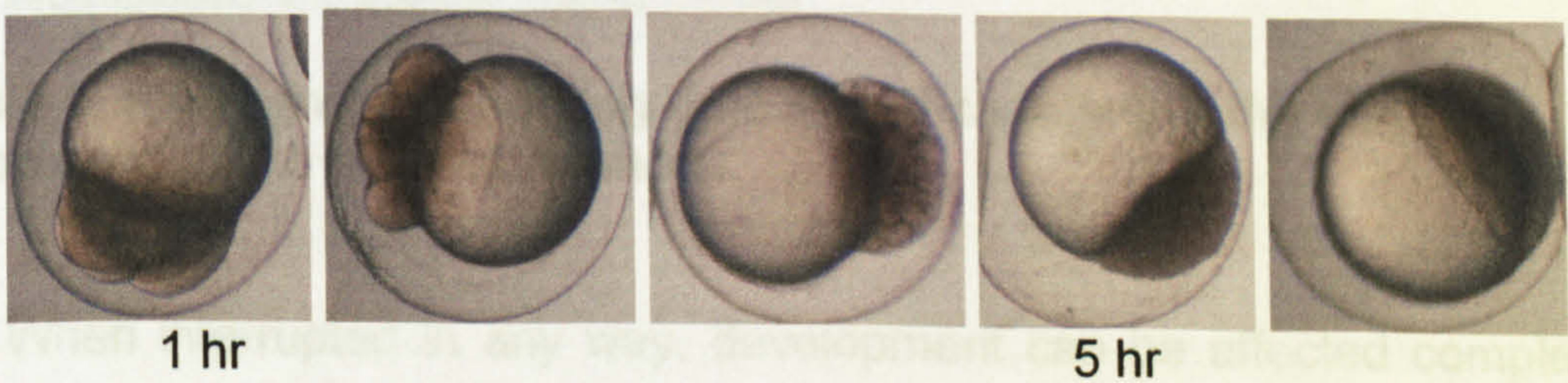
### 3.1.1 Basic embryo development

It is important when looking at the effects of different pollutants on embryo development to consider normal development first. Fathead minnow embryos develop like those of all teleost fish. The normally observed developmental events include: blastulation, gastrulation, completion of somites and optic cup formation, spontaneous contraction and tail detachment, retinal pigmentation, heart beating, body pigmentation and commencement of hatching (Figures 3.1, 1-6).

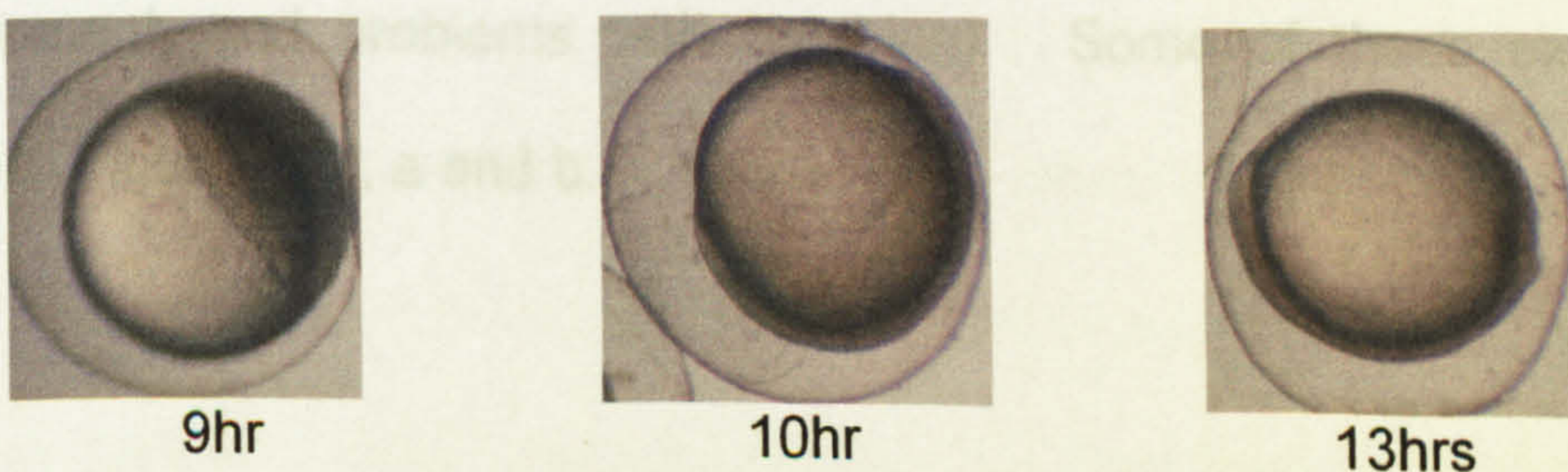
0) Unfertilised egg



1) Blastulation



2) Completion of gastrulation



3) Completion of somites and optic cup formation



15hrs



17hrs

4) Spontaneous movement and tail detachment



23hrs



29hrs

5) Retinal pigmentation/heart beating/body pigmentation



46hrs

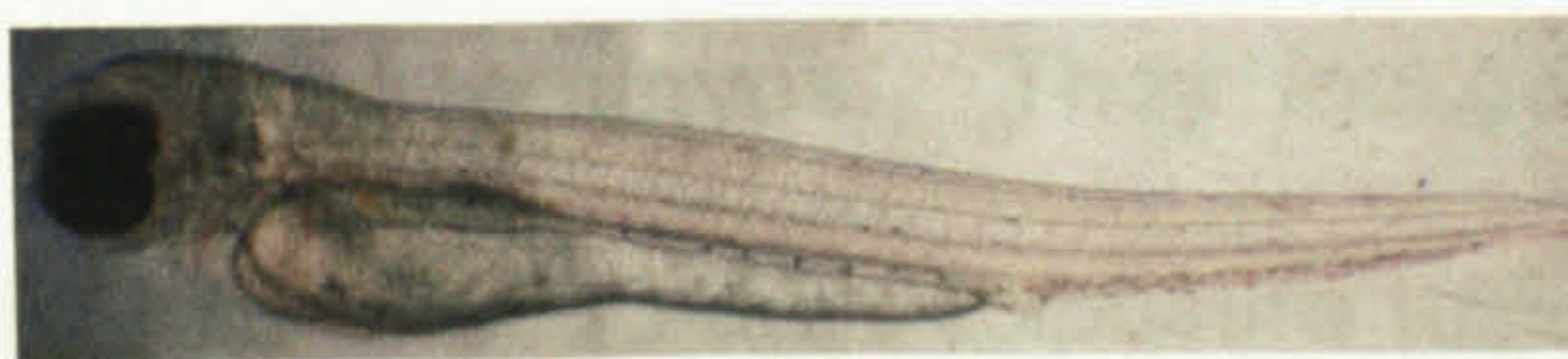


69hrs



78hrs

6) Commencement of hatching



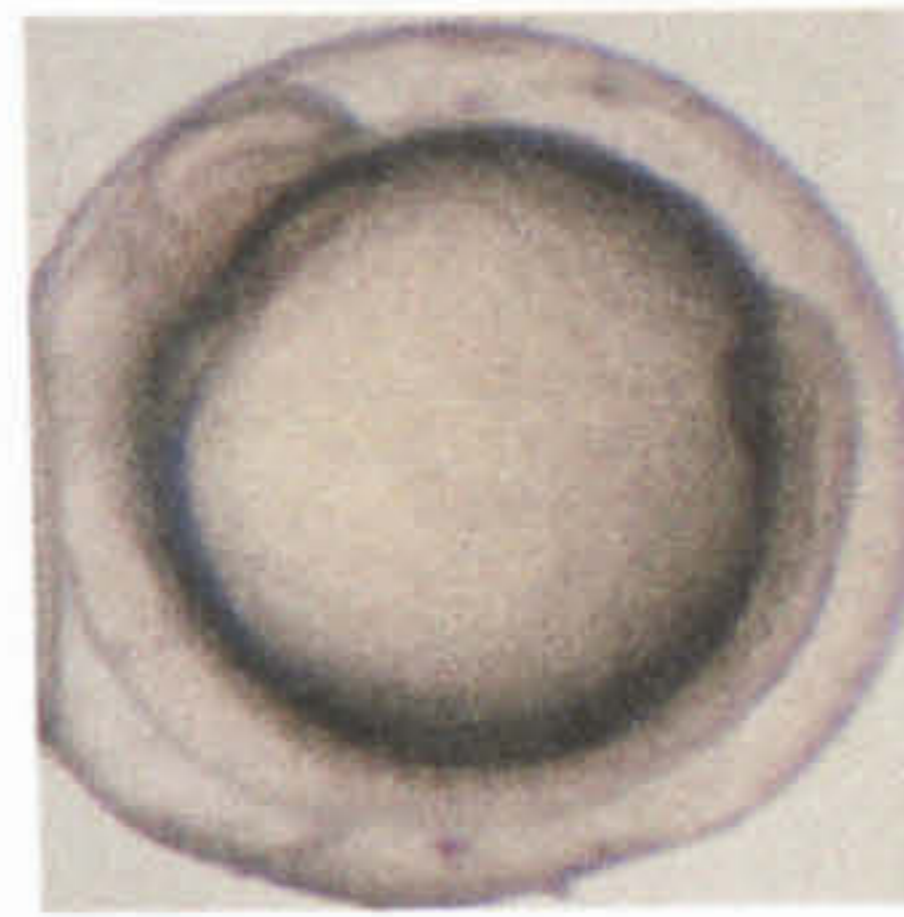
**Figure 3.1: Photographs showing time specific stages in the early development of the fathead minnow.**

When interrupted in any way, development can be affected completely (immediate death) or to various degrees (leading to malformations, delays in development, and problems with hatching). Some of these examples are shown in Figures 3.2, a and b.

3) Completion of somites and optic cup formation



15hrs

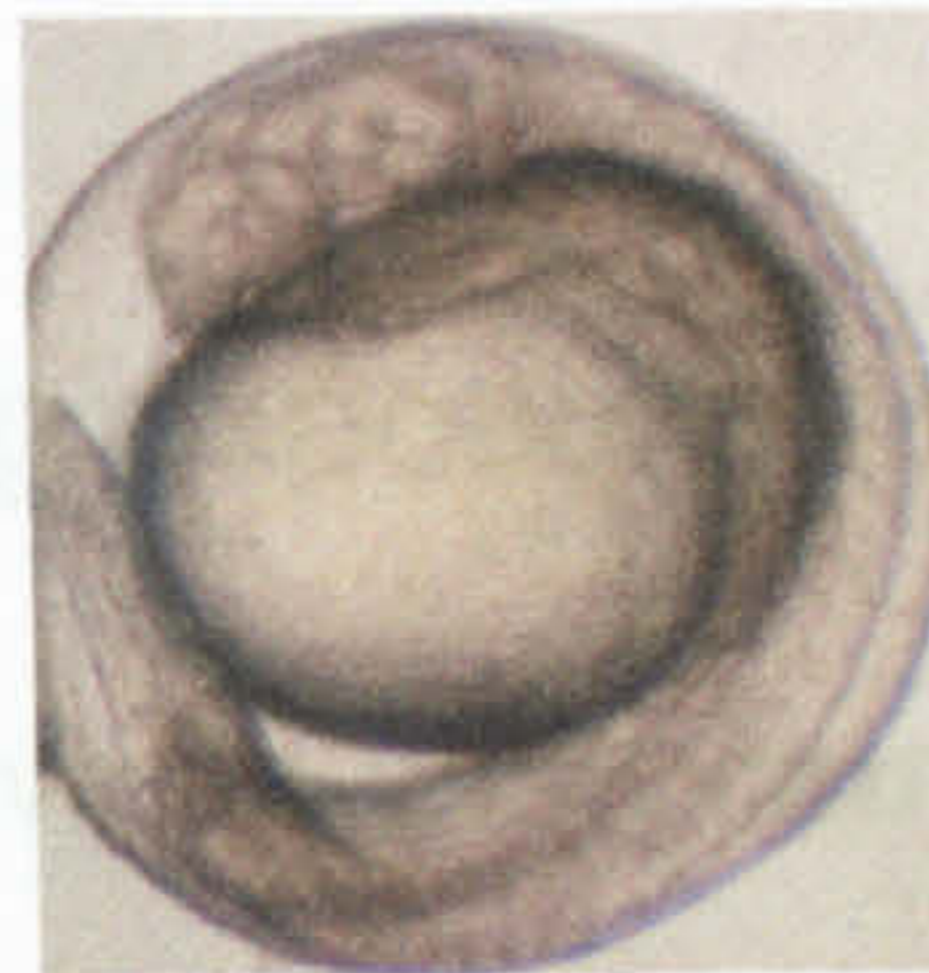


17hrs

4) Spontaneous movement and tail detachment



23hrs



29hrs

5) Retinal pigmentation/heart beating/body pigmentation



46hrs



69hrs



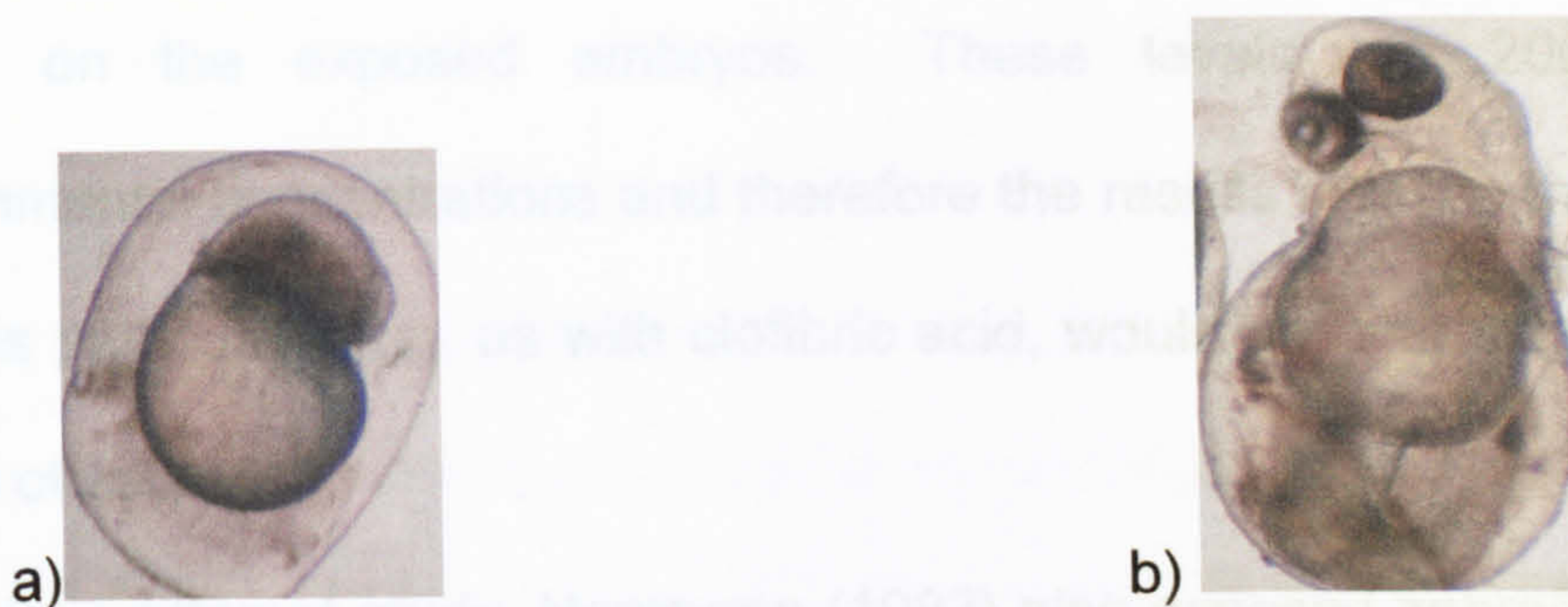
78hrs

6) Commencement of hatching



**Figure 3.1: Photographs showing time specific stages in the early development of the fathead minnow.**

When interrupted in any way, development can be affected completely (immediate death) or to various degrees (leading to malformations, delays in development, and problems with hatching). Some of these examples are shown in Figures 3.2, a and b.



