THE EFFECT OF ENVIRONMENTAL FACTORS AND HORMONE TREATMENTS ON OVULATION RATE AND SPAWNING SUCCESS IN CARDINAL TETRAS, *PARACHEIRODON AXELRODI* (PISCES: CHARACIDAE).

THESIS

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by

SIMON MARK BURTON

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DECLARATION

I declare that this dissertation describes my original work, and has not been submitted for a degree at any other university.

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ABSTRACT

Cardinal tetras, *Paracheirodon axelrodi* (Pisces: Characidae), are among the most popular tropical ornamental fish in the world. Millions of cardinal tetras are removed from their natural habitat, the upper tributaries of the Rio Negro in Brazil, each year and exported to North America and Europe where they are abundantly available at low cost. This, combined with the fact that they do not spawn freely in captivity has excluded any incentive to culture this fish on a commercial scale in these areas. However, the demand for cardinal tetras in South Africa is high, but their availability is limited to sporadic imports of wild caught fish which has resulted in an increase in their relative value. Thus, there is an appreciable incentive to investigate methods for inducing this species to reproduce in captivity. Information on methods for inducing spawning would form an integral part of a potential production plan for the culture of this species in South Africa.

This study consisted of a number of experiments designed to investigate factors related to the spawning of cardinal tetras in captivity. The first series of experiments investigated the effect of variables such as the water chemistry of the experimental system, the sex ratio of the fish, the effect of conditioning and the effect of different spawning media on spawning success.

The second series of experiments tested the effects of various environmental stimuli or hormonal treatments on spawning in cardinal tetras. Environmental stimuli tested included a rise in temperature, a rise in water level and a fresh rain water flush. Hormonal treatments included feeding GnRHa enriched brine shrimps to the fish or the application of hormone injections (hypophysation). Neither environmental stimuli nor brine shrimp enrichment induced cardinals to spawn in a repeatable manner. The same was true for hypophysation, but in this case a low incedence of ovulation of injected females was observed.

Oocytes from ovulated and non-ovulated females were examined under a microscope and from this it was determined that cardinal tetras are group synchronous spawners, containing two distinct cohorts of oocytes in the ovary.

The effect of a number of environmental stimuli on ovulation rate in cardinal tetras were then tested but did not induce ovulation in any females.

A series of three experiments was performed investigating the effect of conditioning the sexes together on ovulation rate and spawning success. Conditioning the sexes together had no effect on spawning success, but a significantly higher ovulation rate was observed in females that were conditioned together with males. Male cardinals did not follow this trend and lost condition when conditioned together with females.

The next experiment tested if pheromones from red serpae tetras, *Hyphessobrycon serpae*, could be used to induce ovulation and spawning in cardinal tetras. This was found not to be the case.

The final series of experiments tested the effect of Aquaspawn^{R} enriched *Artemia* on ovulation rates in female cardinal tetras. Aquaspawn^{R} is a water soluble preparation of GnRHa and dopamine. Aquaspawn^{R} enriched *Artemia* were found to be effective for inducing female cardinal tetras to ovulate. However, the results from this method of enrichment were not entirely reliable and further research is warranted.

Finally, a protocol for evaluating spawning in topical, freshwater egg-laying fish was proposed, based on the results from this study. The advantages and disadvantages of such a protocol were discussed.

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CHAPTER 1: INTRODUCTION

1.1 BIOLOGY AND ECOLOGY

Description

The cardinal tetra *Paracheirodon axelrodi,* belongs to the order Cypriniformes and the family Characidae. The family Characidae comprises of a large group of small to medium sized fish that are distributed throughout tropical South America and Africa (Axelrod & Schultz 1990). The size of the family is not known since it is thought that a large proportion of characin species have yet to be described (Axelrod & Schultz 1990). This is due to the fact that the majority of characins are small (<50mm) and are found in small tropical forest streams (Axelrod & Schultz 1990). The cardinal tetra was first described as *Cheirodon axelrodi* (Schultz 1956), but later changed to *Paracheirodon axelrodi* (Weitzman & Fink 1983). In the wild they reach a maximum size of 33mm TL and age of 18 months (Geisler & Annibal 1987). In captivity, however, they may reach a maximum length of 50mm and live for three to four years (Geisler & Annibal 1987). Cardinal tetras are easily recognisable by their metallic blue-green stripe along their lateral line. Below this line they are bright crimson for the entire length of the body. This, along with their slightly larger size, distinguishes them from the closely related neon tetra, *Paracheirodon innesi,* which is only red on the posterior half of the body. All fins of the cardinal tetra are transparent. Sexes are not easy to distinguish although females are generally larger, fuller bodied (Axelrod & Shaw 1967; Geisler & Annibal 1987; Richter 1991) and the margin of the anal fin is strongly concave in females and almost straight in males (Noznov 1986; Richter 1991). Cardinal tetras show strong shoaling tendencies, forming mixed-sex shoals of 12-30 fish in the wild (Chao 1992).

Distribution

The cardinal tetra is endemic to the tributaries of the upper Rio Negro in Brazil (Fig 1.1.1. and Fig. 1.1.2). Here they occur in heavily shaded forest streams, in still, shallow areas near the water's edge (Geisler & Annibal, 1987).

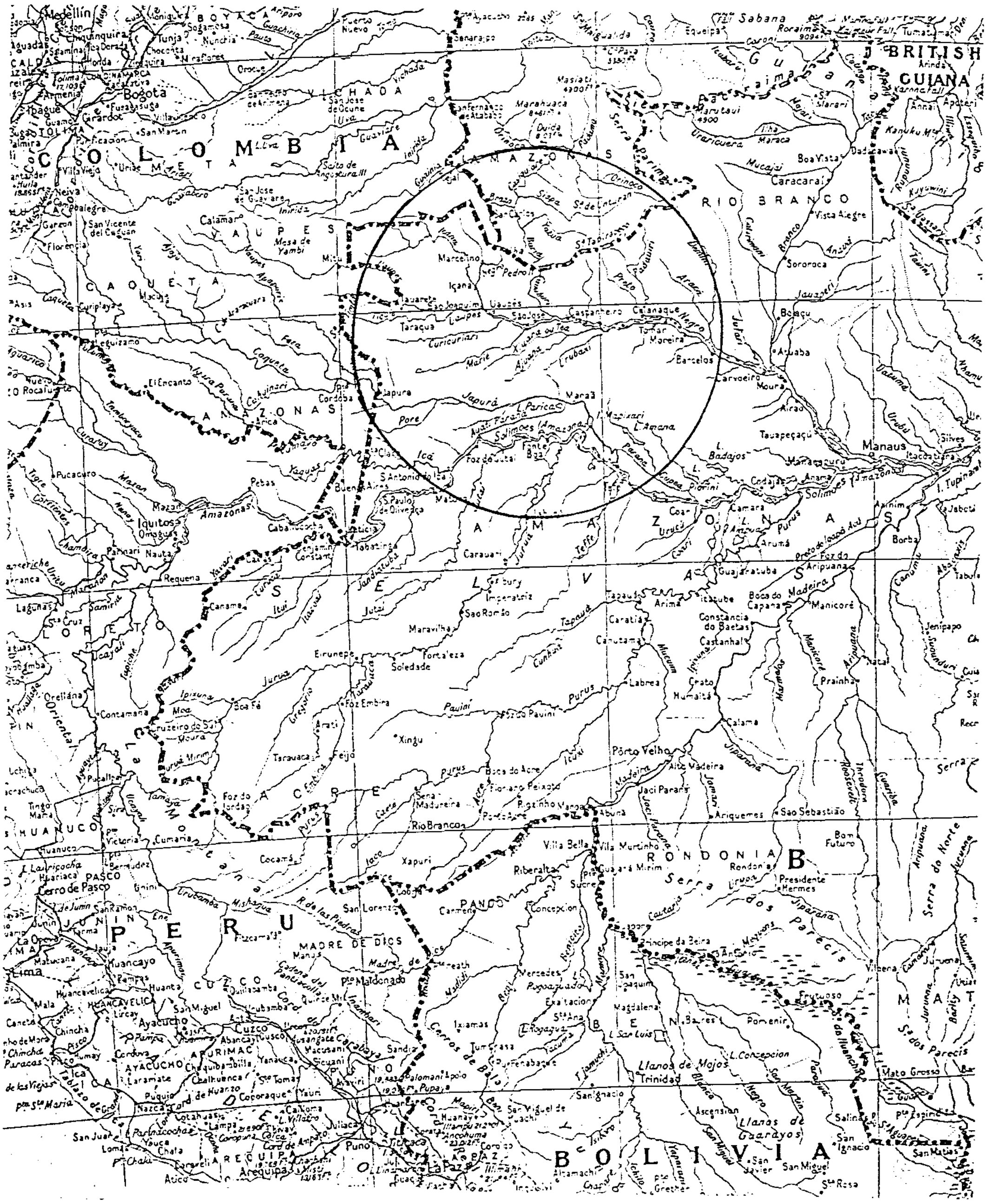


Fig 1.1.1 Distribution area of *Paracheirodon axelrodii* in northwest Amazona.

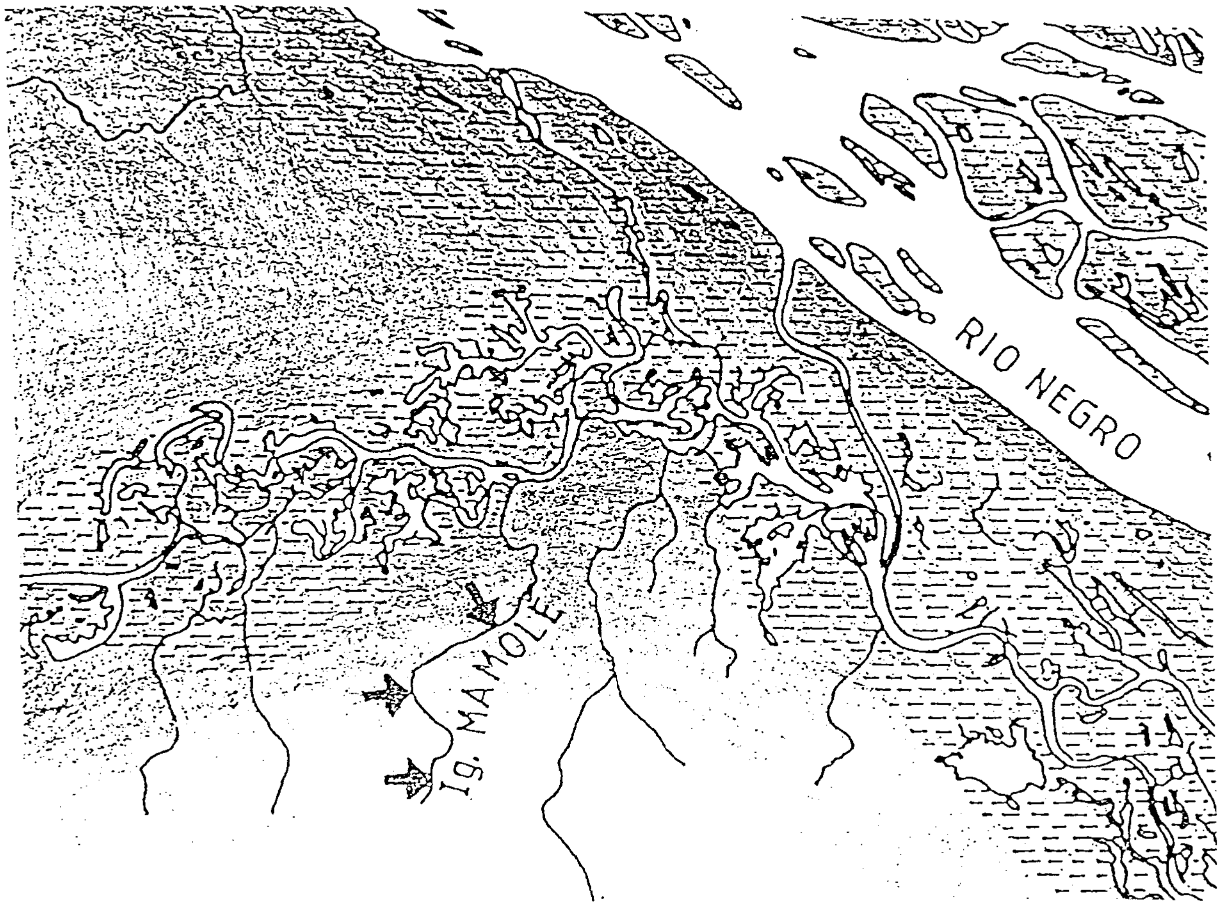


Fig 1.1.2 *P. axelrodi* distribution. Arrows indicate likely areas in the Igarapé Mamolé (basin of the Rio Cuiuni around Barcelos) (FromGeisler & Annibal 1987).

Biotope Limnochemistry

The Rio Negro is a typical example of a "black water" river. Black water rivers are defined by Sioli (1968) as: "Rivers with clear, very transparent but dark brown water,..." The physical and chemical properties of the water varies seasonally and between tributaries but the typical Amazonian black water stream can be defined by the parameters in Table 1.1.1. The range of physical and limnochemical parameters of *P. axelrodi* biotopes is shown in Table 1.1.2.

Table 1.1.1 Physical and limnochemical properties of a typical Amazonian black water stream (Geisler & Annibal 1987)

Parameter	Measured Values
pH	<4.3
Conductivity µS/cm	<12
Colour Hazen	>50
Organic substance as $KMnO_4$ consumption	>50
Ca^{2+} and Mg^{2+}, $mg.l^{-1}$	<1

Table 1.1.2 Physical and limnochemical parameters of *P. axelrodi* biotopes (Geisler & Annibal 1987).

Paramter	Range
Temperature	24 - 30^{0}C
Ca^{2+}, ug/l	0 - 88
pH values	3.97 - 5.1
Mg^{2+}, ug/l	0 - 49
Conductivity in µS/cm	3.4 - 41
Colour Hazen	15 - >300
Total Hardness dH^0	0.00 - 0.03
$KMnO_4$-distribution, mg/l	15 - 190

Cardinal tetras are photophobic, strongly avoiding bright light or sunshine. This photophobia is pronounced in young fish and larvae (Geisler & Annibal 1987). Cardinals therefore inhabit heavily shaded areas where the light intensity during the day ranges between 25 and 300 lux (Geisler & Annibal 1987). Cardinals also avoid strong currents, favouring quiet, shallow (<1m deep) backwaters just off the main current where the water flow is just a few centimeters per second. These biotopes typically have a thick carpet of decaying leaves as a substrate with very few, if any, aquatic plants.

Diet

Due to the extreme lack of nutrients in blackwater streams, there is very little for cardinals to feed on. Geisler and Annibal (1987) found; "...no zooplankton in several 100 L scoop samples from the free running water." They did, however, find a small quantity of benthic copepods, tiny ephemerid (mayfly) larvae and the occasional shrimp of the genus *Macrobrachium*, in the upper layers of the leaf litter. Hence, according to Geisler & Annibal (1987) wild cardinals are typically in;"...poorer condition than those kept in captivity under optimum feeding conditions".