**Figure 3.2: Some examples of problems during development: a) malformed during blastulation b) difficulty in hatching.**

### 3.1.2 Results of previous egg exposure experiments

There has been some previous work carried out to determine the effects of clofibric acid on fish embryos. In 1997, Henschel *et al* carried out an acute toxicity test using zebrafish embryos. Using this they found embryos to be sensitive to clofibric acid, with an  $EC_{50}$  of 86mg/l. They also looked at sublethal effects of clofibric acid, including examining heartbeat in the embryos. They found an  $EC_{50}$  on pulse rate at 100mg/l clofibric acid and determined that a drop in heartbeat frequency of greater than 40%, when compared to that of the controls, was considered lethal. Ferrari *et al* (2003) also carried out a study using zebrafish embryos. They looked at hatching rate and embryolarval mortality rate after 10 days exposure to 5 concentrations of clofibric acid. After 24hrs the dead eggs were counted and survival recorded. They found a NOEC of 70mg/l, and a LOEC of 140 mg/l. Thus, it seems as though fish embryo development is relatively unaffected by clofibric acid, at least until very high concentrations are used.

In embryo studies using other types of pharmaceuticals, Hallare *et al* (2004), looked at the effect of diclofenac on zebrafish embryos at six different concentrations (from 0-2000 $\mu$ g/l). They found an apparent delay in hatching at the later part of a 96hr exposure period, but they found no other significant

effects on the exposed embryos. These levels are 2000 times the environmental concentrations and therefore the results indicate that it is unlikely that this particular drug, as with clofibric acid, would pose a hazard to early life stages of zebrafish.

In a different study, Herrmann (1993) also exposed zebrafish embryos to a different drug, an anti-epileptic drug called valproic acid (VPA), a known teratogen. Exposure to VPA was found to cause retardation and cessation of development of the embryos. It generated malformations such as oedema, brain deformities, a shortened and bent tail, and bipartite axiation of the posterior trunk.

Nagel (2002) exposed zebrafish embryos to propranolol (an antagonist of adrenaline/noradrenaline and the  $\beta$ -adrenergic receptors that reduces the frequency of the heart beat and power of contraction in mammals). He found a significant reduction in the heartbeat frequency with increasing exposure to propranolol.

It is clear from the results of these studies that the use of fish embryos for routine toxicological testing is increasing and can be a sensitive tool for monitoring the sublethal effects of different pharmaceuticals that are in the environment.

### 3.2 Experimental protocol

A preliminary study was carried out to look at the effects of clofibric acid on the development of the fathead minnow embryo directly after fertilization (1hr post) to hatch (normal fertilization to hatch take about 4 days at 25°C). This experimental design was adapted from the zebrafish embryo test (Schulte and

Nagel, 1994; Henschel, 1997), in which sublethal effects on embryo development are examined.

A batch of adult fathead minnows was set up to spawn in pairs in 30 litre tanks containing breeding tiles, with a 16 hour light/8 hour dark photoperiod. The adults were fed 3 times per day, once with gamma irradiated, adult Brine shrimp (Tropical Marine Centre, Chorleywood, Herts) and twice with flake food (King British Tropical flake fish food, Lillicos, Surrey). The fish were monitored and at one hour post spawning (to ensure they were water hardened) the eggs were collected by carefully rolling them from the spawning tiles. A twelve well plate was set up such that wells 1 to 3 acted as controls, 4 to 6 contained clofibric acid at 1ng/l, wells 5 to 9 clofibric acid at 1µg/l, and wells 10 to 12 clofibric acid at 1mg/l (as shown in Figure 3.3), each well containing 5ml of solution (see Section 2.1.2 for the method used to make up the stock solutions). In this experiment, all stock solutions of clofibric acid were made up using borehole water (supplied on site at Calverton Fish Farm, Nottingham).

Embryos were placed into the wells, 2 per well, and hence there were six embryos per treatment. Photographs were then taken and the embryos monitored every hour using a Cannon digital camera, connected to a Kyowa microscope (x4 magnification, 1/60 light, focus 3.5) from one-hour post fertilisation until the embryos hatched or died. The water was changed daily, by static renewal, and the temperature was maintained at 24±2°C.





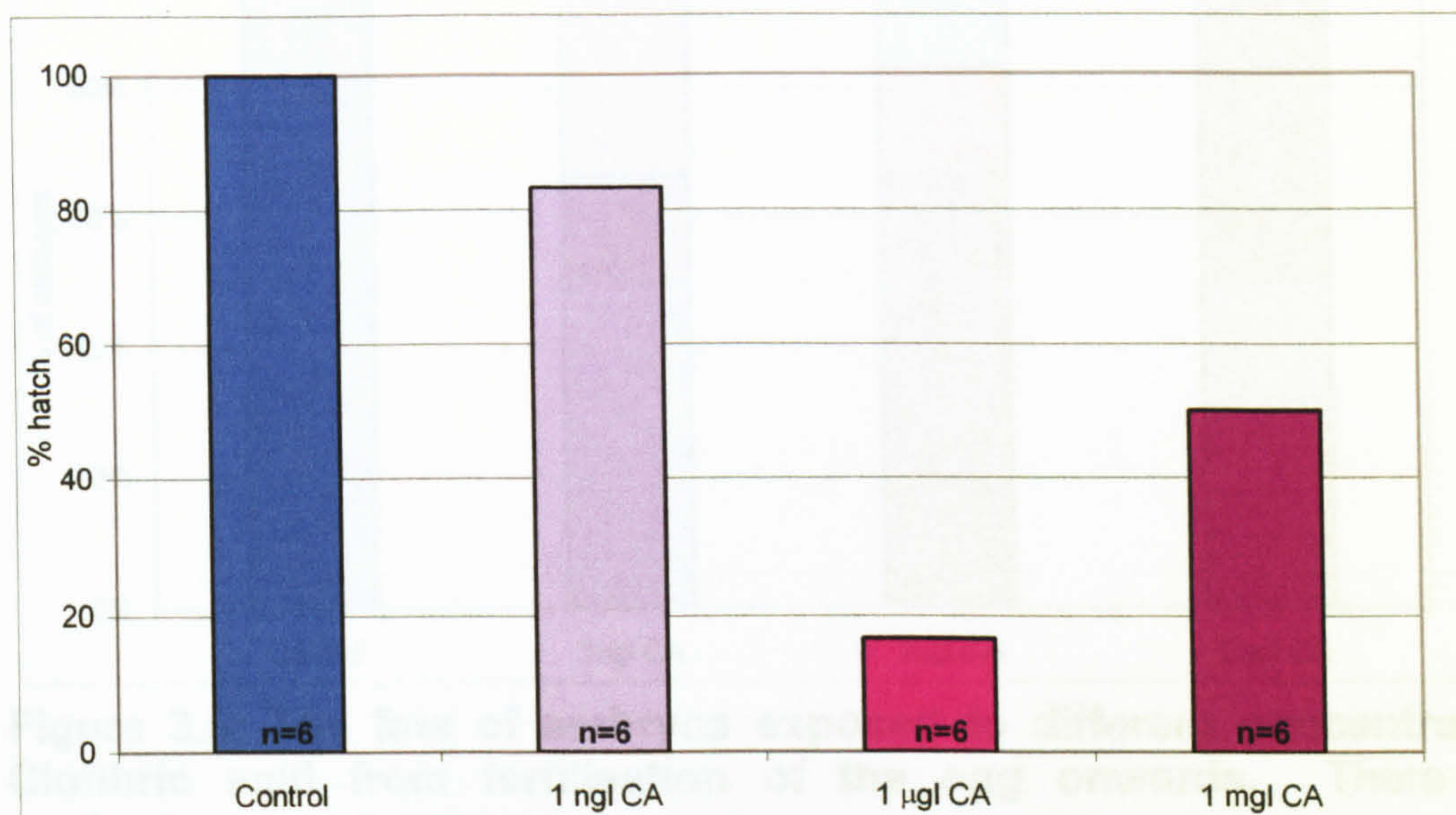
**Figure 3.3: A 12 well plate showing the layout of treatments in the plate.** There were 3 control wells, and 3 wells for each of the three treatments of clofibric acid (1ng, 1 $\mu$ g and 1mg/litre). Two eggs (not visible here) were added to each well, and hence there were 6 eggs per treatment.

The photographs (1247 in total) were subsequently analysed and different parameters quantified, including the number of deaths, time to hatch, hatchability, size of the egg, time to development of the vitelline vein, spleen, and eye darkening.

The size of the egg was measured in two ways, both using the photographs previously taken. Initially, the diameters of the eggs were measured using Microsoft Paint (Version 5), where two diameter measurements were taken per egg, for each egg in the treatment, at every hour until hatch. The numbers produced gave the two diameters in pixels, and consequently all were relative to one other. These data were then analysed statistically. Measurements were also carried out on the egg photographs by hand, (using a ruler) to obtain the mean diameter (mm) at fertilisation and just before hatch. Two measurements per egg were again taken for each egg within each treatment. These data were again relative (rather than actual diameters) as the measurements were from the photographs.

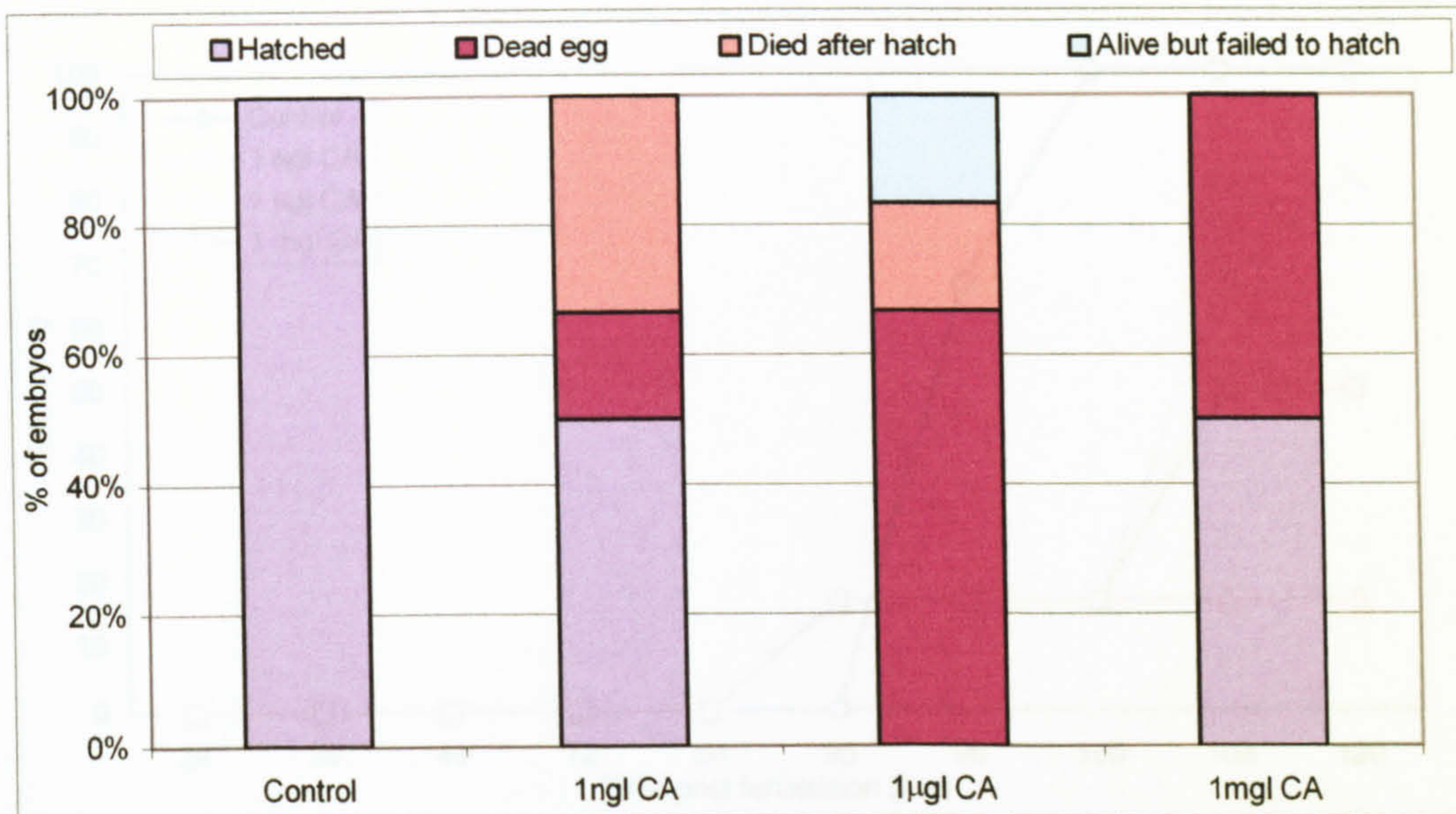
The data were analysed statistically to see if there were any significant differences when clofibric acid-exposed embryos were compared to the controls.

### 3.3 Results



**Figure 3.4: The effect of different concentrations of Clofibric acid on the hatching success of fathead minnow embryos exposed from fertilisation to hatch.**

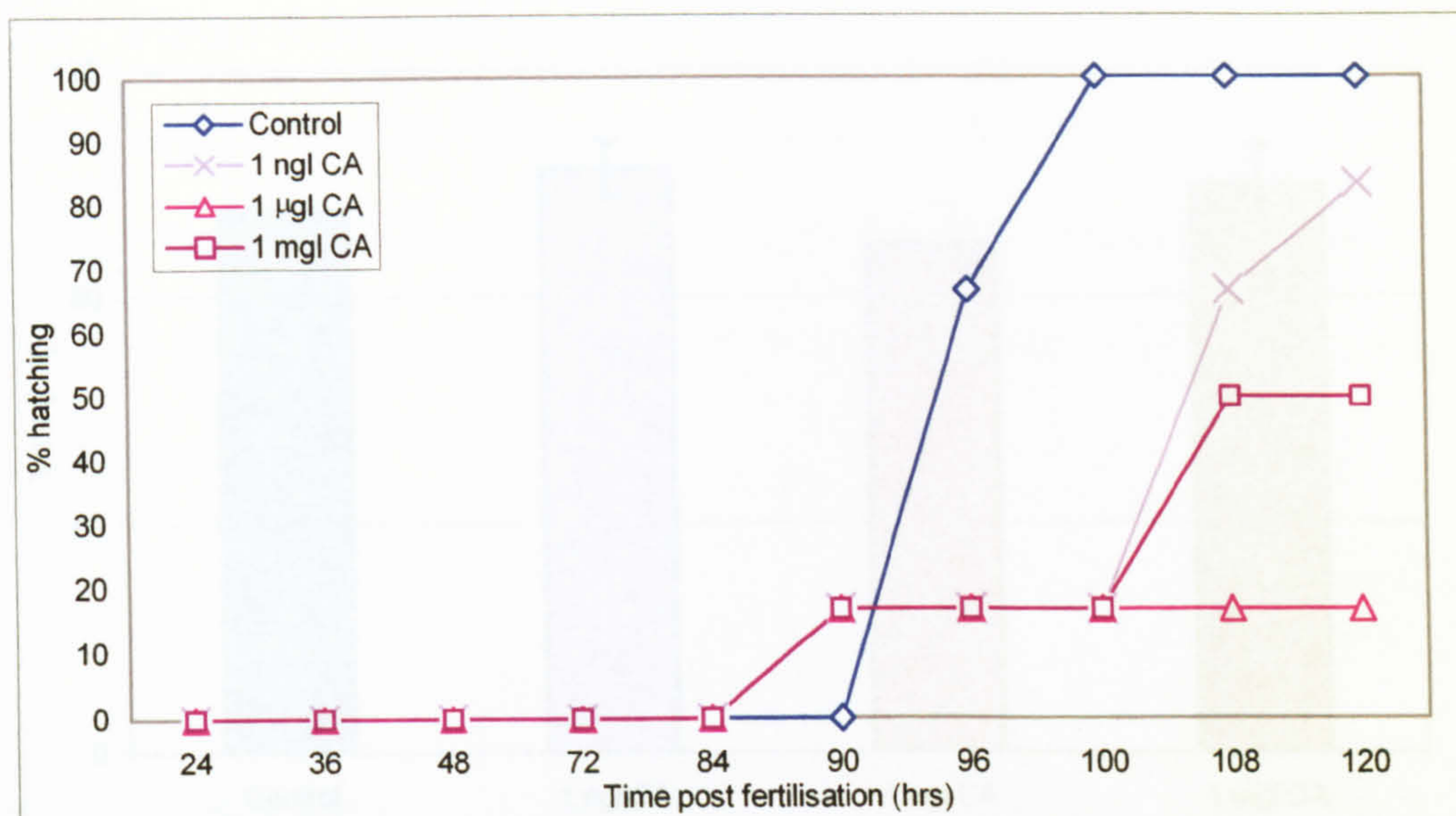
As Figure 3.4 shows, there was a definite effect of clofibric acid on the percentage of embryos that hatched. In the controls, all of the embryos hatched, whereas in the 1ng/l dose, 83% hatched, in the 1µg/l concentration, 16% hatched and in the 1mg/l concentration, 50% hatched. The results indicate that there may be a biphasic dose response of clofibric acid on the hatchability of the embryos: it may be that at the lower two doses, clofibric acid accumulates and causes mortality in a dose responsive way; however, at the highest dose, those protection systems which prevent toxic actions might be triggered and upregulated and consequently may lead to more embryos surviving. However, due to the low numbers of embryos it was not possible to demonstrate this statistically. Additional studies are necessary in order to investigate this phenomenon further.



**Figure 3.5: The fate of embryos exposed to different concentrations of Clofibric acid from fertilisation of the egg onwards.** There were 6 egg/embryos per treatment.

As can be seen from Figure 3.5 (which shows the fate of embryos exposed to clofibric acid), there seemed to be an effect of clofibric acid on hatchability of the embryos. In the controls there was 100% hatch, in the 1ng/l dose, however, 50% hatched normally, 33% died after hatching, and 17% died before hatching. In the 1µg/l dose, 66% died before hatch, 16% died after hatch and 16% did not hatch at all, but stayed alive inside the egg until termination of the exposure (at 120 hours; normal hatching occurs around 96hrs). In the 1mg/l dose, 50% hatched normally, and 50% died before hatching. Interestingly, these preliminary results indicate that there is more of an effect at the lower doses than at the highest concentration of clofibric acid.

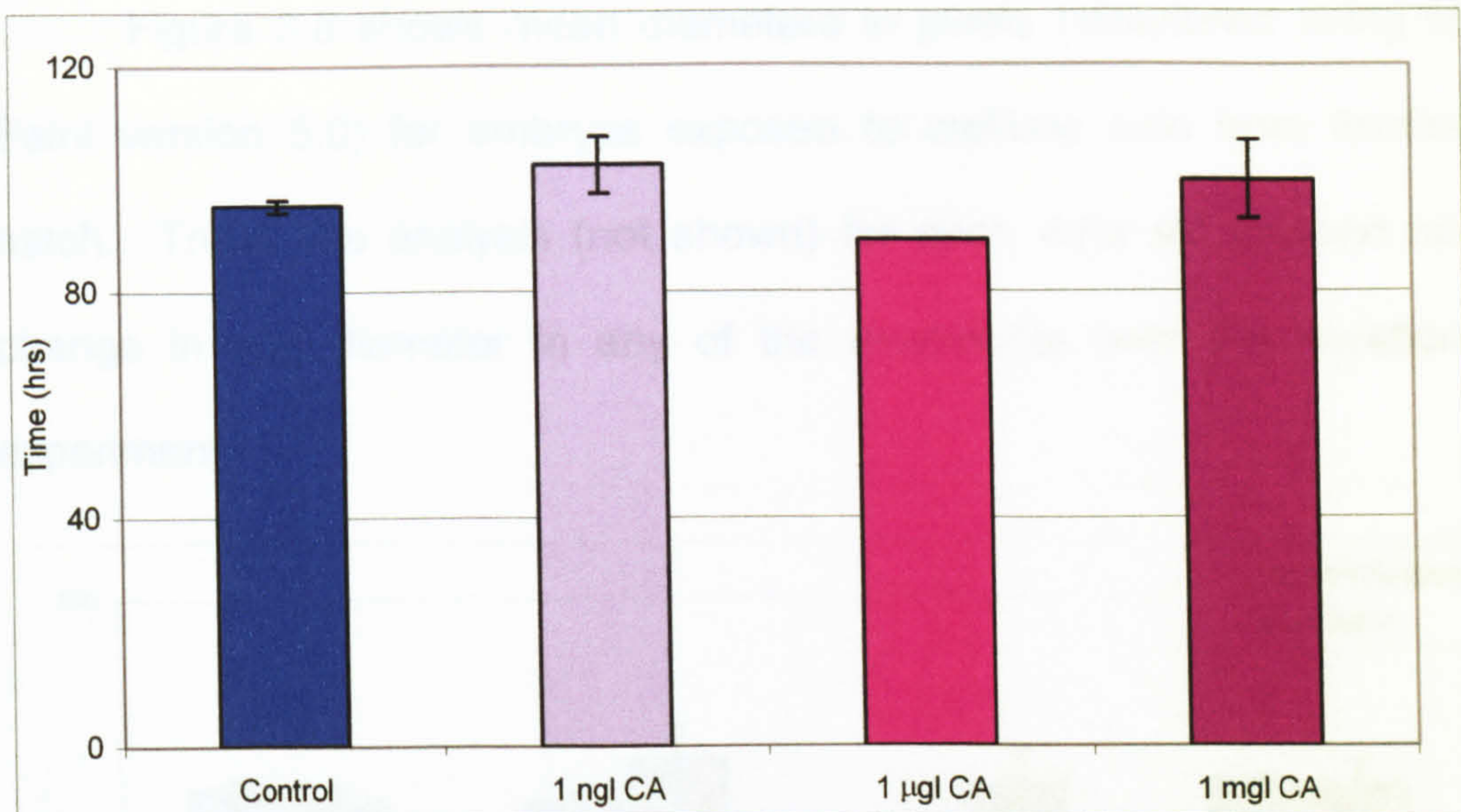
On a qualitative note, the late hatching embryos in my opinion tended to look 'less healthy' early on in their development; however this was difficult to quantify. There were definite differences in the yolk composition (the yolk seemed more opaque and appeared to contain lipid droplets) and differences in heart rate were also noted in the embryos exposed to the higher concentrations of clofibric acid.



**Figure 3.6: Timing of successful hatching of fathead minnow embryos exposed from fertilisation of the eggs to different concentrations of Clofibric acid (CA).** There were 6 eggs/embryos per treatment. All 6 control embryos hatched, 5 treated with 1ng/l CA, 1 treated with 1µg/l CA, and 3 treated with 1mg/l CA hatched.

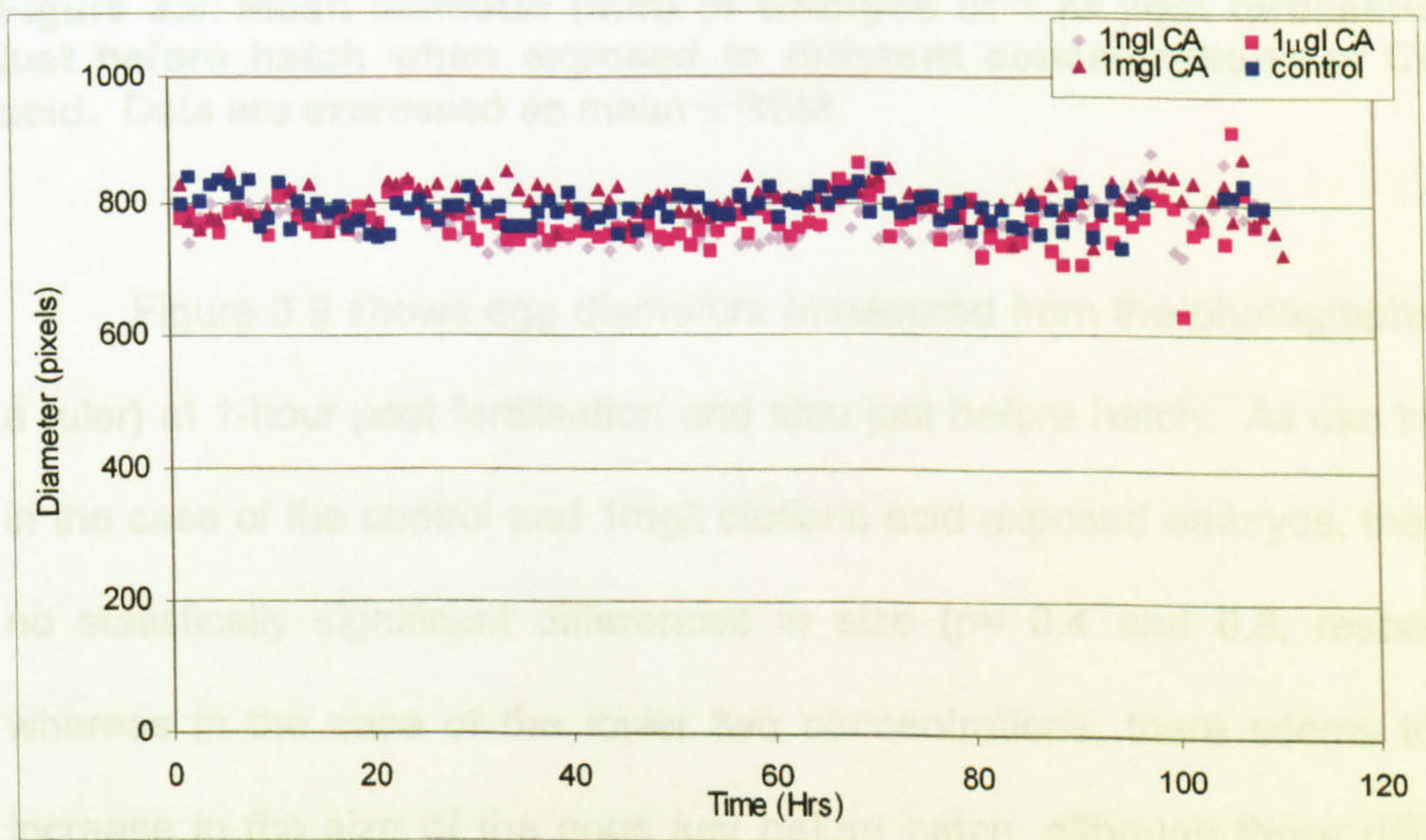
Figure 3.6 shows the relationship between time and the hatching success rate (%) of fathead minnow embryos exposed to the differing concentrations of clofibric acid. As can be seen, there was an apparent delay in hatching due to exposure to clofibric acid. In the controls, all embryos hatched within a 4-hour window of the 'normal' 96hrs, whereas in the embryos exposed to clofibric acid there was great variability in the time of hatch; it occurred between 18-30 hrs after the time the first embryo hatched (which in all the concentrations occurred by 90hrs).