Seasonal Migrations

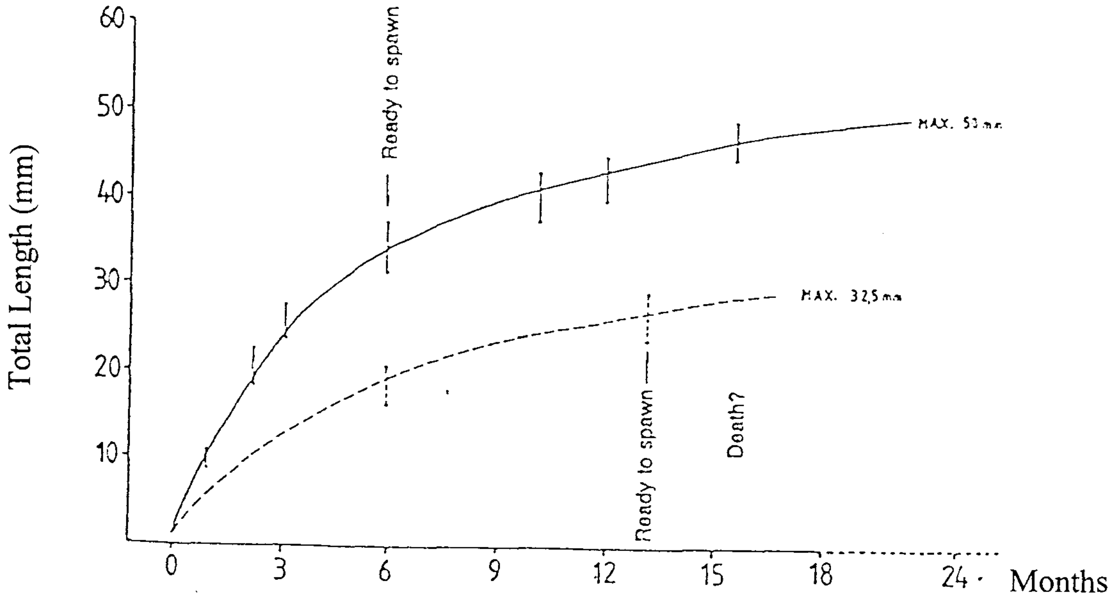
During the dry season (December - March), cardinals move downstream to the mouths of the streams or to the margins of lower reach lakes (Chao 1992; Geisler & Annibal 1987). In extreme dry seasons they may even move downriver to within 20-50km from the Rio Negro itself (Chao 1992). With the start of the rainy season the water levels in the lower reaches rise and flood the cardinal biotopes. The water level may rise as much as 5m above the normal dry season level in the lower reaches of the tributaries. Hence at the start of the rainy season (April) the cardinals migrate upstream until they find suitable biotopes, in terms of biotope morphology, in the headwaters of the tributaries. Thereafter, as the water level falls, the cardinals migrate downstream (Chao 1992; Geisler & Annibal 1987).

Spawning season

The spawning season of cardinal tetras begins with the rising water levels and lasts while the water levels are high (Geisler & Annibal 1987; Chao 1992; Munro 1995). Hence, the changes in water level and water quality determine the start and the duration of the cardinal spawning season. Generally the spawning season begins in late March, early April and lasts until June (Geisler & Annibal 1987; Munro 1995). Although spawning behaviour has never been observed in the wild, cardinals ready to spawn, together with young fish, have been found in typical cardinal biotopes in the upper reaches during the spawning season (Geisler & Annibal 1987). Cardinals are group- synchronous spawners, spawning a number of times in one spawning season (Geisler & Annibal 1987).

Growth

There are no continuous growth measurements for cardinal tetras in the wild. However, by plotting length distributions of cardinals six months and a year after the spawning season, Geisler & Annibal (1987) propose the growth curve shown in Fig. 1.1.3. Cardinals in the wild grow much slower and reach a smaller maximum size than those spawned and reared in captivity. Geisler & Annibal (1987) relate this to the lack of food in the wild. In the wild, cardinals reach sexual maturity in a year and few, if any, survive longer than 18 months (Geisler & Annibal 1987).



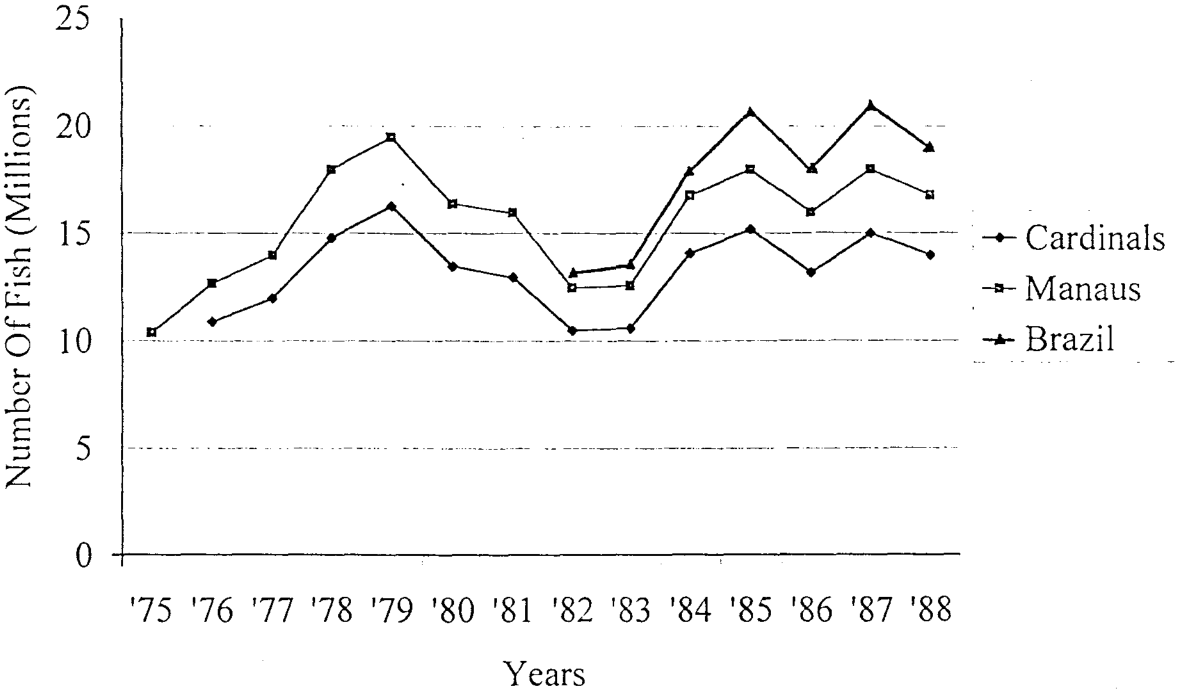
1.1.3 Growth curves of P. axelrodi in natural biotopes (broken line) and fish bred in captivity (continuous line)(from Geisler & Annibal 1987).

Relevance of biological and ecological information to this study

Detailed information on the biology and ecology of any fish is necesssary before an artificial breeding program can commence. Information on the biology is needed such that the researcher knows the details relevant to breeding the fish, such as the size at sexual maturity, size at first spawning, reproductive strategy and fecundity, as well as details relevant to keeping the fish in captivity, such as the diet and social interactions. Ecological information is necessary for understanding the environmental parameters, such as water quality, temperature or photoperiod, required to keep the fish in captivity. Further, fish will typically spawn when conditions are most favourable for the survival of their offspring (Hontela & Stacey 1990). Hence, to achieve "natural" spawning in captivity, the conditions experienced by wild fish during their spawning season should be simulated as close as possible. Thus the need for detailed information on the biology and ecology of a fish before commencing an artificial breeding program.

1.2 ECONOMIC IMPORTANCE

Cardinal tetras are among the most popular freshwater ornamental fish in the world (Axelrod & Shaw 1967). Hence there is a significant demand for this species world wide. This demand is taken up predominantly by wild caught fish. According to Chao (1992) the forest streams and lakes in the tributaries of the mid and upper Rio Negro are the main fishing grounds for the 10-20 million fish exported through Manaus annually. Of these, 80% are cardinal tetras. Total fish exports and cardinal tetra exports are displayed in Fig. 1.2.1 (Geisler & Annibal 1987; Chao 1992). However, the fish suffer 30-40% mortality in the hands of the fishermen, which means that 20-40 million cardinals are removed from their habitat each year (Chao 1992). Chao represents the data as follows:



1.2.1 Number of ornamental fishes exported from Brazil and Manaus, Amazonas (from Chao 1992).

The vast majority of cardinal tetras exported from Brazil are sold in the United States, Europe and the Far East, where they are abundantly available at very low cost. Hence, there has been no incentive to breed cardinals on any significant scale in these areas. However, very few if any cardinals are exported to other parts of the world. In South Africa, for example, the supply of cardinals to the ornamental trade is sporadic and very limited. Further, most of the cardinals entering the country are in poor condition and appear to suffer high mortality, although no exact figures are available. The result is that cardinals are scarce and relatively expensive, retailing at around R15 each for small fish. Therefore, since the demand and the value of cardinals in South Africa is high, there is a significant incentive to develop a culture protocol for this species. This would provide a steady supply of healthy fish to the local trade.

Further, there has been increasing awareness of the dangers of overexploiting limited resources, and some authors believe that the fishes of the Amazon are threatened by habitat destruction and over fishing (Richter 1991; Chao 1992; Axelrod 1994). Hence, there has been a trend towards developing breeding programs for many such fish species. A culture protocol for cardinal tetras would contribute towards this trend and possibly to a reduction in fishing pressure on wild stocks of cardinal tetras.

1.3 A REVIEW OF SPAWNING CARDINALS IN CAPTIVITY

Until recently it was thought that spawning cardinal tetras in captivity was extremely difficult (Axelrod & Shaw 1967). However, since Geisler & Annibal (1987) investigated the water quality of cardinal biotopes in the wild the water quality characteristics required for spawning have been replicated, and a number of authors have documented successful spawnings (Roloff, in Noznov 1986; Axelrod & Shaw 1967; Geisler 1972; Kuskow, in Noznov 1986; Pinter, in Noznov 1986; Tsilinszky, in Noznov 1986; Noznov 1986; Richter 1991; Benninger 1996). However, spawnings are still typically random and once off spawnings are difficult to repeat. This is a major problem for any potential aquaculture species since the fish must be produced on a regular basis on a sufficient scale.

The literature pertaining to spawning cardinal tetras in captivity is very limited with the majority of publications being the observations of hobbyists. Hence, these observations are non-scientific with few, if any, references. Therefore, most recommend methods are little more than the opinions of the respective authors with little if any scientific evidence to back them. Further, many observations were not published by the original authors but included in the publications of others. Noznov (1986), for example mentions the work of Roloff, Tsilinszky, Pinter and Kuskow, but does not include any references. Very few observations have been scientifically published with complete reference lists.

However, from the observations published, a general spawning procedure can be outlined, although many of the authors differ in opinion on many of the details. Almost all agree, though, that the most important factor to consider when attempting to spawn cardinals is the water chemistry in the spawning tank. These water quality parameters are summarised in Table 1.3.1

Table 1.3.1 Physical and chemical properties of "breeding water" for Cardinal tetras.

Author	Temperature°C	pH	Conductivity μS/cm
Axelrod & Shaw (1967)	24	5.0-6.8	<100
Geisler (1972)	25	5.8	10-65
Frank (1980)	---	5.5	----
Tsilinszky*	27	6.0-6.5	<60
Kuskow*	24	6.2	<80
Noznov (1986)	24	5.5	<80
Hadrava**	25	5	60
Zemanek**	25	5.8	40
Richter (1991)	25	---	<60
Benninger (1996)	---	6.5-6.7	<80

* In Noznov (1986)

** In Geisler & Annibal (1987)

Before attempting a spawning trial the fish should be separated by sex (Noznov 1986; Richter 1991) and conditioned for one to two weeks. Prior to spawning, Axelrod & Shaw (1967), Noznov (1986), and Geisler & Annibal (1987) recommend that the fish should be fed large quantities of live food. However, they do not mention how long prior to spawning this feeding regimen should begin or how much to feed.

Thereafter a small (15-20 L) spawning tank should be set up in a dimly lit area (Tsilinszky, in Noznov 1986; Kuskow, in Noznov 1986; Geisler & Annibal 1987), and filled to about 10- 15cm (Axelrod & Shaw 1967; Tsilinszky, in Noznov 1986) with suitable water and maintained at

24-25^{0}C (Roloff, in Noznov 1986; Axelrod & Shaw 1967; Geisler 1972; Kuskow, in Noznov 1986; Richter 1991). Axelrod & Shaw (1967), Tsilinszky (in Noznov 1986), Kuskow (in Noznov 1986), and Richter (1991) suggest placing a small amount of spawning grass such as *Nitella* or *Fontinalis* into the spawning tank, but Roloff (in Noznov 1986) and Noznov (1986) leave the tank empty. Once the spawning tank is ready, one pair of cardinals can be introduced (Roloff, in Noznov 1986; Axelrod & Shaw 1967; Tsilinszky, in Noznov 1986; Richter 1991; Benninger 1996).

In captivity, cardinals spawn only at night, either in complete darkness or very low light intensity (Roloff, in Noznov 1986; Noznov 1986; Geisler & Annibal 1987; Richter 1991), although none of the authors mention any figures. During spawning the pair scatter 100-300 (Axelrod & Shaw 1967; Geisler & Annibal 1987), small (<1mm), transparent to reddish brown, eggs (Roloff, in Noznov 1986; Tsilinszky, in Noznov 1986; Richter 1991). Courtship is active with both partners chasing each other. Richter (1991) recommends that the morning after spawning has occurred the adults should be removed from the spawning tank. Benninger (1996), however, states that the adults do not prey on the eggs. None of the other authors mention whether or not the adults should be removed from the spawning tank after spawning.

According to Axelrod & Shaw (1967) the eggs hatch after two days at 25°C, while Richter (1991) and Tsilinszky (in Noznov 1986) observed that the eggs hatched after 20 hours at 26-27°C and Roloff (in Noznov 1986) reported that the eggs hatch after 24-36 hours at 26°C. These appear to be observations without scientific basis. For the first few days the fry remain adhered to the plants or sides of the tank, by means of a mucous thread, while they use up their yolk sacs (Axelrod & Shaw 1967; Tsilinszky, in Noznov 1986; Richter 1991). However, none of the authors mention how many days. Thereafter they become free swimming and begin feeding on infusoria (Axelrod & Shaw 1967; Kuskow, in Noznov 1986), minute copepods (Kuskow, in Noznov 1986; Richter 1991) or brine shrimp (*Artemia salina*) nauplii (Roloff, in Noznov 1986). Growth thereafter is rapid and the young cardinals reach sexual maturity in six months at a size of 33mm (Geisler & Annibal 1987).