On a qualitative note, the late hatching embryos in my opinion tended to look 'less healthy' early on in their development; however this was difficult to quantify. There were definite differences in the yolk composition (the yolk seemed more opaque and appeared to contain lipid droplets) and differences in heart rate were also noted in the embryos exposed to the higher concentrations of clofibric acid.



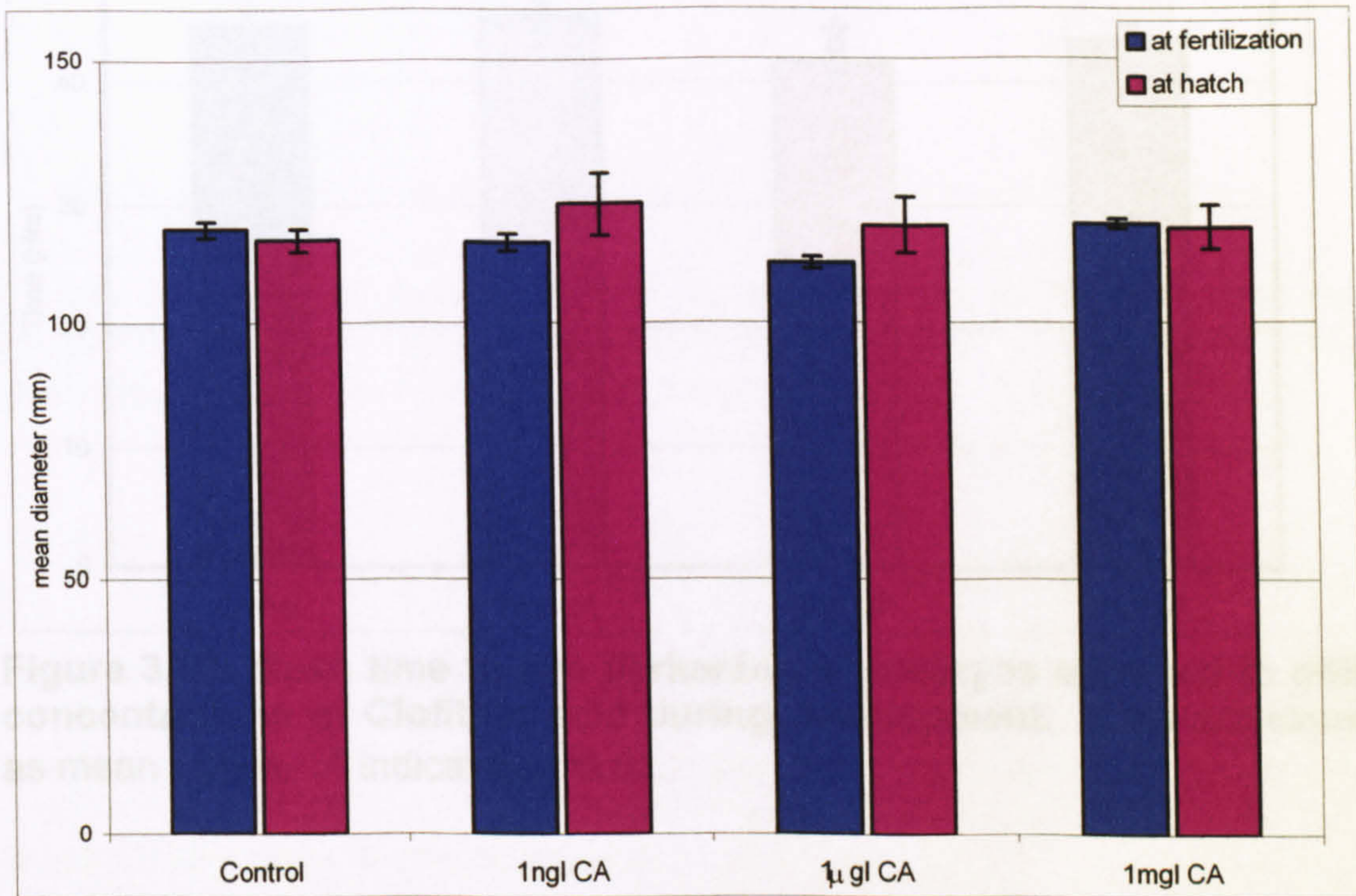
**Figure 3.7: The average time taken for embryos to hatch after exposure to different concentrations of Clofibric acid during their development. Data are expressed as mean  $\pm$  SEM.**

Figure 3.7 shows the average time to hatch of the embryos exposed to the different concentrations of clofibric acid. When analysing these data statistically, there were no significant differences when each concentration was compared to the control, and also to each other.



**Figure 3.8: The mean diameter (pixels) of fertilized eggs during their development when exposed to different concentrations of Clofibric acid from 1hr post fertilisation to hatch.**

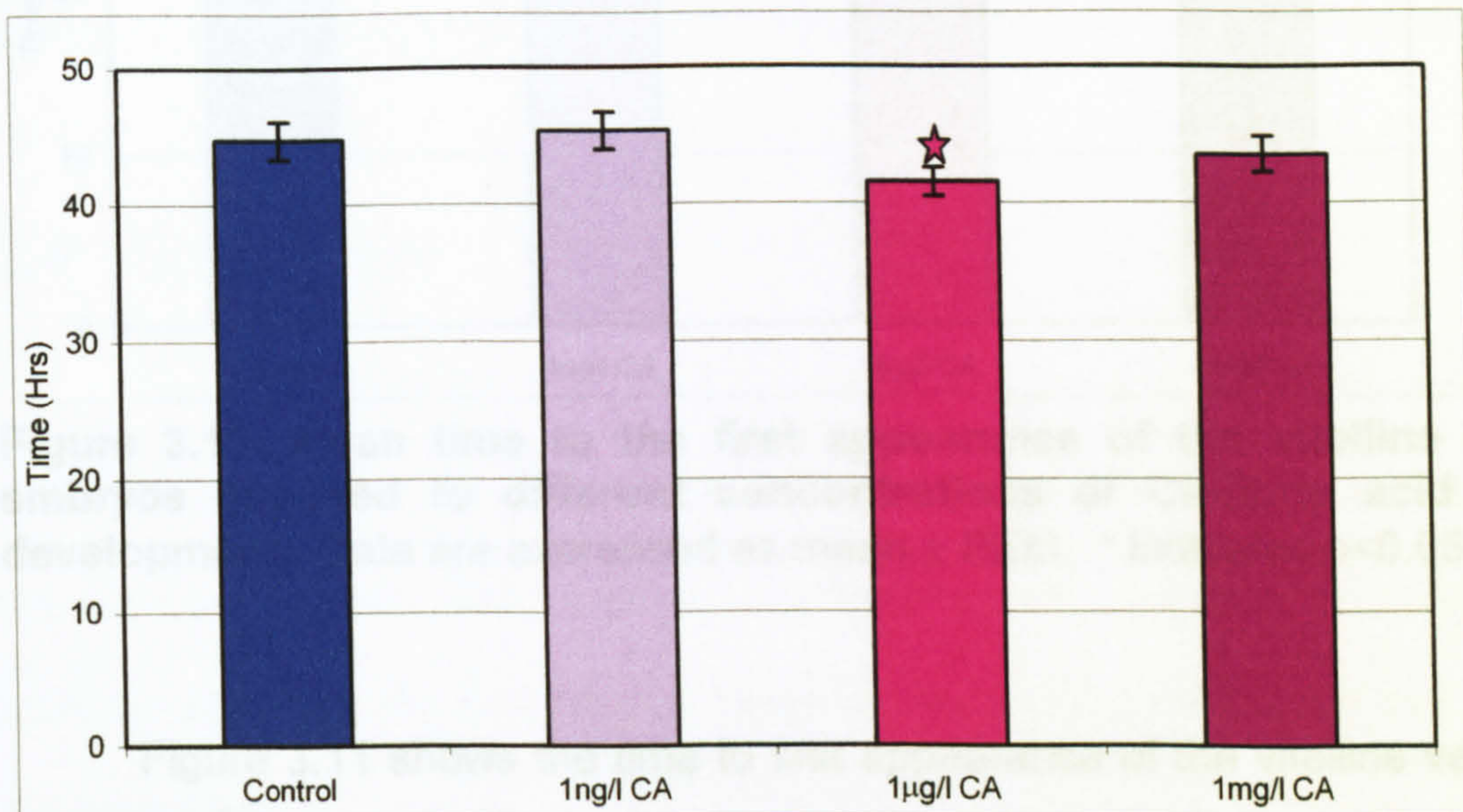
Figure 3.8 shows mean diameters in pixels (measured using Microsoft Paint version 5.0) for embryos exposed to clofibrac acid from fertilisation to hatch. Trend line analysis (not shown) for each data set showed no overall change in egg diameter in any of the treatments over the duration of the experiment.



**Figure 3.9: Mean diameter (mm) of embryos at 1 hr post fertilisation and just before hatch when exposed to different concentrations of Clofibrac acid. Data are expressed as mean  $\pm$  SEM.**

Figure 3.9 shows egg diameters (measured from the photographs, using a ruler) at 1-hour post fertilisation and also just before hatch. As can be seen, in the case of the control and 1mg/l clofibrac acid exposed embryos, there were no statistically significant differences in size ( $p=0.4$  and  $0.8$ , respectively), whereas in the case of the lower two concentrations, there seems to be an increase in the size of the eggs just before hatch, although these differences are still not significant ( $p=0.6$  and  $0.2$ , respectively). At 1-hour post fertilisation, the error bars (in all 4 treatments) were very small, whereas at hatch

(particularly in clofibric acid exposed fish) the variance (error bars) was much greater, perhaps indicative of possible effects of clofibric acid on the eggshell structure.

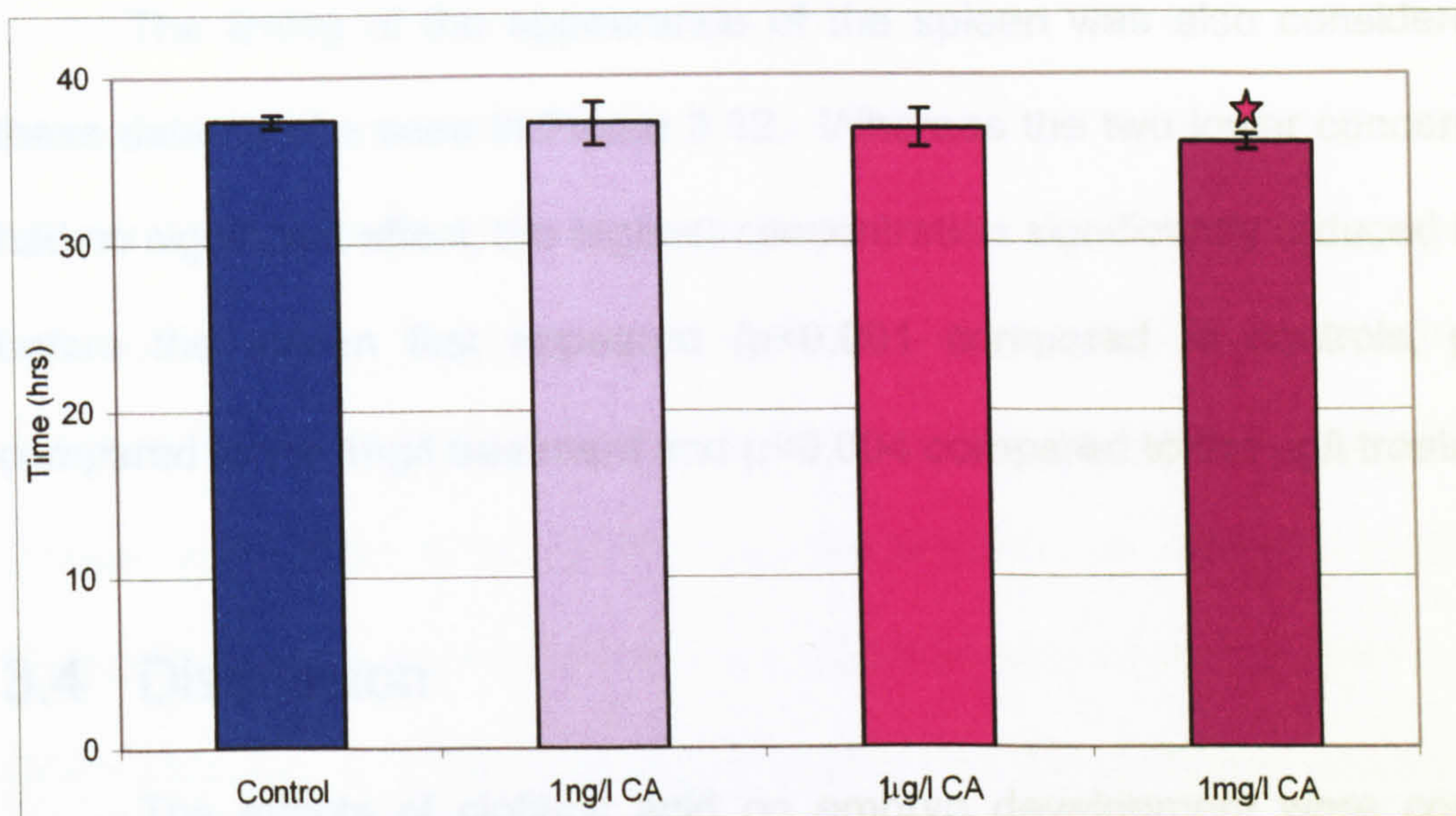


**Figure 3.10: Mean time to eye darkening of embryos exposed to different concentrations of Clofibric acid during development.** Data are expressed as mean  $\pm$ SEM. \* indicates  $p=0.01$ .

taken for the vitelline yolk to appear.