Under optimum conditions in an aquarium, cardinals can spawn every 8-14 days producing about 120-350 eggs per female, respectively (Geisler & Annibal 1987). However, it is not explained how these results were obtained. According to Geisler & Annibal (1987), optimum breeding results are achieved by selecting 'well matched' fish with the males being slightly older, but they do not mention how to judge a well matched pair, their age or how much older "slightly older" is. A female can spawn up to 30 times (Geisler & Annibal 1987).

Pinter (in Noznov 1986), Kuskow (in Noznov 1986) and Noznov (1986) report that sexually mature cardinal tetras will spawn in small shoals. This overcomes the problem of mismatched pairs. In this case a number of pairs (although no mention is made of how many) should be placed in a larger, 30 L, tank (Pinter, in Noznov 1986) or one fish per 5 L (Noznov 1986), spawning tank furnished as described above. Pairs that have spawned together should not be separated (Noznov 1986).

Roloff (in Noznov 1986), the first author to document spawning behaviour of cardinal tetras, left his pair in the spawning tank for four days without result. Only when the temperature was raised from 22°C to 25°C did the pair spawn. Noznov (1986) believes that this rise in temperature is essential. He recommends a raise from 25°C to 30°C in the morning, after which the temperature should be allowed to gradually fall during the second half of the day. He does not mention, however, what methods were used to derive these recommendations and hence it is not possible to judge how valid they are.

Since cardinals come from nutrient deficient waters, Richter (1991) believes that cardinals should be fed very sparingly, even starving them for a few days at a time. This, he believes, is particularly important when attempting to breed them. He then feeds them "exceptionally well" a few days prior to the spawning attempt. However, once again, this appears to be his own personal opinion with no scientific evidence to back these statements. Benninger (1996) believes that the fish should be fed extremely well, and is of the opinion that feeding is more important than water quality when attempting to spawn cardinal tetras.

Requirements of a protocol for spawning cardinal tetras

From this brief review it is evident that there is no clear and proven protocol for spawning cardinal tetras. The few authors who have documented spawning events, used a number of different methods and many of the authors attribute their success to different factors. Further, most of the observations were non-scientific and therefore the validity of these recommendations is doubtful. Therefore,u any experimental protocol should fulfill the requirements of a scientific study. It should be reliable and repeatable, feasible and practical, logical and sequential, efficient, intuitive, and analysable and documentable.

1.4 SYSTEM DESIGN

The system used for this study was a closed recirculating system (Fig. 1.4.1) consisting of 24 X 25 L spawning tanks, three 100 L holding tanks, three (70 L) trickle filters, a submerged filter (100 L) and a 400 L sump. Water was pumped by a Nocchi VIP 130/6 submersible pump (0.3Kw). Half the water was pumped directly to the spawning tanks and holding tanks, ¼ was pumped through the trickle filters and the final ¼ was pumped through the submerged filter. All the water was then returned to the sump via PVC guttering. Water flow through the spawning tanks was 1.7 $L.min^{-1}$ or four theoretical exchanges per hour and water flow through the holding tanks was 5.1 $L.min^{-1}$ or three exchanges per hour. All tanks contained an air stone with light aeration.

Water pH and conductivity were monitored at the start and end of each experiment using a HANNA HI9023CN portable µP meter and a HANNA HI933100 microprocessor, 4-ring ATC conductivity meter, respectively. Ammonia and oxygen levels were monitored each week using a Hach DR2000 spectrophotometer and an OxyGuard Handy MK III portable DO meter, respectively. Nitrite, nitrate, phosphate, and hardness were monitored bi-weekly using a Hach DR2000 spectrophotometer. Water quality was maintained within the levels shown in Table 1.4.1.

The brood stock holding tanks contained strips of green shadecloth tied together and weighted, for cover and were stocked at 1 $fish.L^{-1}$ when all the brood stock were in the holding tanks. For the duration of the study the water temperature was maintained at $25\pm0.2^{\circ}C$ and the photoperiod was maintained at 12L:12D. The room did not receive any natural light. Fish in the holding tanks were fed to satiation twice per day on finely sieved (800µm) dry pelleted feed for the duration of the study (Table 1.4.2). During some experiments these feedings were supplemented or substituted with live *Artemia* or *Daphnia*. These changes will be discussed later.

Table 1.4.1 Water quality parameters for the recirculating system for the duration of the study.

Parameter	Max Value	Min Value
Temperature	25.2°C	24.8°C
Conductivity	120µS/cm	46µS/cm
pH	6.4	5.6
Oxygen	8.7mg/L	7.8mg/L
Ammonia	0.15mg/L	0.08mg/L
Nitrite	0.001mg/L	0.000mg/L
Nitrate	2.52mg/L	0.24mg/L
Phosphate	0.80mg/L	0.52mg/L

Table 1.4.2 Composition and size of pelleted feed used during this study.

Constituent	Inclusion
Protein	48%
Carbohydrate	30%
Fat	8%
Moisture	10%
Spirulina	2.5%
Vitamins & minerals	1%
Carophyl pink	300ppm
Size of pellets	<0.5mm

Fig. 1.4.1 (a) The entire experimental system.

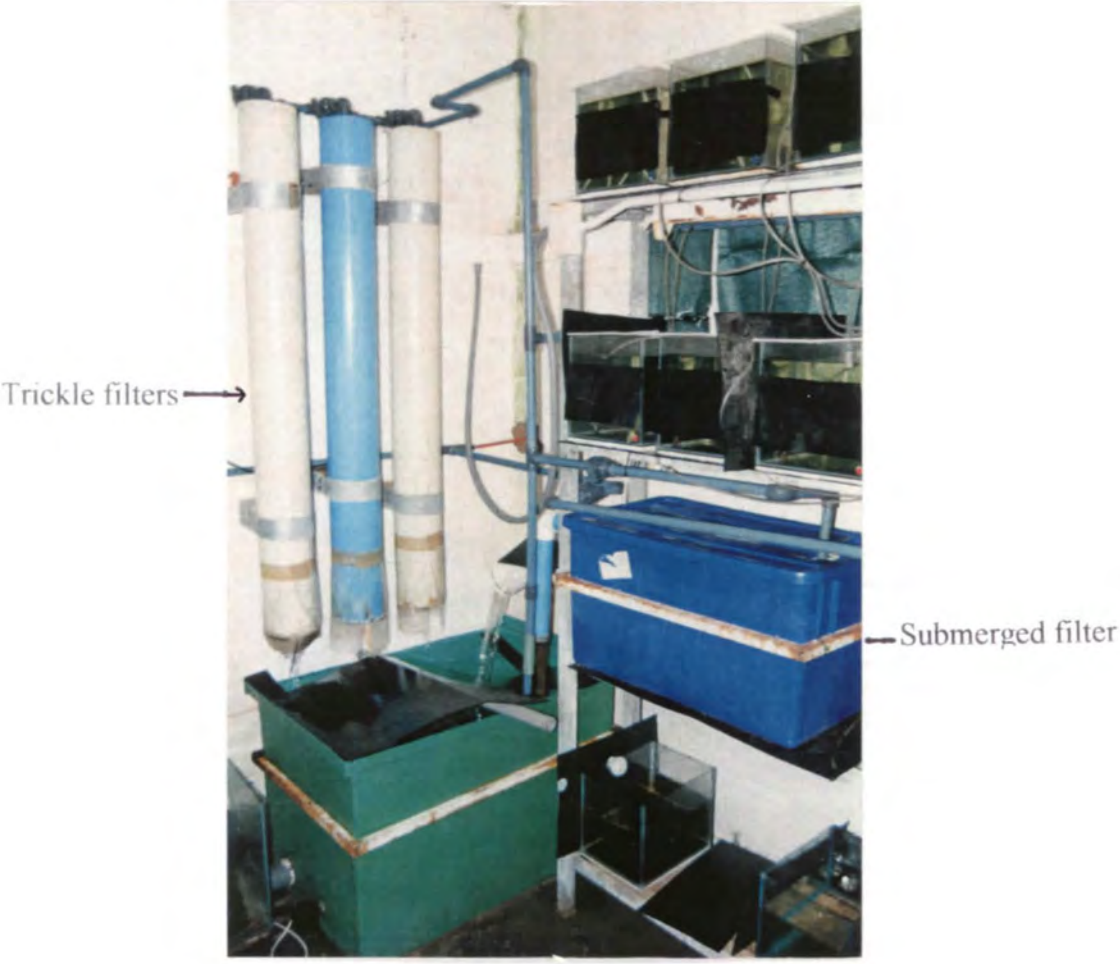
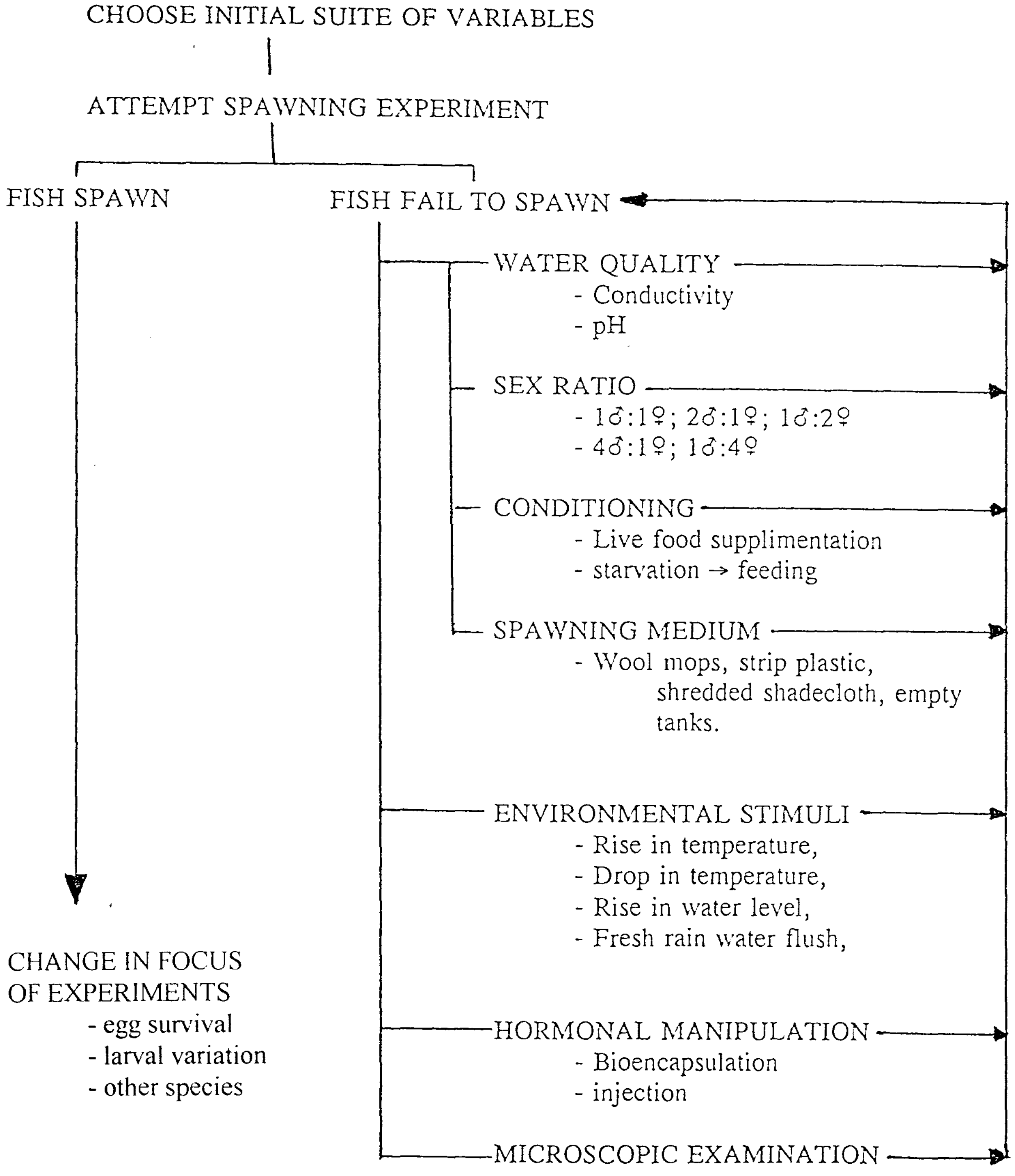


Fig 1.4.1 (b) Close-up of the filtration systems

Fig. 1.4.1 (c) A brood stock holding tank showing artificial weed made from stripped shade-cloth

1.5 EXPERIMENTAL APPROACH

This study was designed to develop a standardized protocol for spawning cardinal tetras, with the aim of obtaining repeatable results. Further, the experimental approach was designed in such a way as to be most practical to potential aquaculturists. Hence, the study was designed to follow a logical sequence of experiments that can be illustrated by means of a flow diagram (Fig. 1.5.1). Water quality was deemed to be the most important factor related to spawning in cardinal tetras (Axelrod & Shaw 1967; Noznov 1986; Geisler & Annibal 1987, Richter 1991), and hence the study began with a series of experiments investigating the effect of water quality on spawning success. Thereafter, if no repeatable spawning success was observed, the study would move on to investigate other factors that could influence spawning in cardinal tetras. If at any stage repeatable spawning events were observed, then the study would, time permitting, expand to include egg survival and larval rearing. It must be stressed, though, that the aim of the study was not exclusively to spawn the fish, but also to understand the process of spawning in cardinal tetras and in the event of the fish not spawning, to investigate the reasons why. The successes and failures observed will form the basis for the guidelines of the spawning protocol.



1.5.1 Flow diagram depicting the proposed sequence of experiments for this study.

CHAPTER 2: NATURAL SPAWNING.

2.1 THE EFFECT OF WATER QUALITY ON SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

Until 1967, spawning cardinal tetras in captivity was thought to be extremely difficult (Axelrod & Shaw 1967). However, since their natural habitat and water quality has been studied (Geisler & Annibal 1987) a number of authors have reported successful spawnings. This has been attributed mainly to understanding and replicating the water quality experienced by wild fish during the spawning season (Noznov 1986, Geisler & Annibal 1987, Richter 1991). As mentioned earlier, cardinal tetras inhabit the extremely soft, acidic waters of the upper tributaries of the Rio Negro (Geisler & Annibal 1987, Chao 1992). Therefore, cardinals have only been reported to spawn in extremely soft acidic water. The optimum water quality for spawning has been investigated and is shown in Table 1.3.1.