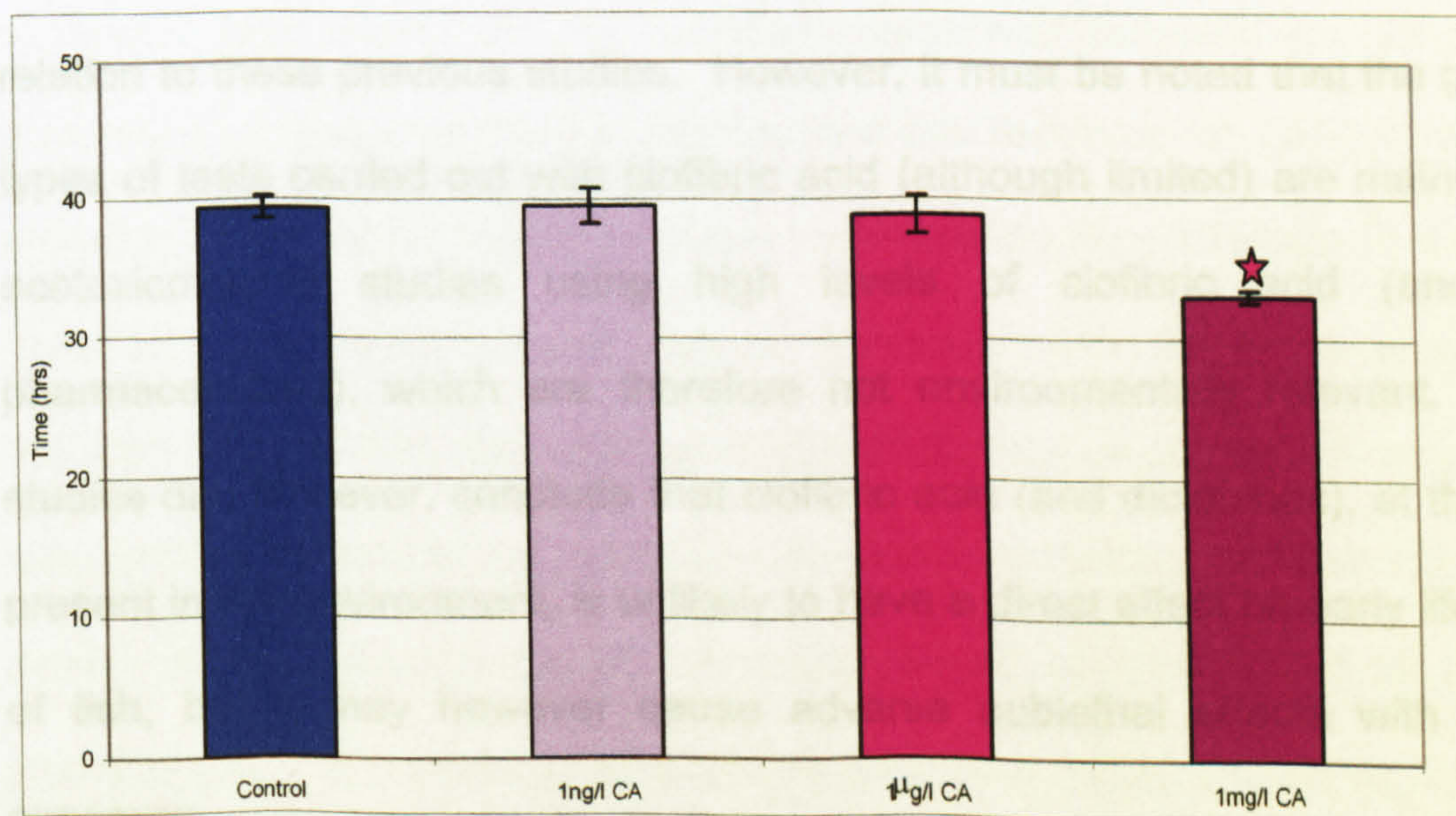
The time taken for the embryos eyes to darken is shown in Figure 3.10. There was a statistically significant difference between the time taken in control and 1µg/l embryos ( $p=0.01$ ), and also a possible trend ( $p=0.06$ ) when comparing 1ng/l and 1µg/l - exposed embryos. There were, however, no significant differences between the other groups of dosed embryos (1ng/l and 1mg/l) when considering this particular stage, so it can be determined there were no dose-related effects observed. Although embryos exposed to 1µg/l clofibric acid had darkened eyes earlier than control embryos or embryos exposed to lower concentrations (1ng/l), this apparent effect of clofibric acid was not observed in the embryos exposed to the highest concentration (1mg/l).

Data are expressed as mean  $\pm$ SEM. \* indicates  $p<0.05$ .



**Figure 3.11: Mean time to the first appearance of the vitelline vein in embryos exposed to different concentrations of Clofibric acid during development.** Data are expressed as mean  $\pm$  SEM. \* indicates  $p < 0.05$ .

Figure 3.11 shows the time to first appearance of the vitelline vein. The two lowest concentrations had no observed effect on this endpoint. However, the highest concentration caused a significant ( $p = 0.05$ ) decrease in the time taken for the vitelline vein to appear.



**Figure 3.12: Mean time to the first appearance of the spleen in embryos exposed to different concentrations of Clofibric acid during development.** Data are expressed as mean  $\pm$  SEM. \* indicates  $p < 0.001$ .



The timing of the appearance of the spleen was also considered, and these data can be seen in Figure 3.12. Whereas the two lower concentrations had no significant effect, the highest concentration significantly reduced the time before the spleen first appeared ( $p < 0.001$  compared to controls,  $p < 0.002$  compared to the 1ng/l treatment and  $p < 0.004$  compared to the  $\mu\text{g/l}$  treatment).

### 3.4 Discussion

The effects of clofibric acid on embryo development were considered and, although the numbers of embryos used within this experiment were low, the indications are that even at environmentally relevant concentrations, clofibric acid has the potential of causing deleterious effects on fish embryo development.

Previous findings from embryo exposure experiments have been reported by Henshel *et al* (1997), Hallare *et al* (2004) and Ferrari *et al* (2003), and these are mentioned in the Introduction to this chapter. When taking into account the results of this embryo experiment, it is important to consider them in relation to these previous studies. However, it must be noted that the previous types of tests carried out with clofibric acid (although limited) are mainly acute ecotoxicological studies using high levels of clofibric acid (and other pharmaceuticals), which are therefore not environmentally relevant. These studies did, however, conclude that clofibric acid (and diclofenac), at the levels present in the environment, is unlikely to have a direct effect on early life stages of fish, but it may however cause adverse sublethal effects with life-long exposure.

The results of this chapter found significant effects on the percentage of embryos that hatched, their hatchability and rate of hatching, and the time taken

to reach different stages of their development. When these results are compared with those of previous authors, it can be seen that the significant effects I have seen are at very much lower concentrations, and most importantly, more environmentally relevant, than previously reported. For example, Henschel *et al*, (1997), reported an EC<sub>50</sub> of 86mg/l and a NOEC of 70mg/l, and Ferrari *et al*, (2003) reported a LOEC of 140mg/l. However, all these values were obtained from acute (short-term) toxicity tests, using mortality as an endpoint. It is therefore very apparent that the traditional ecotoxicological endpoints such as mortality are not sensitive enough to detect more subtle effects on embryos exposed to clofibric acid (though these may still be very significant).

It has been very recently reported in Lahnsteiner *et al* (2004) that G6PDH activity (the enzyme involved in gluconeogenesis) is related to egg quality and therefore very important in the development of the embryo. Monosaccharides are needed for the synthesis of nucleotides (RNA and DNA) – whose levels increase significantly during embryogenesis (as there are extremely high levels of cell division) - and are essential for all anabolic processes. I propose the hypothesis that the reduction in G6PDH that was detected in the adult fish in my later experiments (see Chapter 6) may also occur in these embryos (and may be a consideration for future studies). A reduction in G6PDH may reduce monosaccharide levels, which may in turn reduce DNA synthesis and consequently slow cell division, and that this slowing down of cell division could ultimately result in the delay in hatching. Interestingly, the inhibition of G6PDH has also been shown to affect the production of egg yolk precursor proteins in the liver and also increase an organism's susceptibility to xenobiotic toxicity (Winzer, 2002). These factors

could be manifest in my embryos by the decrease in quality of the yolk and changes in heart rate. Therefore, a reduction of G6PDH induced by clofibric acid may itself make clofibric acid more toxic. Although not xenotoxic in adults, this might have an effect on more susceptible stages of development. In adult fish there is a lower rate of cell division compared with embryos, so the effect of an inhibition of G6PDH may not be as well revealed.

Differences were observed in the yolk of exposed embryos: some of the yolks became more opaque and appeared to have lipid droplets within them (although this was not quantified). Lipoprotein lipase (LPL) has previously been shown to be involved in lipid mobilisation in the egg yolk (Kwon *et al*, 2001) and I have shown a decrease in LPL in my adult fish exposures (as seen in the results of Experiment 5 - Chapter 6). The decrease in LPL seen in the adults may also be true in these embryos, and this may therefore account for the differences in egg yolk seen in this study (although this is only speculative as neither LPL expression or egg yolk quality were measured; this may be subject for future work). However, the previous report has shown that this effect of LPL is during oogenesis and not during embryogenesis in Rainbow trout (Kwon *et al*, 2001) however, this may not be the case in fathead minnow.

More specific effects of clofibric acid on embryogenesis were also noted. For example, the time taken for the development of the spleen was affected by the highest concentration of clofibric acid, which is important for immune responses. These effects have not previously been reported in the literature, and consequently may need replication. One possible link may be via PPAR $\alpha$  which is well known to be activated by fibrates, and is expressed in macrophages which are produced by the spleen. One theory is that fibrates induce PPAR $\alpha$  and a toxic response leading to apoptosis in the spleen – seen

as an enlargement of the spleen, which in the developing embryo may make it easier to see, and hence it may be seen earlier in development. In rats, however, it has been noted that exposure to fibrates decreases the size of the spleen (Cleary *et al*, 1987). Based on my observation, it would be interesting to examine the spleen from exposed embryos allowed to grow on, to see if there were any changes in development of the spleen/macrophage production. Perturbations in macrophage number would have a detrimental effect on the immune response and overall fitness.

It was also found that the vitelline vein developed significantly faster in the embryos exposed to the highest concentration of clofibric acid. The vitelline vein is crucial for embryo development as it links the yolk to the embryo, and consequently feeds the embryo during development. The time to eye darkening was also faster in the embryos exposed to the  $\mu\text{g/l}$  concentration of clofibric acid when compared with the control. However, I am unsure how biologically relevant this is. Although the development seemed to be faster in the exposed embryos, the time taken for them to hatch was longer. It was also noted in the clofibric acid exposed embryos that the heart rate seemed slower, although this was not quantified, which ties in with results from rats studies, which also showed a decrease in heart rate (Cleary *et al*, 1987).

When compared with previous studies using different pharmaceuticals, it can be concluded that clofibric acid, even at the highest concentration tested (1mg/l), had few, if any, pronounced effects on fathead minnow embryos (relative to other pharmaceuticals that have been reported). The lack of a clear dose-response relationship with some of the endpoints indicates that some of the effects observed may be more likely to be due to chance than 'real' effects of the drug.

It is very clear from this preliminary experiment that further work needs to be carried out to determine if the putative effects of clofibric acid on fish embryos that I observed are reproducible. Ferrari *et al* 2003, also highlights the need for more in-depth chronic studies to be carried out, using more sensitive and specific endpoints such as narcosis, developmental abnormalities, and sex ratios.

Chapter 4: Potential effects of Clofibrilic acid  
on lipid metabolism in Bream (*Abramis  
brama*).

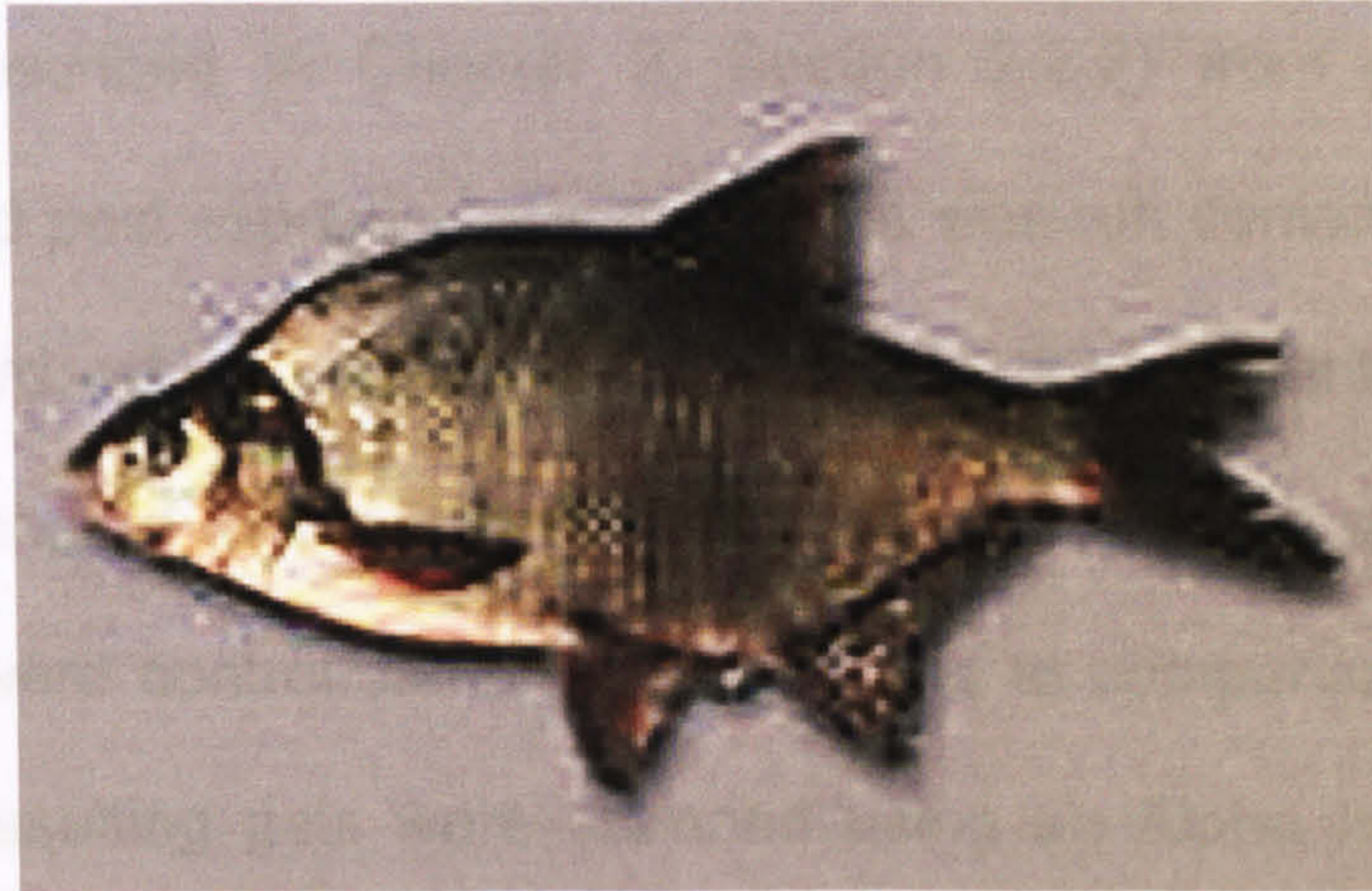
## 4.1 Adult Experiment 1

The aim of this thesis was to utilise different methods to determine the effects, if any, of clofibric acid on fish, both in the embryo and adult. This chapter reports the results of a single exposure study that involved developing and then utilising a suite of potential methods to address this goal. The exposure study involved the use of adult, reasonably large fish (compared to regulatory ecotoxicology test species) which could be blood sampled both pre- and post-exposure, thus enabling the effect of clofibric acid to be assessed on each fish individually, and not on groups of fish, as is studied in subsequent experiments. The pre-sample serves as an internal control for each post-sample. Such an approach often allows small changes to be observed, which might be masked in more traditional approaches (when only post-treatment values are available), especially if the parameters under study vary markedly from fish to fish.

### 4.1.1 Exposure Protocol

Adult bream (*Abramis brama*, 2+ years of age; sex unknown; Figure 4.1), kindly supplied by Calverton Fish Farm (Calverton, Nottingham), were held in a continuous flow through system in twenty 30-litre tanks (3 fish per tank) for 21 days. The fish within each of the tanks were individually marked with either a tail cut or a fin cut or no cut, to ensure that pre and post blood samples could be assigned correctly. Stock solutions of clofibric acid were prepared as described in Chapter 2, Section 2.1.2 and ten of the tanks were dosed one week prior to entry of the fish, to ensure equilibrium of the chemical within the tanks, with 1mg/l clofibric acid. All of the tanks were monitored daily for dissolved oxygen concentration and temperature (maintained at  $12\pm 1^{\circ}\text{C}$ ), and the flow rate of the

clofibrinic acid stock solution was also checked daily to ensure consistent dosing. The photoperiod was maintained at 16h light:8hr dark throughout, incorporating a 20 min dawn:dusk transition period. The bream were fed carp pellets (Coppens BV Cyprinid diet (36% protein, 7% oil)) twice per day.



**Figure 4.1: The bream (*Abramis brama*).**

At the commencement of the exposure study, the bream were lightly anaesthetised and blood samples were taken (Chapter 2, Section 2.1.3) and stored at 4°C ready for analyses (Chapter 2, Section 2.2). After the 21-day exposure period, fish were terminally anaesthetised, as described in Chapter 2, Section 2.1.3. Length, weight, liver weight and gonad weight measurements were taken and blood was again collected for measurement of total cholesterol and triglyceride levels, VTG concentration and lipoprotein analysis. Each blood sample was spun and stored appropriately for each type of analysis (for lipoprotein, it was stored at 4°C for a maximum of three days, whilst for VTG, cholesterol and triglyceride it was stored at -20°C).



#### 4.1.2 Measurement of Cholesterol within Lipoproteins

As fibrates have been shown in humans to have effects on serum lipoprotein levels (this is the intended and desired effect), it was considered that lipoprotein levels in bream could also be affected. The Hydrigel LDL/HDL CHOL Direct procedure was used to quantify cholesterol within LDL and HDL fractions (as described in Chapter 2, Section 2.2.2) from serum samples collected pre and post exposure. Each gel that was run contained six samples and one standard, which was run on all gels (in this experiment fish 64 was used as the standard in the pre samples and fish 24 was used in the post samples (both were control samples)). This was to compensate for gel-to-gel variation. The resulting gels were scanned using an Alpha Imager 1220 and analysis system V5.5. Densitometry provided an area under the curve and in each case, this was divided by the area of the control band to normalise results between the gels. This gave a standardised value, which could then be analysed statistically.

#### 4.1.3 Manual Cholesterol and Triglyceride assays

Fibrates are also known to have effects on total plasma triglyceride and cholesterol levels in humans (also desired and intended results), and consequently these parameters were also measured in this experiment. Enzymatic methods were used to quantify both cholesterol and triglyceride levels from plasma samples collected from this experiment. This was done using colorimetric assay kits purchased from ThermoTrace DMA (Victoria, Australia, as previously described in Chapter 2, Section 2.2.3). Total cholesterol measurement was based on the methodologies of Allain *et al*, 1974 and Roeschlau *et al*, 1974, and triglyceride measurement on a method

developed by Waco Pure Chemicals Industry Ltd, with modifications from McGowan *et al*, 1983 and Fossati, *et al*, 1982. Both assays also had modifications making them appropriate for use with fish serum (Jensen and Taylor, 2002; Jensen, 2003, personal communication), which were necessary because of the lipid-rich nature of fish plasma. All assays were performed in non-absorbent Titertek 96-multiwell flat-bottomed plates (Flow labs, Irvine). Standard curves were also run in parallel to the samples, examples of which are shown in Chapter 2, Section 2.2.2.

#### 4.1.4 VTG ELISA

Lipid lowering drugs are known to lower lipoprotein concentration in humans, and this might be true in fish also. As vitellogenin is a lipoprotein, I hypothesised that these drugs may affect the levels of this lipoprotein in the blood. If the VTG concentration was affected, this could lead to effects on oocyte development. It could also be hypothesised that if effects were seen on the HSI, the production of VTG could be affected, as the liver is the organ where it is synthesised. At the extreme, this may in turn affect the viability of the offspring of the exposed fish and, extrapolating further, whole populations.

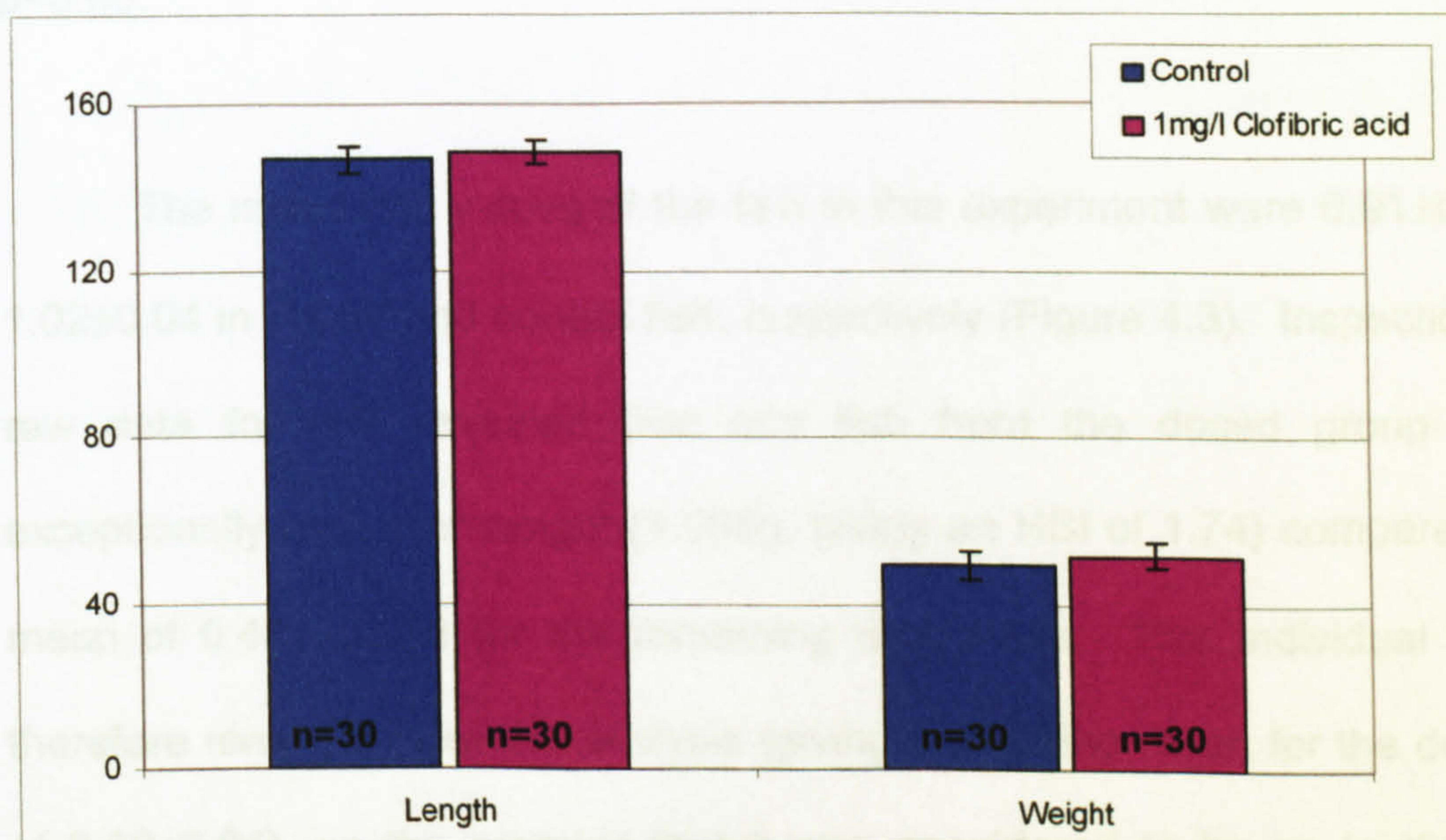
The VTG Enzyme-Linked-ImmunoSorbant-Assay (ELISA) based on carp (*Cyprinus carpio*) vitellogenin (Tyler *et al*, 1999) was used to determine the amount of vitellogenin present in bream before and after exposure to clofibrac acid, to identify if clofibrac acid had an effect on the concentration of VTG. The method is described in detail in Chapter 2, Section 2.2.4.

### 4.1.5 Statistical analyses

In all cases, differences between control and dosed fish, pre and post exposure, were analysed using SigmaStat v2.03 (Section 2.4.2). Where data were shown to be normally distributed, a t-test was used. Where normality was not met, data were analysed using a suitable non-parametric test.