For the purpose of this study, the first priority was to determine the simplest possible method for making up large quantities of suitable water. The simplest method for softening and acidifying water is by filtering it through peat. Peat acts as a natural ion exchanger, taking up calcium and magnesium ions from the water and exchanging them for hydrogen ions (Schiff 1994). The effectiveness of the peat in softening and acidifying water depends on the type of peat used, the duration of use and the initial conditions of the water being used (Schiff 1994). The more peat used, the quicker it works and the greater the volume of water it can treat. Peat can not soften water completely (Schiff 1994).

In addition three different sex ratios were investigated and the experiment was designed to test for any influence of pheromones on other cardinals in the system.

This experiment tested the hypothesis that water quality does not influence spawning success in cardinal tetras. Further, it also tested the hypotheses that sex ratio has no effect on spawning success in cardinal tetras and pheromones do not influence spawning of other fish in the same system.

Materials and Methods

Thirty males and thirty females were selected randomly from the holding tanks and conditioned in 25-L spawning tanks, with ten fish in each of six tanks, three tanks for males and three tanks for females. The fish were then conditioned for two weeks on dry pelleted feed (Table 1.4.2). The fish were fed to satiation twice per day at 09:00 and 16:30 for the duration of the conditioning period.

At the end of the conditioning period, 24 spawning tanks were set up with 5mm plastic mesh false bottoms, and spawning mops made from colourfast green wool tied to the false bottoms. The back and sides, and the lower half of the front of the tanks were covered with black plastic and the tanks were covered with white plastic cardboard lids.

Three sex ratios were then chosen; 1♂: 1♀, 2♂: 1♀ and 1♂: 2♀. Six replications of each sex ratio were then placed into randomly selected spawning tanks. This was to eliminate any possible bias from tank position or height. A further six, randomly chosen, spawning tanks were isolated from the system and one male and one female placed into each. These six isolated tanks were used to test for any pheromonal influence between the rest of the tanks in the system. This is because the system water passed through all the other tanks and any pheromones released by one pair spawning could influence others.

This sex ratio study was "repeated" five times under different water quality conditions. These five runs have been summarised into Table 2.1.1. Before each run the system was drained and then filled with the respective water type. For the final two runs the water was only filtered through peat until a pH of 5.5 was obtained. During all five runs the temperature was maintained at 25±0.2°C.

Table 2.1.1 Type of water used with the respective pH and conductivity values, and the volume of peat used for each run.

Run	Water Type	pH	Conductivity µS/cm	Peat Volume dm^3
1	Tap water	8.1	>500	25
2	Aged Rain Water	7.2	100	25
3	Aged Rain Water	7.2	100	25
4	Fresh Rain Water	7.3	48	25 until pH 5.5 was reached.
5	Fresh Rain Water	7.3	48	25 until pH 5.5 was reached.

All tanks were checked for eggs every morning for the duration of the experiment. Noznov (1986), Richter (1991) and Benninger (1996) found that cardinals usually spawned on their third night in the spawning tank. If the fish fail to spawn on the third night they recommend that the fish should be removed. Benninger (1996) found that, out of 15 spawning observations, 13 occurred on the third night, one occurred on the first night and one occurred on the fourth night. Hence, the fish were left in the spawning tanks for four nights and removed in the morning of the fifth day.

The spawning results were transformed by a factor of $\sqrt{x}+1$ to eliminate any zeros. A non-parametric Kruskal-Wallis test was performed to test for significant differences between the transformed data. If significant differences were found then a Student-Neuman-Keuls multiple comparison was performed to identify which treatments differed significantly from the others.

Results

The resulting water quality in each of the five runs are shown in Table 2.1.2. The values for pH and conductivity were obtained by measuring at the start and end of the experiment and when the fish were put into the spawning tanks. Only in the third run did the pH rise from 5.4 to 5.8 while the others all remained constant.

Table 2.1.2 Observed pH and conductivity values during the five experimental runs.

Run	pH	conductivity µS/cm
1	6.5	>400
2	6.5	270-300
3	5.4-5.8	130-170
4	5.5	48-56
5	5.5	56-60

No spawnings were observed in runs one to three. One spawning was observed in run four. This occurred in a tank containing one male and two females and resulted in around 60 eggs of which 35 were unfertilised or nonviable. It was not possible to count exact numbers of eggs since the fertilised eggs were almost completely transparent and were difficult to detect amongst the fine peat dust that settled on the bottom of the spawning tanks during the experiment. Five spawnings were observed in run five. Three of which occurred in tanks containing one male and two females and all resulted in fewer than 50 eggs per female. One spawning was observed in a tank containing one male and one female and resulted in around 90 eggs, of which 24 were unfertilised. The final spawning occurred in one of the isolated tanks and resulted in around 50 eggs per female of which seven were unfertilised. There was no significant difference in spawning success between the three sex ratios and too few fish spawned to test for significant differences between the isolated and non-isolated tanks. All the above spawnings occurred on the third night that the fish were in the spawning tanks.

The spawning results for the five runs were transformed by the factor $\sqrt{x}+1$ and compared by means of a Kruskal-Wallis Test. The results are summarised in Table 2.1.3.

Table 2.1.3 Output from a Kruskal-Wallis test performed on the spawning results of the five runs.

Run	No. Spawned	Scores	Test Statistic	Significance Level
1	0	57.5		
2	0	57.5		
3	0	57.5		
4	1	60		
5	5	70	16.3538	0.002579*

* denotes a significant difference.

The final run was significantly different to all the others (P=0.00258). There were no other significant differences between runs one to four.

Discussion

The first run of this experiment demonstrated that even a large volume of peat was not sufficient to soften or acidify the local tap water to levels recommended in the literature for spawning cardinal tetras. Hence, in the second run, the system was drained and filled with aged rain water.

However, some tap water still remained in some inaccessible areas of the system, such as in the bottom of the filters. This combined with the relatively high conductivity of the aged rain water resulted in pH and conductivity values that were too high, according to the literature, despite being filtered through a large volume of peat. For the third run the system was drained completely and filled with aged rain water. Once again the peat was not effective in softening and acidifying the water to suitable levels, according to the literature. For the fourth and fifth run, the system was completely drained and filled with fresh rain water and filtered through peat until the correct pH was obtained. Thereafter the peat was removed and the pH remained stable at 5.5. In all cases the peat coloured the water a clear yellow/brown. This is due to the release of tannins and lignins into the water (Geisler & Annibal 1987; Schiff 1994) and closely resembles the water in the natural cardinal biotopes (Geisler & Annibal 1987). Hence, although the peat did not soften the water to the required levels, it was very effective in acidifying the water. Therefore, water with the correct hardness and conductivity, such as fresh rain water or deionised water, should be used and then acidified with acid peat. Deionised water is ideal but is expensive to produce and fresh rainwater is a good substitute. For the purpose of this study it was not possible to deionise enough water to fill the system.

The results of this experiment match those found by other authors (Axelrod & Shaw 1967; Geisler 1972; Frank 1980; Tsilinszky, in Noznov 1986; Kuskow, in Noznov 1986; Richter 1991), in that spawnings were only observed when the pH was around 5.5 and the conductivity in the range of 40-60µS/cm. Further, the final run showed a significantly higher spawning success than the other treatments. Above these levels, no spawnings were observed. However, relatively few of the fish spawned and the spawnings appeared to be random chance events.

These results match the observations of Noznov (1986) and Richter (1991) that in captivity cardinal tetras spawn on their third night in the spawning tank. However, this is still based on few observations and therefore for the following spawning experiments the fish were still left in the spawning tanks for four nights.

Although there was no significant difference in spawning success between the three different sex ratios tested, this may be biased by the fact that very few fish spawned which resulted in many zeros in the data set. Significant differences might have appeared if a higher proportion of fish had spawned.

Too few fish spawned to determine whether pheromones influenced spawning of other fish in the system. However, this had to be one of the first experiments since all the other experiments could have been affected by the potential artifact created from pheromones.

Although cardinal tetras spawned in fresh rainwater (conductivity 40-60μS/cm) filtered through peat until a pH of 5.5 was obtained, spawning success was low and therefore, some other limiting factor must have been present . Further, the hypothesis that water quality has no effect on spawning success in cardinal tetras could be rejected since the fish only spawned when the water pH was 5.5 and the conductivity was in the range 40-60μS/cm, and the final run showed a significantly higher spawning success than the other runs. However, relatively few fish spawned, and those that did, spawned relatively few eggs with a low fertilisation rate. To investigate reasons for this the next experiment followed the flow diagram (Fig. 1.5.1) to further evaluate the effect of sex ratio on spawning success.

2.2 THE EFFECT OF SEX RATIO ON SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

In small characins, the number of fish in the spawning tank and their sex ratio, has been reported to be particularly pertinent to spawning some species in captivity. Although most small characins are spawned in pairs (Axelrod 1971; Axelrod & Burgess 1973; Palicka 1989; Axelrod & Schultz 1990) many will not spawn in pairs and require higher numbers or different sex ratios for successful spawning. The optimum sex ratio for head-and-tail light tetras, *Hemigrammus ocellifer*, is two males to one female (Axelrod 1971). According to Axelrod & Burgess (1973) the optimum sex ratio for black tetras, *Gymnocorymbus ternetzi*, is two females to every male. The same is true for neon tetras, *P. innesi* (Penrith 1997). Axelrod & Schultz (1990) recommend a sex ratio of two males to three females for rummy-nosed tetras, *Hemigrammus rhodostomus*, and a sex ratio of two males to two females for yellow tetras, *Hyphessobrycon bifasciatus*. Further, many small characins only spawn in small shoals. These include the black lined tetra, *Hyphessobrycon scholzei* (Axelrod 1971), the emperor tetra, *Nematobrycon palmeri* (Nejmark 1987), the copeland tetra, *Hyphessobrycon copelandi* (Böhm 1996), the congo tetra, *Phenecogrammus interruptus* (Johnson 1995), and the dawn tetra, *Hyphessobrycon eos* (Axelrod & Schultz 1990). Red serpae tetras, *Hyphessobrycon serpae*, will normally spawn in pairs. However, if a pair fails to spawn, the addition of another male or another two males and a female may result in successful spawning (Burton 1997). However, these are recommendations of hobbyists and are not based on scientific evidence.

The optimum sex ratio for cardinal tetras has not been determined experimentally. Axelrod & Shaw (1967), Roloff (in Noznov 1986), Tsilinszky (in Noznov 1986), Richter (1991) and Benninger (1996) recommend that cardinals should be spawned in pairs. Pinter (in Noznov 1986) and Noznov (1986), however, recommend that cardinals should be spawned in small shoals. Geisler & Annibal (1987) report that some pairs will not spawn together and once a successful pair is found it should not be split up. Noznov (1986) agrees with this idea and recommends spawning the fish in a small shoal, such that there is a greater chance of finding compatible pairs. However, all the above recommendations were not based on scientific evidence. Further, there are no reported attempts of spawning trials using two males to one female or vice versa or even higher sex ratios such as four males to one female or vice versa.

The aim of this experiment was to test if sex ratios different from the previous experiment would yield a higher spawning success in cardinal tetras. Spawning success being the number of fish successfully spawning in each treatment. This experiment tests the hypothesis that sex ratio does not influence spawning success in cardinal tetras.

Materials and Methods

The experimental system was filled with fresh rain water (conductivity 59µS/cm) filtered through peat until a pH of 5.5 was obtained, and the temperature set at 25°C. Thirty males and 30 females were then selected and the sexes were conditioned separately for two weeks. During this time they were fed to satiation twice per day on dry pelleted feed (Table 1.4.2). For the spawning trial, 18 spawning tanks were furnished with 5mm plastic mesh false bottoms and woolen spawning mops. Five sex ratios were tested (Table 2.2.1).

Table 2.2.1 Sex ratios and replications used in this experiment.

Male: Female	Replications
1:1	6
2:1	3
4:1	3
1:2	3
1:4	3

The fish for each replication of each treatment were placed into randomly chosen spawning tanks and left for four nights, as described in Noznov (1986) and Richter (1991). All tanks were checked for eggs each morning.

Results

The water quality in the system remained constant at a pH of 5.53 and a conductivity of 58µS/cm for the duration of the experiment. No fish spawned.

Discussion

Although the water quality in the system was in the recommended range for spawning cardinal tetras, no fish spawned. This was not deemed to be a consequence of the wrong sex ratios being selected since spawnings had been recorded in two of the selected sex ratios previously in this study. Hence, some other factor other than sex ratio must have been responsible for the fish's failure to spawn. It was not possible, therefore, to statistically determine whether sex ratio had any effect on spawning success. The next step on the flow diagram (Fig. 1.5.1) was to investigate the effect of conditioning on spawning success.

2.3 THE EFFECT OF CONDITIONING THE BROODSTOCK ON LIVE FOOD, OR THE EFFECT OF A PERIOD OF STARVATION, ON SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

It is well documented that the nutritional status of a fish affects its reproductive success (Woodhead 1960; Scott 1962; Hester 1964; Wooton 1973; Hislop *et al.* 1978; Watanabe *et al.* 1984a; Watanabe *et al.* 1984b; Luquet & Watanabe 1986). Nutritional status has been related to reproductive factors such as fecundity, fertility, hatch rate and early larval survival. Nutritional status also affects egg size, spawning interval and egg resorption (Luquet & Watanabe 1986). Bagenal (1969) found that, in brown trout, *Salmo trutta*, the better-fed fish contained significantly more but smaller eggs, while Scott (1962) found that a reduced diet led to a lower fecundity due to follicular atresia, in rainbow trout, *Oncorhynchus mykiss*, but had no effect on egg size. Burton (1994) found that although iteroparous reproduction in fish involves regular repetition, some fish species, such as the winter flounder, *Pleuronectes americanus*, have irregular reproductive omission related to poor nutritional status. In this case the flounder fail to develop oocytes in spring if insufficient food was available in winter. Hence, poor nutritional status could be a causal factor in fish species that fail to spawn in captivity.

According to Benninger (1996) proper feeding is more important than water quality for spawning cardinal tetras. By this he refers particularly to a variety of live food diets and not just dry feeds alone. Most authors (Axelrod & Shaw 1967; Pinter, in Noznov 1986; Noznov 1986; Geisler & Annibal 1987; Benninger 1996) recommend that the fish should be fed a large proportion of live food during the conditioning period. Geisler & Annibal (1987) recommend copepods, but mention that *Artemia* makes a good substitute. Pinter (in Noznov 1986) recommends feeding copepods while Axelrod & Shaw (1967) recommend *Artemia*. Noznov (1986) recommends mosquito larvae, but also uses copepods and *Daphnia*.

In the wild, however, cardinal tetras inhabit extremely nutrient poor waters with very little food for the cardinals to feed on (Geisler & Annibal 1987). Richter (1991) therefore, suggests that cardinals should be fed very sparingly, even starving them for several days at a time. According to Richter (1991), feeding them well just prior to a spawning trial triggers the fish to spawn. In the wild one would expect cardinals to spawn when there is most food available for their offspring. Although it has not been documented, it is possible that there is an increase in food availability for cardinals during their spawning season since the rising flood waters would generate food, as is the case for *Brycinus leuciscus* in the Niger River basin (Bénech & Ouattara 1990). This could lead to the hypothesis that spawning could be triggered by a sudden increase in food availability.

For the purpose of this study, the most practical live food had to be chosen since it would have to be cultured on a large scale. Hence, the brine shrimp, *Artemia salina*, was chosen. *Artemia* is ideal for this purpose since it is available as disease free eggs that can be hatched in the laboratory and can either be fed as nauplii or grown on until they are of a suitable size for feeding. Further, *Artemia* is extensively used as a food source in fish culture systems and its nutritional value is well studied (Lavens & Sorgeloos 1996).

The objective of the first experiment was to investigate whether the supplementation or complete substitution of pelleted feed with *Artemia* had any effect on spawning success in cardinal tetras. This experiment tested the hypothesis that the supplementation or substitution of pelleted feed with *Artemia* has no effect on spawning success in cardinal tetras.

The objective of the second experiment was to determine whether a period of starvation followed by regular feeding would trigger spawning in cardinal tetras. This experiment tested the hypothesis that a period of starvation has no effect on spawning success in cardinal tetras.

Materials and Methods

Experiment 1

The *Artemia* used for this experiment were hatched in the laboratory and ongrown in 400 L porta pools for one week. During this time the *Artemia* were fed the algae *Isochrysis galbana* and grew to a size large enough for the fish to feed on. Thereafter, they were harvested using a fine meshed net, rinsed under fresh rain water and fed to the fish.

The system was filled with fresh rain water and filtered through peat until the pH reached 5.5. The conductivity of the rain water source had edged up to 65μS/cm and no other fresh rain water was available. The temperature of the system was maintained at $25^{\circ}\pm0.2$C for the duration of the experiment.

Twelve unfurnished spawning tanks were stocked with six fish in each for conditioning. Six tanks contained males and the other six contained females. Three feeding regimes, dry pelleted feed only, dry pelleted feed and *Artemia*, and *Artemia* only, were chosen and fed at two different rates, once per day and three times per day, each time to satiation. This results in six treatments (Table 2.3.1, Treatments 1-6) which were then applied to the six tanks of males and the six tanks of females for a period of two weeks.

Table 2.3.1 The combination of three diets and two feeding rates resulting in six treatments.

Diet	Once per day	Three times per day
Pelleted feed only	Treatment 1	Treatment 2
Pellets & Artemia	Treatment 3	Treatment 4
Artemia only	Treatment 5	Treatment 6

At the end of the conditioning period 18 spawning tanks were set up with 5mm plastic mesh false bottoms and woolen spawning mops. Three male fish from each treatment were then randomly selected and each was paired up with a randomly selected female from the corresponding treatment and placed into a spawning tank. The pairs were then left in the spawning tanks for four nights and removed on the morning of the fifth day. During this period the tanks were checked for eggs every morning.

Experiment 2

Eight conditioning tanks were set up. Four were stocked with eight randomly selected males in each and the remaining four were stocked with eight randomly selected females in each. The conditioning period lasted two weeks. During the conditioning period the fish were fed twice per day to satiation on fine pelleted feed unless being starved. One tank of males and one tank of females were fed every day during the conditioning period. The remaining three tanks of males were starved for four, five and six days respectively prior to the spawning trial. The same was applied to the remaining three tanks of females.

Thereafter, 24 spawning tanks were set up in the same manner as for experiment 1, with six tanks for each treatment. Six fish in each treatment were then randomly selected and used for the spawning trial. Each male was paired with its corresponding female and placed into a randomly selected spawning tank. The spawning procedure was the same as for experiment 1.

Results

The conductivity of the system rose from 65µS/cm to 72µS/cm during the experiments. The pH remained constant at 5.5. No fish spawned.

Discussion

Supplementing or substituting the pelleted feed with *Artemia*, was not more effective in stimulating the fish to spawn than dry pelleted feed alone. Neither was a period of starvation followed by feeding and, hence, some other factors must be involved. As mentioned previously, Axelrod & Shaw (1967), Pinter (in Noznov 1986), Noznov (1986), Geisler & Annibal (1987) and Benninger (1996) recommend the use of live food for the development of oocytes and Geisler & Annibal (1987) relate feeding with fecundity, the better the feeding the more eggs spawned. Examination of the fish used for this experiment revealed that all the females contained oocytes. These oocytes were visible by holding the female up to the light in a glass container. Hence the fish had already built up their oocyte supply but still failed to spawn. However, the number of oocytes in each female could not be counted but it would appear that supplementing or substituting the pelleted feed with *Artemia* did not have any effect since the fish already had oocytes.

Starved fish became visibly more nervous and appeared to lose condition compared to the control fish. This was not quantified. Starvation followed by feeding therefore does not seem to have a positive effect on spawning in cardinals.

Feeding cardinals *Artemia* during a spawning trial was not ideal since the *Artemia* were cultured in sea water and although they were rinsed thoroughly in fresh rain water they still caused a rise in the water conductivity. However, the conductivity remained <100μS/cm, recommended by Axelrod & Shaw (1967), and <80μS/cm recommended by Kuskow (in Noznov 1986), Noznov (1986) and Benninger (1996), and so was not considered problematic in this experiment. Using *Artemia* could prove problematic for large scale commercial breeders because of the large quantity of fresh rain water required to reduce the salt levels in *Artemia*.

What was missing in both experiments was the trigger for spawning behavior. Discovering this trigger was the objective of the following experiments.

2.4 THE EFFECT OF SPAWNING MEDIUM ON SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

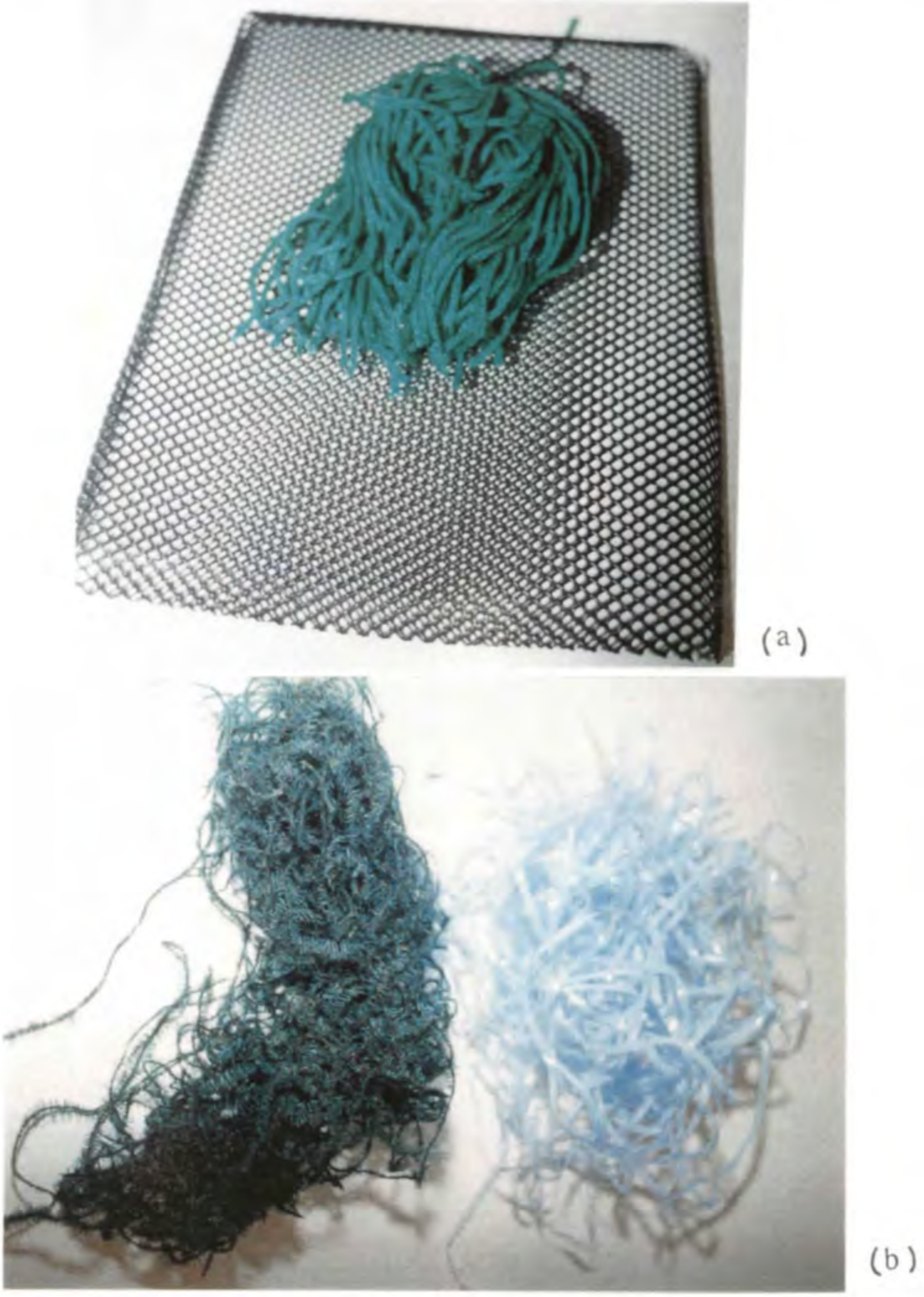
Another controversial factor in the literature pertaining to spawning cardinal tetras in captivity is the use of spawning medium. Most tetra species are successfully spawned on various types of spawning media (Axelrod & Shaw 1967; Nejmark 1987; Palicka 1989; Axelrod & Schultz 1990). Axelrod & Shaw (1967), Tsilinszky (in Noznov 1986), Kuskow (in Noznov 1986), and Richter (1991) recommend that half of the spawning tank should be planted with spawning grass such as *Nitella spp.* or *Fontinalis spp.* In the wild, however, cardinal biotopes contain very few if any green plants and the substrate is made up of leaf litter (Geisler & Annibal 1987). Roloff (in Noznov 1986), Noznov (1986) and Benninger (1996), therefore, leave the spawning tank empty. Hence, it would be useful to determine whether or not spawning medium should be placed in the spawning tank.

For a commercial venture, however, it is not particularly practical to use live green plants since they are difficult to sterilize and maintain in a healthy condition. This would be particularly relevant to cardinal tetras since their eggs and larvae are particularly susceptible to disease. Hence, this experiment tests three types of artificial spawning media; colourfast green wool, strip plastic, and shredded shadecloth (Fig 2.4.1 a, b). All these would be easily available in large quantities and would be simple to sterilize.

This experiment tested the hypothesis that the type of spawning medium has no effect on spawning success in cardinal tetras.

Materials and Methods

The system was filled with fresh rain water (conductivity 59µS/cm) and filtered through peat until the pH reached 5.5. For the duration of the experiment the temperature was maintained at 25±0.2°C.



2.4.1 Photographs of artificial media:(a) Wool mop on 5mm plastic mesh false bottom; (b) Strip plastic and shredded shadecloth.

Twenty-four males and 24 females were randomly selected from the holding tanks and placed in four conditioning tanks, two with 12 males in each and two with 12 females in each. The fish were then conditioned for two weeks. During this period the fish were fed to satiation twice per day with fine pelleted feed (Table 1.4.2).

Twenty-four spawning tanks were set up with 5mm plastic mesh false bottoms. Six tanks were left empty, six contained strip plastic, six contained wool mops and six contained shredded shadecloth. The males were then pooled and one was randomly chosen and placed in each tank. The same was done with the females such that each tank contained one pair of randomly selected fish. The fish were then left in the spawning tanks for four nights and the tanks checked each morning for eggs.

Spawning data were transformed by the factor $\sqrt{x+1}$ and then a Kruskal-Wallis test was performed on the transformed data.

Results

The conductivity and pH remained at 59μS/cm and 5.5 respectively for the duration of the experiment. One pair of fish spawned in a tank containing wool mops. The spawning resulted in an estimated 100 eggs of which 67 were unfertilized. All the eggs were found in the front of the tank and not in the spawning substrate.

There were no significant differences in spawning success between the four treatments ($P>0.391$).

Discussion

The hypothesis that spawning medium has no effect on spawning success in cardinal tetras, could not be rejected. This was due to the fact that too few fish spawned. Significant differences may have appeared if higher spawning rates were observed. Hence, spawning medium was not seen to be the factor limiting spawning success. The only pair to spawn spawned in the open area of the tank and only used the wool mop for cover during the day. It would therefore appear that this result matches the recommendation of Noznov (1986) and Benninger (1996) that spawning medium is not important. Despite the inconclusive results wool mops were used in all the following spawning experiments since they provide refuge for the fish during the day.

CHAPTER 3: ENVIRONMENTAL AND HORMONAL STIMULI

3.1 THE EFFECT OF ENVIRONMENTAL STIMULI ON SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

The reproductive cycles of many teleost species each comprise a number of discrete stages of differing duration. These stages include; gametogenesis, gonad maintenance, final gamete maturation, ovulation or spermiation, spawning and regression (Wallace & Selman 1981; Munro 1990a; Tyler & Sumpter 1996). Each of these stages may be under different external and internal control (Sumpter 1990; Hontela & Stacey 1990; Munro 1990a). The precise timing of reproductive cycles in freshwater fish may be controlled by: (1) endogenous rhythms; (2) exogenous control by reliable environmental predictive factors such as photoperiod or temperature; or (3) the entrainment of one or more endogenous rhythms by these predictive factors (Liley 1980; Sumpter 1990; Munro 1990a).

Predictive factors, most commonly photoperiod and temperature (DeVlamming 1974; Stacey *et al.* 1979; Liley 1980; Peter 1981; Munro 1990a; Cornish & Smit 1995), are particularly salient in the control of reproductive cycles in many temperate species (Peter 1981). These include; rainbow trout, *Onchorhyncus mykiss* (Pavladis *et al.* 1992; Bon et al. 1995; Bromage *et al.* 1995; Davies *et al.* 1995), smallmouth bass, *Micropterus dolomieui* (Cantin 1987), Atlantic cod, *Gadus morhua*, (Norberg *et al.* 1995; Hansen et al. 1995), mummichog, *Fundulus heteroclitus*, (Shimizu 1995), barbel, *Barbus barbus* (Poncin 1989; Baras 1995) and common carp, *Cyprinus carpio* (Davies *et al.* 1986a and b). In temperate areas, seasonal cycles of photoperiod and temperature are pronounced and reliable. In most temperate species, such as those mentioned above, gonadal recrudescence is triggered by these predictive factors and gonad development continues under their direct influence (Munro 1990a). Final maturation and spawning then follow spontaneously (Munro 1990a).