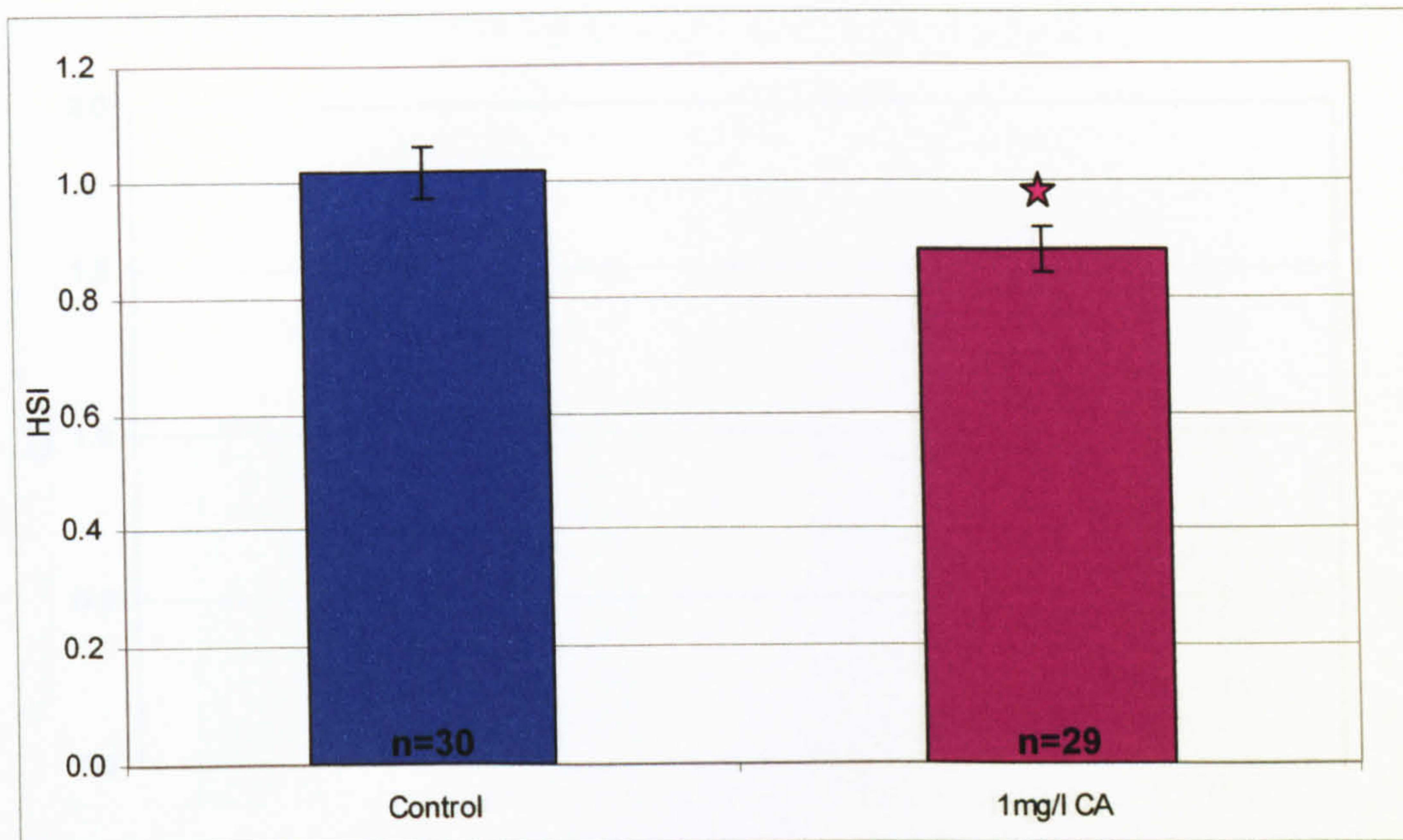
## 4.2 Results

### 4.2.1 The effects of clofibric acid (1mg/litre) on morphometric measurements of Bream



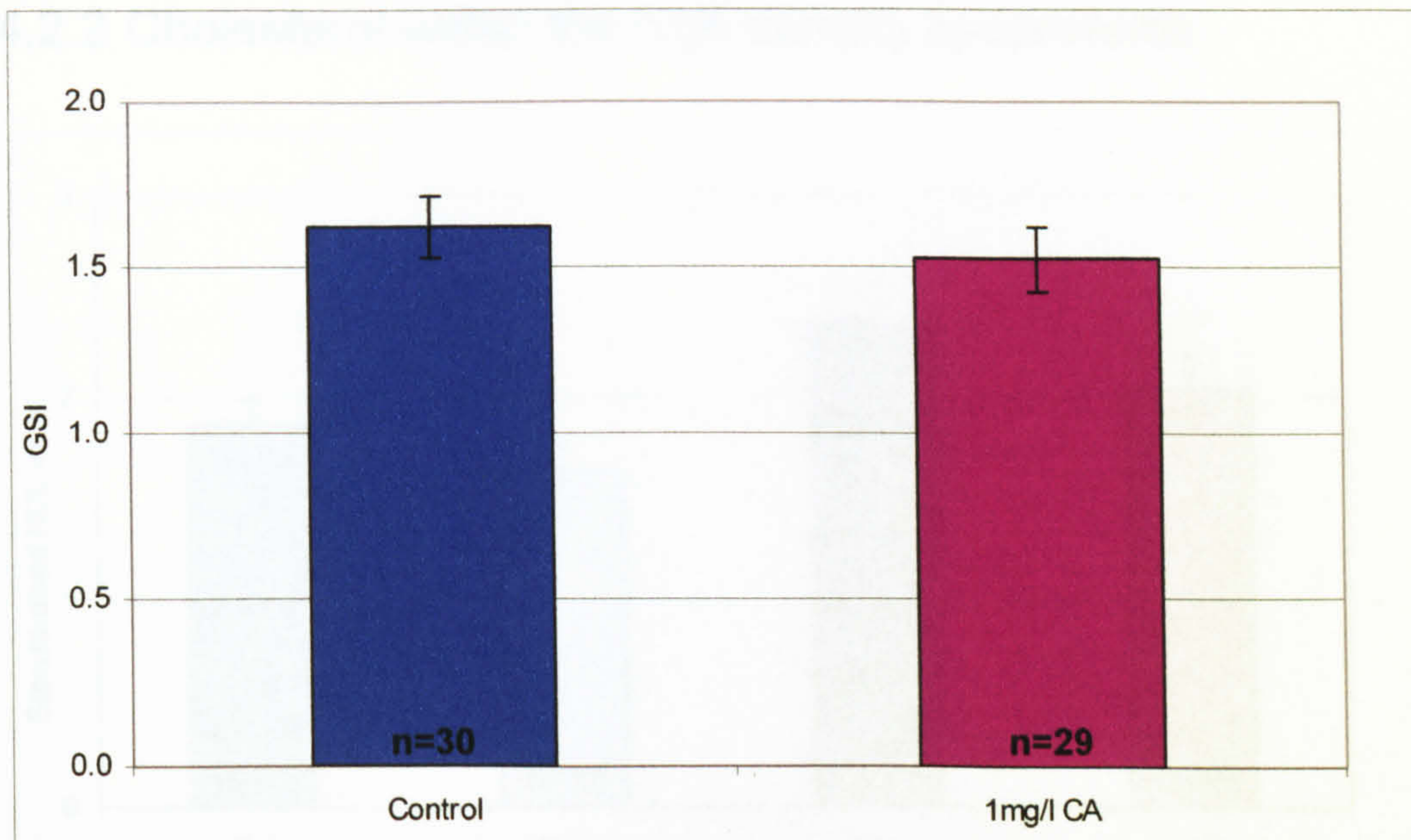
**Figure 4.2: Mean length and weight of bream exposed to a nominal concentration of 1mg/l Clofibric acid for 21 days in Experiment 1. Data are expressed as mean  $\pm$  SEM.**

The control fish had a mean weight of  $49.6 \pm 3.4$ g, and a mean length of  $146.8 \pm 3.2$ mm, whereas the exposed fish had a mean weight of  $51.6 \pm 2.97$ g and a mean length of  $148.6 \pm 2.65$ mm, as shown in Figure 4.2. When control fish were compared to exposed fish, they were found to be not statistically significantly different in length or weight ( $p=0.6$  in both cases).



**Figure 4.3: The effect of Clofibrlic acid on the hepatosomatic index (HSI) of bream in Experiment 1.** Data are expressed as mean  $\pm$  SEM. \* indicates  $p < 0.05$ .

The mean HSI of the control and exposed fish were found to be 1.02 and 0.91, respectively. The mean HSI values of the fish in this experiment were  $0.91 \pm 0.04$  and  $1.02 \pm 0.04$  in dosed and control fish, respectively (Figure 4.3). Inspection of the raw data for HSI revealed that one fish from the dosed group had an exceptionally high liver weight (1.005g, giving an HSI of 1.74) compared with a mean of  $0.46g \pm 0.036$  for the remaining dosed fish. This individual fish was therefore removed from the analysis (giving a resulting mean for the dosed fish of  $0.88 \pm 0.04$ ), on the premise that it was considered to be an 'outlier,' in an attempt to improve homogeneity of the variance, this 'outlier' was also removed from subsequent analysis. These results then indicated that there was a statistically significant decrease ( $p = 0.02$ ) in HSI in dosed fish. Further inspection of the data from this individual fish showed that it also had the highest level of VTG – suggesting it could be beginning to mature sexually – and it could therefore be that synthesis of VTG was responsible for the increased liver size of this fish (it is well established that as VTG synthesis increases during sexual maturation, the HSI increases).



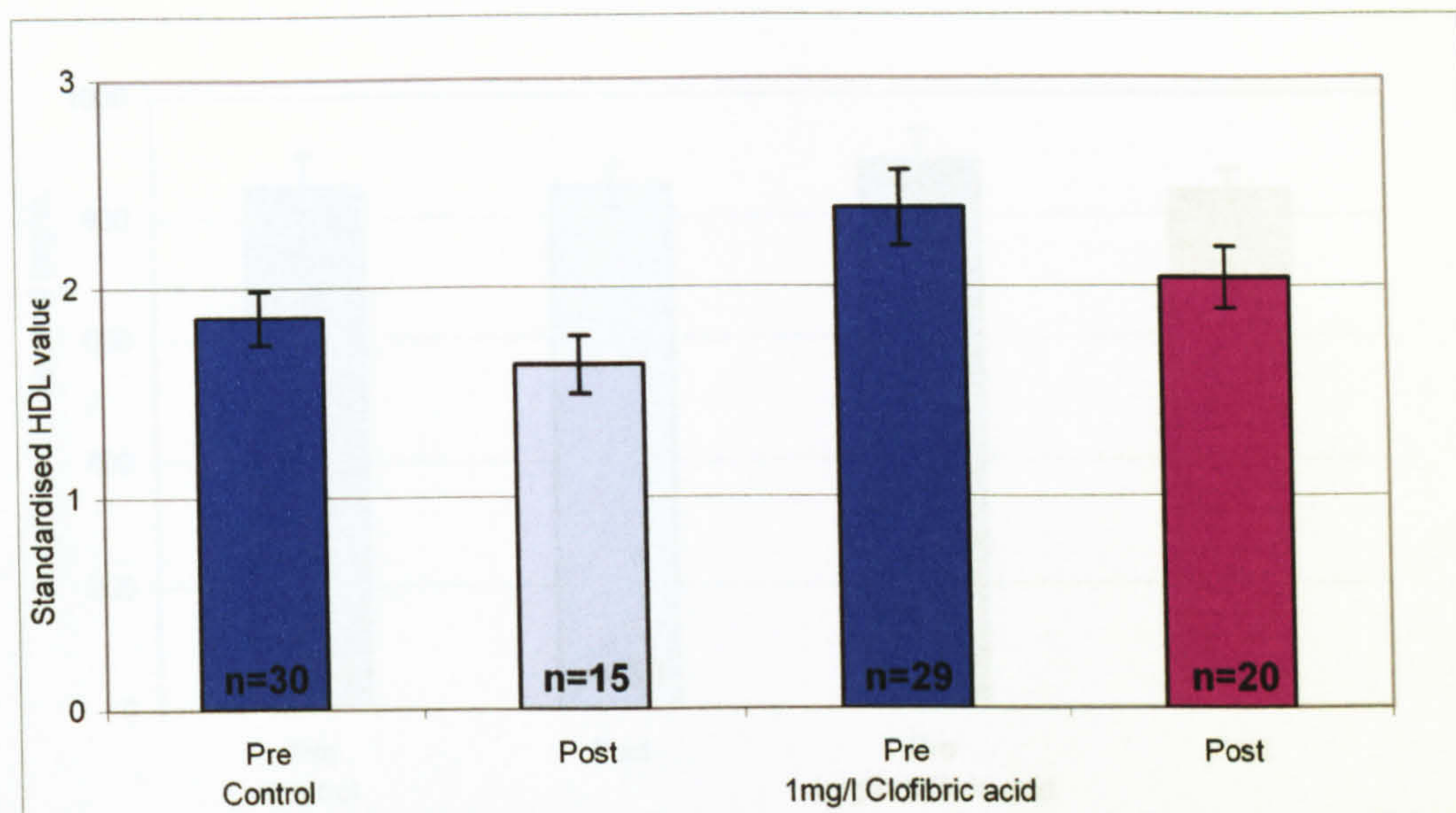
**Figure 4.4: The effect of Clofibrinic acid on the gonadosomatic index (GSI) of bream from Experiment 1. Data are expressed as mean  $\pm$  SEM.**

The mean GSI of the control and exposed fish were found to be  $1.61 \pm 0.09$  and  $1.52 \pm 0.09$ , respectively (Figure 4.4), values which were not significantly different ( $p=0.4$ ). During dissection, it was difficult to distinguish the gonads from the fat within the body cavity, and hence what was weighed may not have been only gonadal tissue. Consequently, the GSI values are possibly inaccurate and should be interpreted cautiously.

The mean percentage change in GSI was found between  $2.40 \pm 0.19$  and  $2.07 \pm 0.15$ . There were no statistically significant differences between pre and post exposure samples of the controls or between the pre and post exposure samples of the fish ( $p > 0.2$  in both cases). A *t*-test and ANOVA were also carried out subsequently to look at the mean percentage change within the control and dosed groups and this was similarly found to be not significantly different ( $p > 0.1$ ) between treatments.

It was found, however, perhaps surprisingly (given the large sample size), that the cholesterol levels within the lipoproteins of the pre samples of the controls differed significantly ( $p=0.02$ ) from the pre samples of the clofibrinic acid exposed fish.

## 4.2.2 Cholesterol within the high density lipoproteins

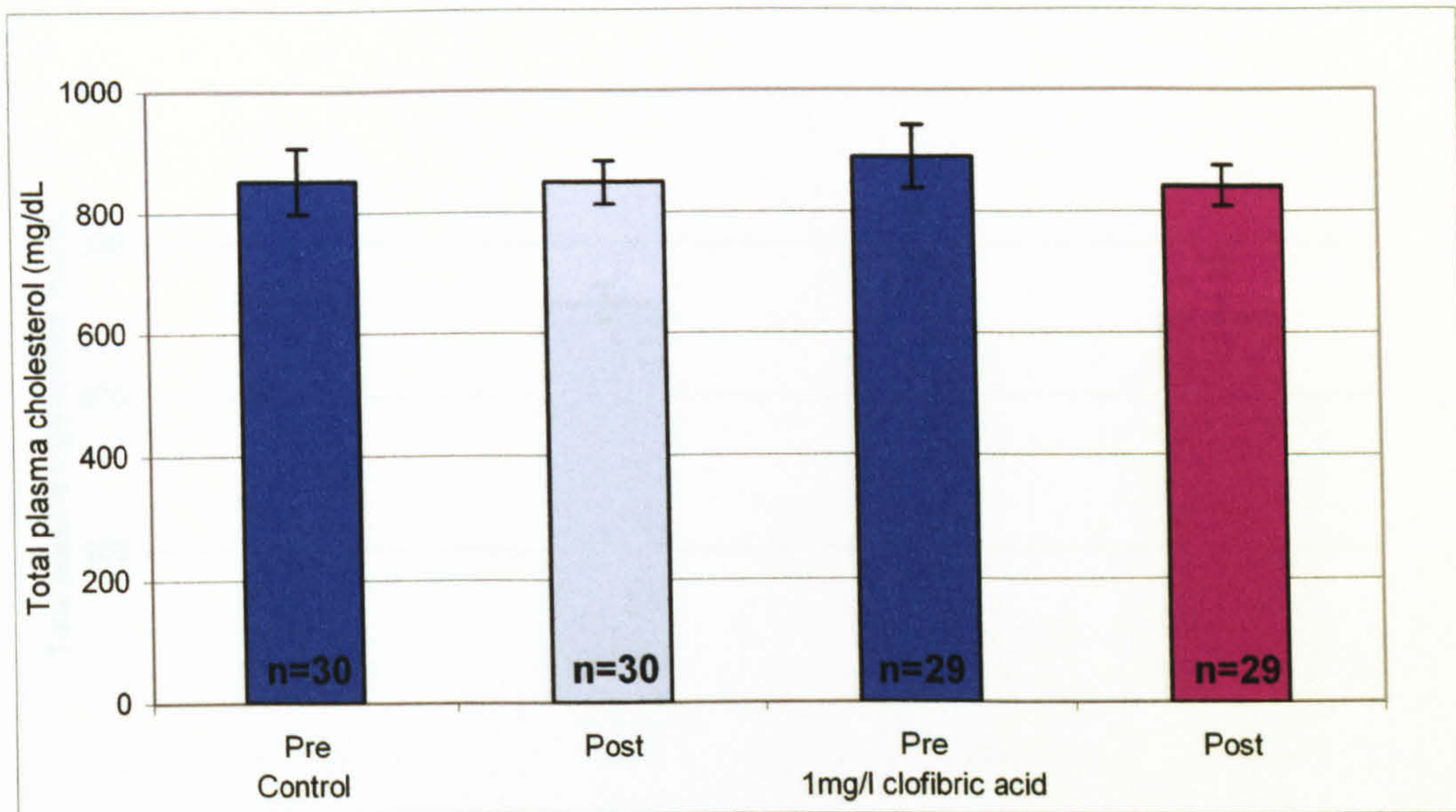


**Figure 4.5: The effects of Clofibrac acid at a nominal concentration of 1mg/l on the levels of cholesterol within the HDL of bream exposed to clofibrac acid for 21 days. Data are expressed as mean  $\pm$  SEM. There were no statistically significant differences within or between treatments.**

The total plasma cholesterol levels found in control bream, pre and post exposure, were  $852.7 \pm 50 \text{ mg/dL}$  and  $848.5 \pm 35.8 \text{ mg/dL}$ , respectively ( $p=0.9$ ), and the levels in dosed fish were  $850 \pm 50 \text{ mg/dL}$  and  $841.3 \pm 3.8 \text{ mg/dL}$ , control pre and post fish, whereas in the pre and post dosed fish, mean cholesterol levels within the lipoproteins varied between  $2.40 \pm 0.19$  and  $2.07 \pm 0.15$ . There were no statistically significant differences between pre and post exposure samples of the controls or between the pre and post exposure samples of the fish ( $p=0.2$  in both cases). A t-test and ANOVA were also carried out subsequently to look at the mean percentage change within the control and dosed groups and this was similarly found to be not significantly different ( $p=0.1$ ) between treatments.

It was found, however, perhaps surprisingly (given the large sample size), that the cholesterol levels within the lipoproteins of the pre samples of the controls differed significantly ( $p=0.02$ ) from the pre samples of the clofibrac acid exposed fish.

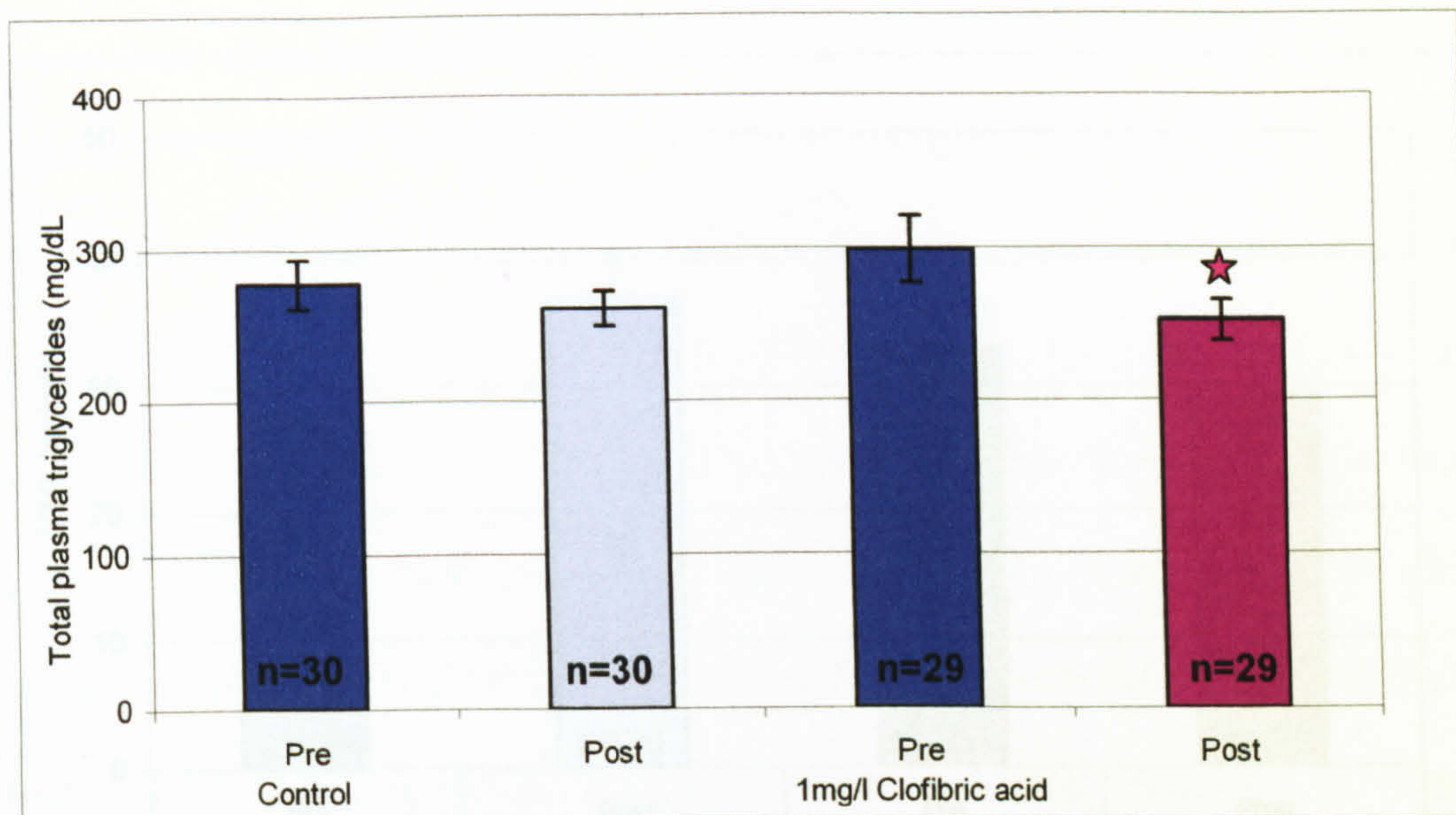
### 4.2.3 Cholesterol levels



**Figure 4.6: The effects of Clofibrac acid on total plasma cholesterol levels in bream in Experiment 1. Data are expressed as mean  $\pm$  SEM. There were no statistically significant differences between groups.**

The total plasma cholesterol levels found in control bream, pre and post exposure, were  $852.7 \pm 55 \text{ mg/dL}$  and  $848.5 \pm 35.8 \text{ mg/dL}$ , respectively ( $p=0.9$ ), and the levels in dosed fish were  $889 \pm 50.6 \text{ mg/dL}$  and  $841.3 \pm 33.6 \text{ mg/dL}$ , respectively ( $p=0.42$ ). In both groups of fish there were no statistically significant differences between pre and post values.

#### 4.2.4 Plasma Triglyceride levels

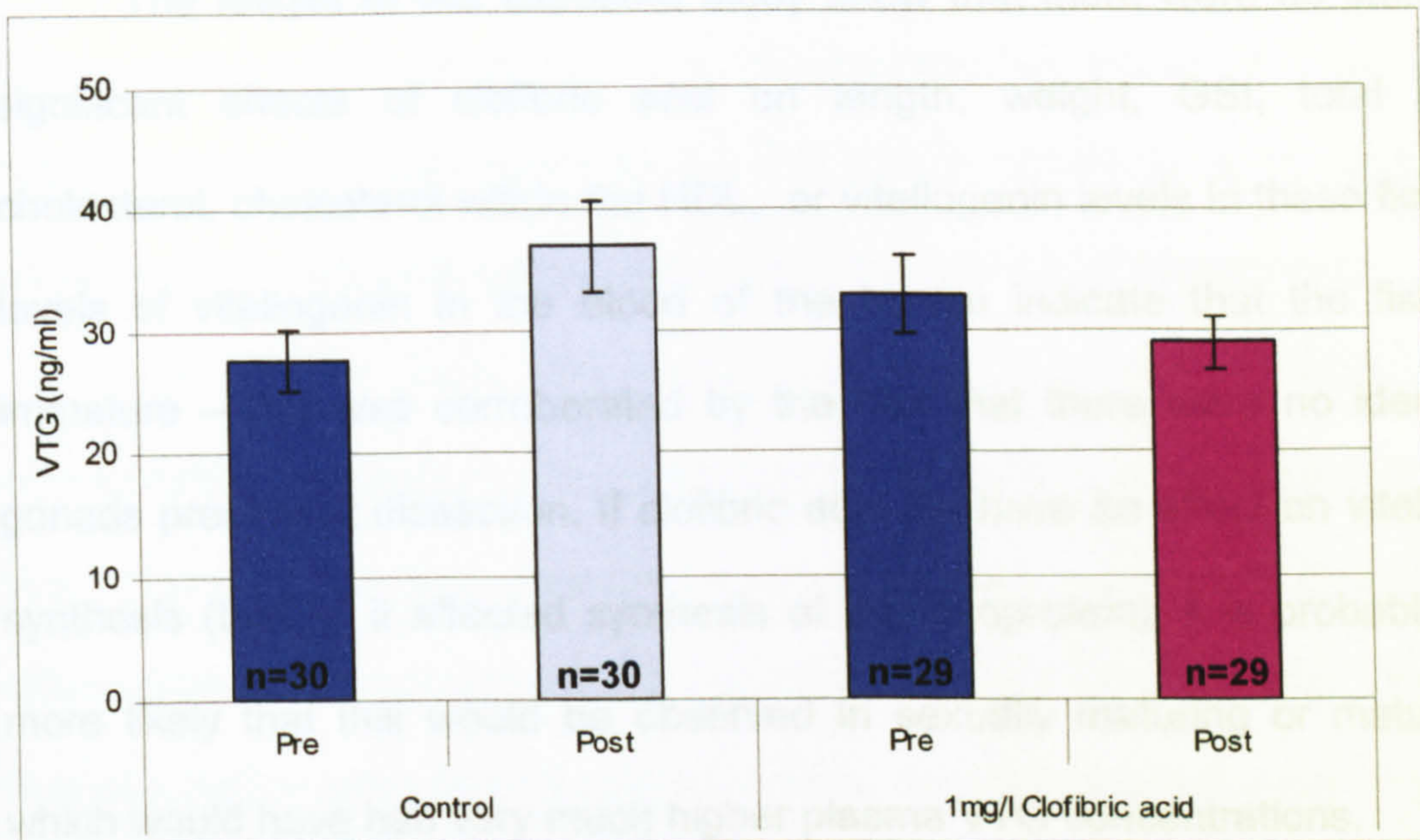


**Figure 4.7: The effects of Clofibrac acid on total plasma triglyceride levels in bream in Experiment 1.** Data are expressed as mean  $\pm$  SEM. \* indicates  $p < 0.01$ .

The mean levels of plasma triglyceride in the bream samples were between  $276.9 \pm 16.39$  mg/dL and  $259.9 \pm 11.29$  mg/dL in the control fish pre and post exposure, respectively ( $p = 0.39$ ), and  $298.25 \pm 22.3$  mg/dL and  $244.54 \pm 11.27$  mg/dL in the dosed fish pre and post exposure, respectively ( $p = 0.01$ ). As can be seen from the  $p$  values, there was not a significant difference between pre and post values in the control group, however there was a significant decrease in the levels of triglyceride between the pre and post dosed groups of fish.



#### 4.2.5 Plasma Vitellogenin concentrations



**Figure 4.8: VTG levels of bream exposed to Clofibrac acid at a nominal concentration of 1mg/l in Experiment 1.** Data are expressed as mean  $\pm$  SEM. Exposure to clofibrac acid had no statistically significant effects.

The levels of VTG found in both exposed and control bream, both pre and post exposure, were found to be very low. Mean levels of the control fish, pre and post exposure, were  $27.7 \pm 2.48$  and  $37.0 \pm 3.76$  ng VTG/ml, respectively. Mean levels in the dosed group pre and post exposure were  $32.9 \pm 3.16$  and  $28.9 \pm 2.13$  ng VTG/ml, respectively. No significant differences were found when comparing the VTG concentrations of control fish pre and post exposure ( $p=0.09$ ) or when comparing the dosed fish pre and post exposure ( $p=0.5$ ). It was determined, however, at termination of the experiment that these fish were all sexually immature (gonads were undifferentiated), and this explains the finding that the levels of vitellogenin in the blood were very low.

### 4.3 Discussion

The results of this exposure study show that there were no statistically significant effects of clofibric acid on length, weight, GSI, total plasma cholesterol, cholesterol within the HDL, or vitellogenin levels in these fish. The levels of vitellogenin in the blood of the bream indicate that the fish were immature – this was corroborated by the fact that there were no identifiable gonads present at dissection. If clofibric acid did have an effect on vitellogenin synthesis (that is, it affected synthesis of this lipoprotein), it is probably much more likely that this would be observed in sexually maturing or mature fish, which would have had very much higher plasma VTG concentrations.

Significant effects were seen, however, on the levels of total triglyceride found in the plasma ( $p=0.01$ ) after exposure to clofibric acid. This result is consistent with the reduction seen in humans (Auwerx *et al*, 1996) and hamsters (Guo, *et al*, 2001). The decrease is thought to occur because fibrates reduce the amount of fatty acid available to the liver for triglyceride synthesis by the up-regulation of the level of lipoprotein lipase (which was observed in male fish in Experiment 4) via PPAR activation.

When the HSI data were examined, there was found to be a statistically significant decrease ( $p=0.02$ ) in the fish exposed to clofibric acid at 1mg/l, so it can be assumed that there was some effect of clofibric acid on the liver of this species. These results agree with those reported in rats exposed to clofibric acid, where liver weight has been shown to decrease (Cleary *et al*, 1987). These results indicate that the exposure to clofibric acid, in both fish and rats, is not simply a toxic one, as this would cause the HSI to go up as a consequence of the up-regulation of the various detoxification mechanisms. However, the reason for the decrease in the HSI caused by clofibric acid is unknown.

Apart from the HSI and triglyceride levels, there were no other observed effects on any other of the parameters measured in this study, and consequently it was decided that a different species would be used (so that higher numbers of fish could be used in future experiments, to determine if there are any subtle effects that were not identified in this experiment).

The results from this chapter suggest that clofibric acid (at the concentration used) may affect lipid metabolism only in a small way (it may be that 1mg/l is on the dose-response curve, but near the bottom of it), and so the effects seen are minimal and are therefore difficult to demonstrate. It may be that at a higher dose there would be a more detectable effect – e.g. at perhaps 10mg/l there may be a greater detectable effect.

As the mechanisms of action of fibrates in fish are unknown, and no evidence of effects has previously been reported, it is very difficult to know if the effects seen in the bream are indicative of what may be expected to occur in other fish species.

The results of this chapter thus led me to carry out further experiments involving exposure to clofibric acid using a different species of fish (the fathead minnow, *Pimephales promelas*), with some additional endpoints incorporated into the experimental design. The results of these experiments, along with some explanations of any effects are in the following chapters.