In tropical areas, however, seasonal cycles of photoperiod and temperature are far less marked and the reproductive cycle of many tropical species is under endogenous control, which may be kept on track by predictive factors (Liley 1980; Munro 1990a). In tropical areas the triggers for final maturation and spawning may be different from those triggering gonadal recrudescence (Munro 1990a). Gonadal recrudescence is triggered either by endogenous rhythms or a combination of endogenous rhythms and predictive factors (Munro 1990a). The gametes then develop under endogenous control and may remain in a fully developed state until the fish is exposed to the relevant synchronising cue (Liley 1980; Munro 1990a; Weitzman *et al.* 1996). These synchronising cues are typically short term factors such as rainfall, floods and their related water quality changes, a sudden rise or fall in water temperature, or the presence of standing water, nutrients, a suitable breeding substrate or the presence of vegetation (DeVlamming 1974; Bruton 1979; Stacey *et al.* 1979; Liley 1980; Munro 1990a; Cornish & Smit 1995; Weitzman *et al.* 1996). Synchronising cues are responsible for final maturation and spawning, provided that the predictive factors are still present. These factors can be referred to as synchronising cues since they synchronise spawning with the optimum conditions for egg and larval survival (Hontela & Stacey 1990; Munro 1990a).

Gamete maturation or the maintenance of mature gametes may cease through one of three mechanisms: (1) the necessary predictive cues are no longer present; (2) an endogenous loss of responsiveness to the continuing presence of appropriate cues; or (3) by the occurrence of exogenous terminating cues which induce gonadal regression despite the continuing presence of appropriate predictive cues (Munro 1990a).

Cardinal tetras are native to the upper tributaries of the Rio Negro in Brazil, between latitudes of 0°and 2°S. Hence, there is no marked seasonal change in photoperiod, and water temperatures seldom fluctuate more than 3°C over the year (Geisler & Annibal 1987; Munro 1990b). One would expect, therefore, that gonadal recrudescence would either be entirely under endogenous control or under endogenous control cued by predictive factors. Under these conditions the gonads would develop and then remain in a fully developed state until final maturation and spawning is triggered by the relevant synchronising cue. This is supported by evidence provided in Geisler & Annibal (1987) for cardinal tetras and Weitzman *et al.* (1996) for cardinal tetras and a number of other small characins. Wild cardinal tetras spawn shortly after the floods of the rainy season (Geisler & Annibal 1987). Hence the synchronising cue(s) is likely to be one, or a combination of changes in the environment related to the floods.

In cardinal tetras it is not known whether gonadal regression occurs after the spawning season and few fish survive more than a few months after their first spawning season (Geisler & Annibal 1987).

The female fish used during this experiment all contained oocytes which were visible in the fish when they were held up to the light in a glass container. Further, the fish that had spawned in previous experiments rapidly replenished their oocyte supply. This matches the result found by Geisler & Annibal (1987) that cardinals can spawn at eight to twelve day intervals. Hence, the initial factor responsible for gametogenesis was not missing. It could therefore be assumed that gonadal recrudescence and development are under internal control and occur as soon as the fish matures or shortly after spawning. The oocytes then remain in a fully developed state until the necessary predictive factor triggers final maturation and spawning. The objective of this experiment was to investigate the effect of various environmental stimuli on spawning success in cardinal tetras.

Two of the environmental stimuli tested in this experiment were related to those likely to occur during a flood event. These were a rise in water level, however this was without any change in water chemistry or temperature, and a fresh rain water exchange with it's associated drop in conductivity and rise in pH, but without any change in temperature. In addition, the effect of a rise in water temperature was also investigated since Noznov (1986) suggests that a rise in water temperature is essential for spawning in cardinal tetras. Three treatments and a control were all that could be tested at once since there were 24 spawning tanks available and six replicates of each treatment had to be performed.

This experiment tested the hypothesis that these environmental stimuli had no effect on spawning success in cardinal tetras.

Materials and Methods

The system was filled with rain water (conductivity 90µS/cm) and filtered through peat until a pH of 5.5 was obtained. Twenty-four males and 24 females were then randomly selected and separately placed in two conditioning tanks, 12 in each. The fish were then conditioned for two weeks during which time they were fed to satiation twice per day on fine pelleted feed.

Twenty-four spawning tanks were set up with 5mm plastic mesh false bottoms and wool mops. The spawning tanks were then isolated from the recirculating system and drained to a depth of 15cm. Heater/thermostats were placed under the 5mm plastic mesh in six randomly selected tanks. Pairs of fish were then randomly chosen and one pair was placed into each spawning tank. After three days, six randomly selected tanks were filled with system water to a depth of 22cm, six were drained to a depth of 10cm and topped up with fresh rain water (conductivity 69µS/cm) back to 15cm, the six with heaters were heated to 27±0.2°C and six remained as controls with a water depth of 15cm and a temperature of 25±0.2°C.

The fish were left in the spawning tanks for a further four nights and removed on the morning of the fifth day. The tanks were checked for eggs each morning.

The spawning data were transformed by the factor $\sqrt{x+1}$ and a Kruskal-Wallis test was performed on the transformed data.

Results

The changes in water chemistry between the different treatments are summarised in Table 3.1.1.

Table 3.1.1 Changes in pH, conductivity and temperature in the three experimental treatments and the control.

Treatment	pH	Conductivity	Temperature	Water Level
Rise in water level	-----*	-----	-----	15→22cm
Rain water exchange	5.5→5.9	90→81µS/cm	-----	-----
Rise in temperature	-----	-----	25→27°C	-----
Control	-----	-----	-----	-----

* ----- indicates no change.

One pair of fish spawned. This occurred in one of the heated tanks and resulted in an estimated 50 eggs, of which 8 were unfertilised.

There were no significant differences in spawning success between the treatments ($P>0.391$). Spawning success being the number of fish that spawned in each treatment.

Discussion

Since only one pair of fish spawned, it was not possible to reject the hypothesis that the environmental stimuli used had no effect on spawning success in cardinal tetras. However, the low proportion of fish spawning indicates that none of the stimuli used were effective in triggering the fish to spawn. It would need to be tested if the exact cues for spawning in cardinal tetras are a combination of factors that may be difficult to simulate in the laboratory. These cues could include a rise in pH, drop in conductivity, drop in temperature, rise in water level, as well as factors such as changes in air pressure (Peterson 1972), a sudden abundance in food (Béneche & Ouattara 1990), or even the increased swimming activity associated with the upstream migration. Simulating one, or a few of these cues may not be sufficient to trigger spawning in cardinal tetras. To solve this problem, multivariate analysis, comparing for example a number of combinations of temperature, fresh water exchange, and water level changes simultaneously, would be ideal. However, this would not be practical or feasible since it would require a far larger sample of fish and number of tanks than was practically possible. It was therefore decided to move on to hormonal manipulation since hormonal manipulation has successfully been employed to induce spawning in a number of species that fail to spawn naturally in captivity (Billard & Marcel 1980; Rowland 1988; Shireman & Gildea 1989; Harmin & Crim 1992; Haider & Rao 1994).

3.2 THE EFFECT OF GnRHa ENRICHED *ARTEMIA* ON SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

In most tropical fish species, final gamete maturation is a distinct process, dissociated from gonadal growth and regulated by synchronising factors (Peter 1981; Hontela & Stacey 1990). This final gamete maturation phase prepares viable gametes for spawning.

The critical event initiating both final maturation and ovulation in female fish is a surge in plasma gonadotropin (GtH) levels (Hontela & Stacey 1990). This GtH increase acts on the ovarian follicles to stimulate synthesis of steroids involved in final maturation (Hontela & Stacey 1990). The most potent maturational steroid in a number of species appears to be 17α,20ß-dihydroxy-4-pregnen-3-one (17,20P) (Hontela & Stacey 1990). With the completion of oocyte maturation, ovarian prostaglandins are synthesized to stimulate follicular rupture, followed by ovulation (Hontela & Stacey 1990). Provided that the relevant synchronising cues are still present, spawning should follow shortly thereafter (Hontela & Stacey 1990). In a number of species, such as goldfish (Hontela & Stacey 1990), females complete vitellogenesis and may remain in a preovulatory state for extended periods if the relevant synchronising cues are not present. The occurrence of these cues will then quickly induce ovulation (Hontela & Stacey 1990).

In males, the germ cells undergo a final maturation process which could be considered homologous to that occurring in females. The rupturing of the spermatic cysts releases spermatozoa into the sperm ducts. This process of increased milt production is termed semen thinning or hydration and can occur weeks or months prior to spawning (Hontela & Stacey 1990). In this process GtH increases milt production by stimulating testicular steroidogenesis. It is not clear, though, which steroids are involved, however, a number of androgens and progestogens have been shown to increase milt volume (Hontela & Stacey 1990). Relatively little is known, however, concerning the proximate factors which trigger the endogenous maturation process. Typical abiotic factors include water temperature, pH, or rainfall.

This has a number of applications to spawning fish that don't normally spawn freely in captivity. In most cases the fish contain fully developed oocytes and spermatocytes in a preovulatory and prespermiatory state, but fail to spawn. This is presumably due to the lack of the relevant synchronising factor that induces final maturation and spawning (Liley 1980; Stacey 1984). Treatment with hormone preparations (hypophysation) causes an artificial GtH surge in the fish and is often sufficient to induce final maturation (Donaldson & Hunter 1983). The treated fish are either allowed to spawn, or are manually stripped (Liley 1980).

Most hormone preparations are made up of either gonadotropin-releasing hormone (GnRH) extracted from fish pituitary glands, such as carp (Rothbard 1981; Hussain 1988; Zonneveld *et al.* 1988) or salmon (Billard & Marcel 1980; Lin *et al.* 1991; Haider & Rao 1994), or artificially produced gonadotropin-releasing hormone analogs (GnRHa) (Harmin & Crim 1992; Krishnan *et al.* 1993; Alok *et al.* 1994). A surge of GnRH acts by inducing a GtH surge which in turn initiates ovulation or spermiation (Halder *et al.* 1991). In fish, GnRH is regulated by dopamine which blocks the action of GnRH (Peter 1982). Hence, many hormone preparations include a dopamine antagonist such as pimozide (PIM) (Lin *et al.* 1991; Halder *et al.* 1991; Tan-Fermin et al. 1993) or domperidone (DOM) (Lin *et al.* 1991; Krishnan *et al.* 1993; Wang *et al.* 1995). Other, preparations that are known to induce final maturation in fish include; human chorionic gonadotropin (Henderson-Arzapalo & Colura 1987; Rowland 1988), luteinizing hormone-releasing hormone (LHRH) and its analogs (LHRHa) (Ngamvongchon *et al.* 1988; Lin *et al.* 1991; Tan-Fermin & Emata 1993), insulin-like growth factors (IGF) (Kagawa & Moriyama 1995) and prostaglandins (King & Young 1995; Tripathi & Singh 1995).

Gonadotropin preparations administered to fish with fully developed gonads usually induces final maturation and spawning. This would bypass the natural trigger mechanisms in species that do not spawn freely in captivity (Liley 1980; Hontela & Stacey 1990). Gonadotropin preparations are usually applied by means of injections (hypophysation). Hypophysation has been widely used in food fish production where brood stock fish are large and are hardly affected by injections. It has seldom been used for ornamental fish as small as cardinal tetras. Most studies testing the effect of hypophysation on spawning success in small fish were performed on goldfish, *Carassius auratus* (Yamamoto *et al.* 1966; Stacey *et al.* 1979; Cook & Peter 1980), but other small fish such as rainbow sharks, *Labeo erythrurus*, and redtail black sharks, *L. bicolor* (Shireman & Gildae 1989) and sticklebacks, *Gasterosteus aculeatus* (Ahsan & Hoar 1963) have also been studied. However, these fish are still relatively large in comparison to cardinal tetras. With cardinal tetras it was feared that injections would be harmful to the fish and cause fairly high mortality levels. Hence, an alternative method was sought. This would involve either placing the fish directly into a hormone solution or incorporating the hormone into their feed. Lockyear (1994) found that the former method was inefficient since it would requires large volumes of expensive hormone preparations. It was, therefore, decided that the hormone would be included into food particles. Possibly the easiest method of incorporating hormone into the food is to bind it into pelleted feed (Garret 1989). However, since the fish in the conditioning tanks did not take pelleted feed freely, much of the food was wasted. It was therefore decided that the hormone should be bioencapsulated into brine shrimp, *Artemia salina,* nauplii before they were fed to the fish.

Artemia has been extensively used as a food source for larval fish. Since *Artemia* is an indiscriminate particle feeder (Touraki et. al. 1995; Lavens & Sorgeloos 1996) it has been used as a carrier for extra nutrients (enrichment) or even chemotherapeutics to larval fish (Aguilar-Aguila et. al. 1994; Dixon et. al. 1995; Touraki et. al 1996). In this process of bioencapsulation, water insoluble nutrients or chemotherapeutics are emulsified and fed to the brine shrimp nauplii which are then fed to the fish (Aguilar-Aguila et. al. 1994; Dixon et. al. 1995; Touraki et. al 1996). Recently, however, liposomes have been used to mediate the delivery of water soluble antibiotics to brine shrimp nauplii (Hontoria et. al. 1994; Touraki et. al. 1995). This process is unfortunately not practical for this study because of its demands on facilities and costs and was therefore not considered. A water insoluble, micropelleted form of GnRHa was obtained from the Laboratory for Ecology and Aquaculture of the Catholic University of Leuven, Belgium, and used for this experiment. These micropellets contained 50µg GnRHa / g micropellets, bound in a starch base.

The objective of the first experiment was to investigate the effect of *Artemia* enriched with different concentrations of GnRHa on spawning in cardinal tetras.

The second experiment was designed to further investigate the effect of brine shrimp nauplii enriched with GnRHa on spawning in cardinals. However, it differed from the first experiment with regard to the enrichment process. In this case a high concentration of hormone (1g/L) was used and the *Artemia* were enriched for different durations.

Both experiments tested the hypothesis that brine shrimp nauplii enriched with GnRHa, had no effect on spawning success in cardinal tetras.

Materials and Methods

Before commencing the experiment a preliminary investigation was performed to ensure that the brine shrimps would assimilate the hormone particles. This involved enriching some brine shrimp nauplii with powdered GnRHa for one hour and then examining the nauplii under a dissecting microscope. At 25X magnification the particles of GnRHa were visible in the gut tracts of the brine shrimps. Further, a brief study investigating the amount of brine shrimps that six fish would eat in two minutes was performed. This was calculated by feeding three replicates of six fish, X amount of brine shrimp nauplii, until the amount that six fish would eat in two minutes was observed three consecutive times. This worked out to be 0.04g *Artemia*/g fish.

Experiment 1

Twenty-four males were randomly caught from the holding tanks and placed into four conditioning tanks at six fish per tank. A further four tanks were stocked each with six randomly selected females. Each tank containing males was randomly paired up with a tank of females by marking the respective tanks. The fish were conditioned for two weeks and fed twice per day to satiation, once on fine pellet feed and once on *Artemia*.

Eight 50ml conical flasks (four each for males and females) were filled to the 20ml mark with filtered sea water. The flasks were well aerated and placed under a 100W incandescent bulb. Thereafter, 0.07g of *Artemia,* ongrown for two weeks, were placed into each of the four flasks for the males. A further 0.12g of ongrown *Artemia* was placed into each of the four flasks for the females. Each of the flasks for the male fish was then paired with one of the flasks for the females. Fine powdered GnRHa micropellets (<120µm) were then added to corresponding pairs of flasks in the concentration of 0, 20, 40 and 60 µg GnRHa /g fish and left for four hours. Ongrown *Artemia* were used since newly hatched nauplii were not large enough to assimilate the hormone particles. Thereafter, the brine shrimps in each flask were fed to the fish in corresponding tank. One hour after feeding, the fish were paired up with respective partners and placed into spawning tanks. Each spawning tank contained a 5mm pastic mesh false bottom and a wool mop. The fish were left in the spawning tanks for four nights and the tanks were checked for eggs each morning.

Experiment 2

The same number of fish were conditioned in the same manner as for experiment 1. The enrichment process was the same as for Experiment 1 except powdered GnRHa micropellets were added to corresponding pairs of flasks at a concentration of 1g.L^{-1} at different times over a four hour period. The first at time 0, then after 2 hours, and 3 hours. No hormone was added to the final pair of flasks. Hence, the *Artemia* were bioencapsulated for 4 hours, 2 hours, 1 hour and 0 hours. The rest of the experiment was the same as for experiment 1.

The temperature of the system was maintained at 25±0.2°C and the pH at 5.5 for the duration of the experiment.

Results

The conductivity of the system increased from 68µs to 80µs during the two experiments. No fish spawned in either experiment.

Discussion

The procedures adopted during these two experiments did not induce the fish to spawn. This means that either the methods of enrichment were ineffective or that the hormone was ineffective. It is unlikely that the methods were ineffective since hormone particles could be seen in the gut tracts of the *Artemia* under magnification. Further, brine shrimps have successfully been enriched with other water insoluble particles such as liposomes and oil emulsions (Hontoria *et al.* 1994; Dixon *et al.* 1995; Touraki *et al.* 1995; Touraki *et al.* 1996; Lavens & Sorgeloos 1996). Hence, the only possibility that the method may have been ineffective is if the brine shrimps did not assimilate enough hormone to be effective. However, this could then be related to the concentration of hormone in the micropellets. It is therefore more likely that the hormone is ineffective. This could be due to a number of reasons. As mentioned the *Artemia* may not have assimilated a high enough concentration of hormone and therefore the fish may have fed to satiation before taking up enough hormone to be effective. Hence, using micropellets with a higher concentration of GnRHa would test this possibility. No such micropellets were available. The *Artemia* may have partly digested the hormone, thus rendering it ineffective. In some cases this has been found to be a problem with ongrown brine shrimps since they have a wider range of digestive hormones than nauplii (Lavens & Sorgeloos 1996). Much of the hormone could have leached out of the starch base that it was bound in. These two possibilities could be tested by performing a bioassay on enriched *Artemia*. Finally, much of the hormone may have passed straight through the fish's gut without being absorbed. This would occur if the fish were not able to digest the starch based micropellets and absorb the GnRHa. I. Roelants (Laboratory of Ecology and Aquaculture, Catholic University of Leuven, pers. comm.) found that goldfish take up food containing the micropellets, but were able to separate the nutrients from the microcapsules and release the microcapsules back into the water. A bioassay performed on the faeces of fish fed enriched brine shrimps would test if the microcapsules were not being digested by the cardinals. These interpretations could not be supported or rejected by the results of other enrichment studies in the literature since this was the first attempt to enrich *Artemia* with GnRHa and the first attempt to enrich *Artemia* with a water soluble compound bound in starch based micropellets. It was, therefore, decided that hormone injections should be attempted since this would ensure that the hormone enters the fish.

3.3 THE EFFECT OF HORMONAL BIOENCAPSULATION VS HYPOPHYSATION ON OVULATION RATE AND SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

The injection of hormone preparations into adult fish in order to trigger final maturation, has been used extensively in food fish culture. This method is used for the propagation of many species of carps (Rothbard 1981; Ngamvongchon *et al.* 1988; Hussain 1988; Halder *et al.* 1991), catfish (Zonneveld *et al.* 1988; Alok *et al.* 1993; Tan-Fermin & Emata 1993; Haider & Rao 1994; Alok *et al.* 1994; Linhart *et al.* 1995; Wang *et al.* 1995; Tripathin & Singh 1995), salmonids (King & Young 1995), as well as fish as diverse as the pike, *Esox lucius* (Billard & Marcel 1980), striped bass, *Morone saxatilis* (Henderson-Arzapalo & Colura 1987), Murray cod, *Maccullochella peeli* (Rowland 1988), Chinese loach, *Paramisgurnus dabryanus* (Lin *et al.* 1991), winter flounder, *Pseudopleuronectes americanus* (Harmin & Crim 1992) and many others.

No record was found of this method being used on ornamental fish as small as cardinal tetras.

This experiment tested the effect of an injection of Aquaspawn^R against the method of bioencapsulation as described in experiment 3.2 and a control of no hormonal treatment, on spawning success in cardinal tetras.

Materials and Methods

Aquaspawn^R hormone preparation was obtained from Spawnrite Ltd, RSA, and is a combination of GnRHa and a dopamine antagonist. No further details are available.

Eighteen males and 18 females were randomly caught from the brood stock holding tanks, anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured. Six males were iplaced in each of three tanks and six females were placed into each of a further three tanks. The fish were conditioned for one week and fed twice per day to satiation on fine sieved pelleted feed (Table 1.4.2).

The mean length of males in the injected group was 27.5±0.957mm (mean±standard deviation) and the mean weight was 0.265±0.027g. The mean length of females in the injection group was 32±1.291mm and the mean weight was 0.538±0.061g. Length measurements were taken as fork length.

After the conditioning period one tank of males was fed GnRHa enriched *Artemia*, enriched in the same manner as in experiment 3.2. The males in the second tank received an injection of Aquaspawn^R at the recommended dose of 0.5ml/kg. Dilutions (with saline) had to be made due to the small size of the fish and the minimum volume of 0.01ml that could be injected. Volumes to be injected were calculated from the mean mass of fish in the treatment. These fish were anaesthetised in a solution of 0.3ml/L 2-phenoxy-ethanol and placed on a wet cloth. Injections were applied by means of 0.5ml disposable U-100 insulin needles (100μm) to the dorsal muscle between the dorsal fin and the lateral stripe. Males were injected with 0.01ml (37.7ml/kg) of the solution while the larger females were injected with 0.02ml (37.2ml/kg). The males in the third tank served as a control. The same procedure was performed on the three tanks of females.

Once treated, the fish were paired up with a partner from a corresponding treatment and placed into a randomly selected spawning tank. All spawning tanks contained a 5mm plastic mesh false bottom and a woolen mop. The system was maintained at 25±0.2°C and a pH of 5.6 for the duration of the experiment. The fish were left in the spawning tanks for four nights and removed on the morning of the fifth day. The tanks were checked for eggs each morning of the five-day period.

Results

The conductivity of the system rose from 82µS/cm at the beginning of the experiment to 98µS/cm at the end.

No fish spawned. Two of the injected fish ovulated. The ovulation data was transformed by the factor $\sqrt{x+1}$ and a Kruskal-Wallis test was performed on the transformed data. There were no significant differences in ovulation rate between the treatments ($P= 0.116$). There were no mortalities.

Discussion

The failure of the fish to spawn could be due to a number of reasons. The hormone injections may have been ineffective because the smallest possible volume of hormone that could be injected (0.01ml) was still great, relative to the body mass of the fish (3.77% of body mass for males and 3.72% for females). Hence, much of the hormone could have leached out of the wound. This has not been recorded in the literature, but is a possibility when dealing with such small fish. Further, the application of a hormone injection may have stressed the fish to such an extent that they failed to spawn since stress has been shown to have a detrimental effect on the reproduction in fish (Pankhurst & van der Kraak 1997). The conductivity of the system may have been too high, at 82-98µS/cm for spawning or some other synchronising cue may have been missing. Hormone treatments are meant to overcome these problems (Liley 1980), however, and they are only likely to inhibit spawning if the hormone treatments are ineffective.

The results of this experiment were promising since two of the injected fish ovulated. Fish that ovulated had an extremely robust appearance, particularly evident from above, and could be induced to extrude eggs when light pressure was applied to their abdomens. Further, when held up to the light in a glass container, the oocytes of ovulated fish were transparent. The low ovulation rate (i.e. proportion of fish ovulating) in the injected group of fish, could have been caused by the factors discussed above.

A possible reason for the induction of ovulation in two of the Aquaspawn^{R}-injected fish compared to the *Artemia*-treated fish is that Aquaspawn^{R} contains a dopamine antagonist while the powdered GnRHa did not. However, neither treatment was more effective than the control in inducing the fish to spawn.

The injected fish did not suffer mortalities and hence the following experiment further tested the effect of an Aquaspawn^{R} injection against a control of a saline injection.

3.4 THE EFFECT OF A HORMONE INJECTION ON OVULATION RATE AND SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

This experiment repeated the hormone injection treatment of the previous experiment (3.3), but included a control of a saline injection to test whether ovulation was in fact caused by the hormone injection.

Materials and Methods

Twelve males and 12 females were randomly caught from the brood stock holding tanks, anaesthetised in 0.3ml/L 2-phenoxyethanol and weighed and measured. Six males were then placed into each of two conditioning tanks and six females were placed into each of a further two conditioning tanks. The fish were conditioned for 1 week and fed twice per day to satiation on fine sieved pelleted feed.

After the conditioning period, six males and six females received an injection of Aquaspawn^R at the recommended dose of 0.5ml/kg. Doses were calculated from the mean mass of fish in the treatment. The mean length of males in the injected group was 27.5±0.764mm and the mean weight was 0.285±0.021g. The mean length of females in the injection group was 31.8±1.1mm and the mean weight was 0.527±0.064g. Length measurements were taken as fork length. The fish were anaesthetised in a solution of 0.3ml/L 2-phenoxyethanol and placed on a wet cloth. Injections were applied by means of 0.5ml disposable U-100 insulin needles (100μm) into the dorsal muscle between the dorsal fin and the lateral stripe. Males were injected 0.01ml while the larger females were injected 0.02ml. The remaining fish were anaesthetized in the same manner as the hormone treated group and injected with an equivalent amount of saline.

Once treated the fish were paired up with a partner from a corresponding treatment and placed into randomly selected spawning tanks. All spawning tanks contained a 5mm plastic mesh false bottom and a woolen mop. The system was maintained at 25±0.2°C and a pH of 5.6 for the duration of the experiment. The fish were left in the spawning tanks for four nights and removed on the morning of the fifth day. The tanks were checked for eggs each morning.

Results

The conductivity of the system rose from 104µS/cm at the beginning of the experiment to 106µS/cm at the end.

No fish spawned, but two hormone injected fish ovulated. The ovulation data were transformed by the factor $\sqrt{x+1}$ and a Kruskal-Wallis test was performed on the transformed data. There were no significant differences in ovulation rate between the treatments ($P=0.1380$). There were no mortalities.

Discussion

Although the results were not significantly different, it appeared that the hormone injection does have an effect on the fish and with larger sample sizes, significant differences may occur.

The failure of the fish to spawn could be due to the same reasons discussed in the previous experiment. A further consideration is that the two females that ovulated didn't spawn. This could be due to the high conductivity or the lack of some exogenous spawning cue, or it could be that all or some of the male fish were at fault. It was not possible to strip any of the males for milt. This could be because either it is not possible to strip males, even if they have spermiated, or that none of the males spermiated. If none of the males spermiated then they would not respond to the female's spawning behaviour and the pair would fail to spawn. Hence, the following experiment was designed to test the effect of a hormone injection on two sex ratios, 1♂:1♀ and 4♂:1♀ against saline injected fish at the same sex ratios, on spawning success in cardinal tetras.

3.5 THE EFFECT OF HORMONE INJECTION AND SEX RATIO ON OVULATION RATE AND SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

This experiment was designed to test the effect of different sex ratios in combination with a hormone injection on spawning success in cardinal tetras. This was to test if some of the males may have been responsible for the lack of spawning success by comparing the effect of a sex ratio of four males to one female to a sex ratio of one male to one female.

This experiment tests the hypothesis that sex ratio has no effect on spawning success in hormone injected fish.

Materials and Methods

Thirty males and 12 females were randomly caught from the brood stock holding tanks, anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured, and placed into three tanks of twelve fish, two for males and one for females. The mean length of males in the injected group was 27.4±0.8mm and the mean weight was 0.280±0.026g. The mean length of females in the injection group was 32.17±1.5mm and the mean weight was 0.540±0.084g. Length measurements were taken as fork length. The fish were conditioned for 1 week and fed twice per day to satiation on pelleted feed (Table 1.2.4). Thereafter, all the fish received an injection of Aquaspawn^R at the recommended dose of 0.5ml/kg. Hormone concentrations were calculated from the mean mass of fish in the treatment. The fish were anaesthetised in a solution of 0.3ml/L 2-phenoxyethanol and placed on a wet cloth. Injections were applied by means of 0.5ml disposable U-100 insulin needles (100µm) into the dorsal muscle between the dorsal fin and the lateral stripe. Males were injected 0.01ml while the larger females were injected 0.02ml.

Once treated the fish were placed into randomly selected spawning tanks, six tanks with one male to one female and six tanks with four males to one female. All spawning tanks contained a 5mm pastic mesh false bottom and a woolen mop. The system was maintained at 25±0.2°C and a pH of 5.5 for the duration of the experiment. The fish were left in the spawning tanks for four nights and removed on the morning of the fifth day. The tanks were checked for eggs each morning.

Results

The conductivity of the system remained at 105µS/cm for the duration of the experiment.

No fish spawned, one female ovulated and there were no mortalities. No sperm could be stripped from any males.

Discussion

The failure of the fish to spawn could be due to a number of reasons that were discussed in the previous experiments. This experiment was designed to test if a sex ratio of four males to one female was more effective in inducing the fish to spawn than a sex ratio of one to one. However, since none of the fish spawned, this was not the case. This means that cardinals will either not spawn at such high sex ratios, or that the hormone injections were ineffective. It is unlikely that the sex ratios were too high based on evidence in Noznov (1986) that cardinals will spawn in groups. Therefore, it is likely that the hormone injections were not effective since only one of the females ovulated and no milt could be stripped from the males. Although spermiation was not specifically checked, it seems likely that hypophysation is ineffective on the males since no males in this, or in the previous experiments, spermiated. A histological study of the males would have helped answer this problem. However, this was not possible due to the shortage of male brood stock (34 fish).

Since the reliability of the males was in doubt, the following experiment was designed to test the effect of a range of hormone treatments on ovulation rates in female cardinal tetras. There was no shortage of females (180 fish) and hence, multivariate analysis was possible. Thereafter, if the ideal treatment was found, then that treatment could be tested on the males.

3.6 THE EFFECT OF TWO HORMONE INJECTION METHODS AND THREE HORMONE DOSES ON OVULATION RATE IN TWO SIZE CLASSES OF CARDINAL TETRAS.

Introduction

Hormone injections can either be applied intramuscularly or intraperitoneally. Although both methods are widely used it is unclear which is more effective for inducing final maturation. Henderson-Arzapalo & Colura (1987), Zonneveld *et al.* (1988), Lin *et al.* (1991), Halder *et al.* (1991), Tan-Fermin & Emata (1993), Alok *et al.* (1993), and Alok *et al.* (1994) successfully used intramuscular injections in their hormone studies, while Ahsan & Hoar (1963), Billard & Marcel (1980), Cook & Peter (1980), Rowland (1988), Shireman & Gildea (1989), Harmin & Crim (1992), and Haider & Rao (1994) successfully used intraperitoneal injections. None of these authors mention why they used the method that they did and none used both methods of hormone application in order to compare the two methods.

In the previous experiments, intramuscular injections with Aquaspawn^R were not effective in inducing the fish to spawn. This experiment was designed to test the effect of two different application methods, intramuscular (IM) and intraperitoneal injection (IP), and three different doses of Aquaspawn^R (1x, 2x and 4x the recommended dose) on ovulation rate in two size classes (> 32mm fork length and <32mm FL) of female cardinal tetras. The objective being to identify a method of hormone treatment that would produce higher ovulation rates in cardinal tetras.

Materials and Methods

Forty-eight females larger than 32mm FL were randomly caught from the brood stock holding tanks and placed into six conditioning tanks, eight in each. A further 48 females smaller than

32mm FL were randomly caught from the brood stock holding tanks and placed into six conditioning tanks, eight in each. The fish were conditioned for one week and fed twice per day to satiation on pelleted feed (Table 1.4.2). Thereafter, the fish in the twelve tanks were treated according to a factorial, 2x size x 2x injection method x 3x dose design (Table 3.6.1). Aquaspawn^R was the hormone preparation used for this experiment. Aquaspawn^R dosages per fish were calculated based on the mean mass of fish in the treatment. The fish were anaesthetised in a solution of 0.3ml/L 2-phenoxyethanol and placed on a wet cloth. Injections of 0.5ml/kg Aquaspawn^R were applied by means of 0.5ml disposable U-100 insulin needles (100μm). Intramuscular injections were applied to the dorsal muscle between the dorsal fin and the lateral stripe. Intraperitoneal injections were applied by a shallow insertion between the pelvic fins and the anus. The females were injected 0.02ml per fish.

Table 3.6.1 Hormone treatments and doses for the two size classes of female cardinal tetras.

Size	Large						Small					
Injection	IM			IP			IM			IP		
Dose	x1	x2	x4	x1	x2	x4	x1	x2	x4	x1	x2	x4
Treatment	1	2	3	4	5	6	7	8	9	10	11	12

Once treated, the fish were placed back into the conditioning tanks and left for five days. The fish were removed from the tanks and anaesthetised, weighed and measured and checked for evidence of ovulation. Ovulated fish had extended abdomens and the eggs could easily be stripped from the fish. Ovulation rate was the number of fish in each treatment that ovulated. Further, each fish was assigned a value according to an ovarian maturation index (OMI). This value was assigned visually according to the state of the ovaries, with 1 being assigned to fish with no or very few visible oocytes, 2 to fish with fully developed ovaries and 3 to fish which had ovulated.

Condition factors (CF) were then calculated according to the equation (Bolger & Connolly 1989):

$$CF = (W/L^3)\ 100\,000$$

where W= weight (g)
and L= fork length (mm)

The mean condition factors for each treatment were then compared by means of multifactor ANOVA, with size, injection type and dose being the main effects. All possible two-way interactions were tested. In the case of significant differences, a Tukey multiple range test was performed to identify which of the treatments differed significantly from each other. OMI distributions between treatments were compared by means of Chi-squared tests at $P<0.05$.

The water in the system was maintained at 25±0.2°C and a pH of 5.5 for the duration of the experiment.

Results

The conductivity of the system remained at 105µS/cm for the duration of the experiment.

There were no significant differences, or interactions between main effects between any of the treatments ($P>0.05$) with regard to CF.

There was a significant difference in the OMI distributions between large and small females with the majority of large females scoring a 2 while the majority of the small females scored a 1 (Fig 3.6.1). There was no significant difference in the OMI distributions between females injected intramuscularly and those injected intraperitoneally. There were no significant differences in the OMI distributions between the three Aquaspawn^R doses (Table 3.6.2).

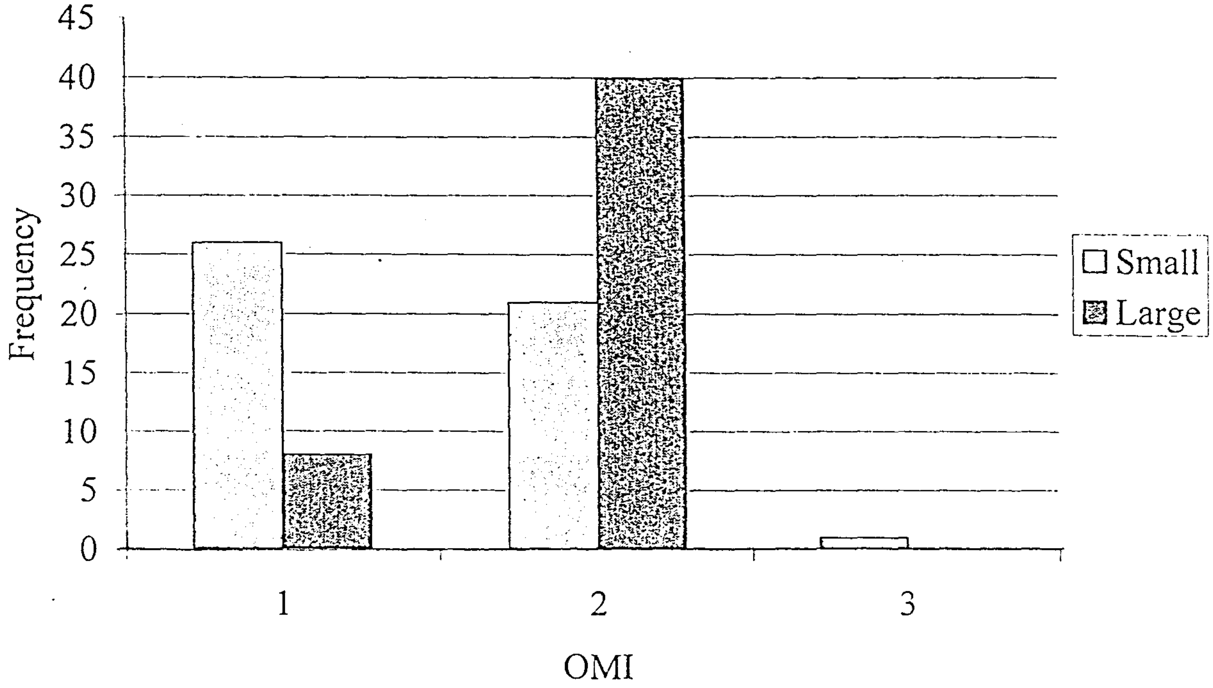


Fig. 3.6.1 (a) Frequency histogram of OMI distribution for size.

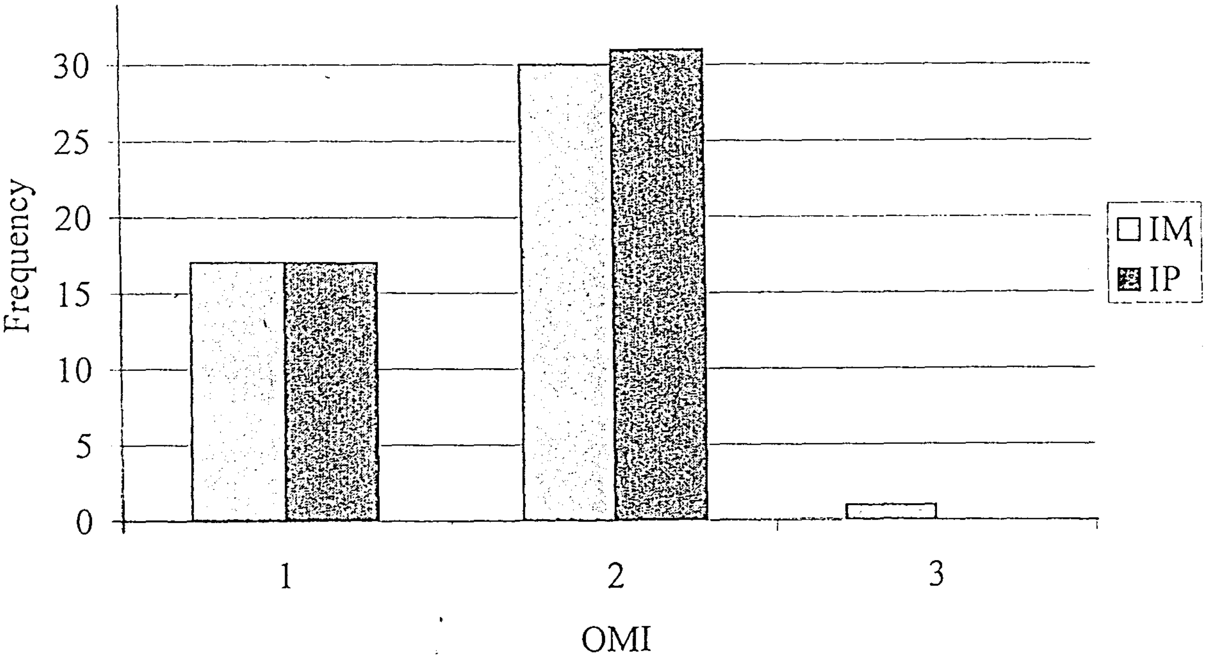


Fig. 3.6.1 (b) Frequency histogram of OMI distribution for injection method.

(c)

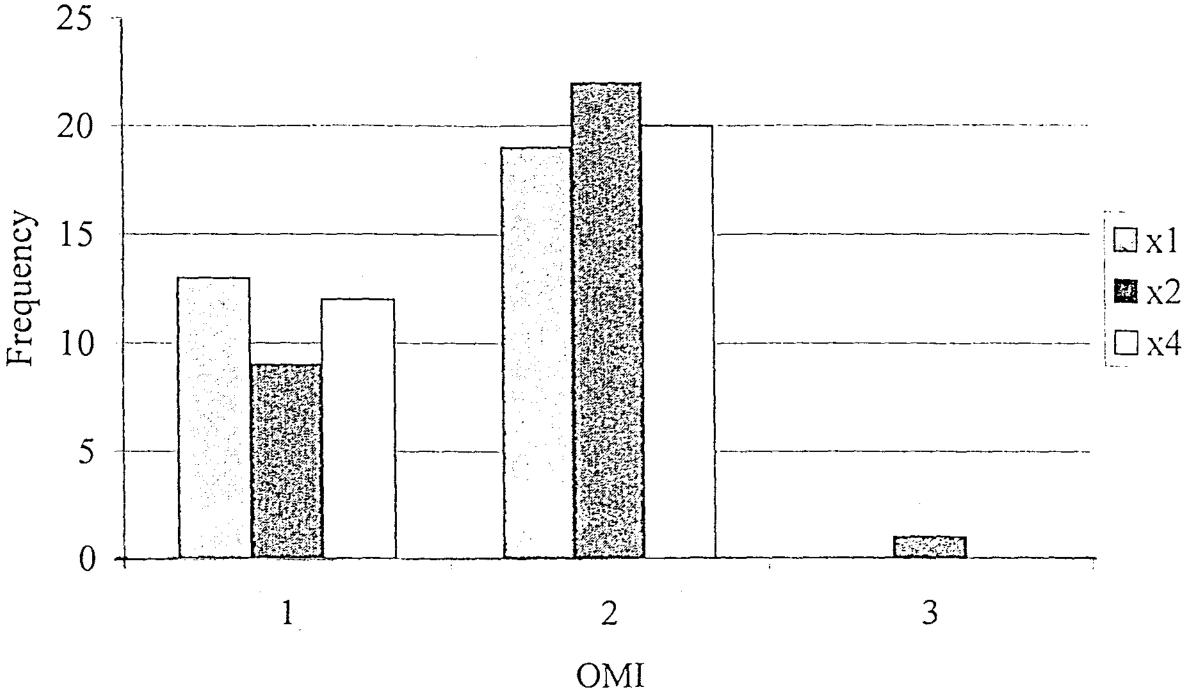


Fig. 3.6.1 (c) Frequency histogram of OMI distribution for hormone dose.

Table 3.6.2 Chi-squared values and degrees of freedom for the ovarian maturation index data.

Treatment	Test Parameters	X^2	df	Rejection region
Size	Small vs Large*	49.525	2	$X^2 > 5.99$
Injection	IM vs IP	0.095	2	$X^2 > 5.99$
Dose	1x vs 2x	2.187	2	$X^2 > 5.99$
	1x vs 4x	0.133	2	$X^2 > 5.99$
	2x vs 4x	1.182	2	$X^2 > 5.99$

* denotes a significant difference ($p>0.05$)

Discussion

In this experiment CFs were not measured prior to the sperming trial so as not to stress the fish unnecessarily. Two size classes were chosen because the larger females appeared to contain proportionally more oocytes than the smaller females and may therefore be induced to ovulate more easily than the smaller females.

The hormone doses were chosen to ensure that sufficient hormone was absorbed by the fish and that any possible effects of leaching were eliminated.

None of the combinations of hormone treatments induced the females to ovulate. This could be due to a number of reasons. The hormone injections may stress the fish to the point where they fail to ovulate, hormone injections may be ineffective on cardinal tetras, as the lack of the relevant synchronising cue may block the effect of the hormone injection and inhibit ovulation, or some social cue from the males may be necessary for the females to ovulate. Hence, this method of injecting cardinal tetras with Aquaspawn^R was not a feasible method for inducing final maturation and spawning. This is probably true for any type of GnRHa preparation since Aquaspawn^R has been successfully used to induce final maturation in other fish such as black phantom tetras, *Megalamphodus megalopterus* (Lockyear 1994) and *Synodontis petricola* (Rouhani 1997). The effects of other methods of hormone treatment, such as the incorporation of a hormone preparation into the feed or hormone baths on final maturation and spawning would have to be investigated in order to identify a more practical method of hormone treatment.

The following experiment was designed to microscopically examine stripped oocytes.

3.7 EXAMINATION OF CARDINAL TETRA OOCYTES

Introduction

Geisler and Annibal (1987) suggest that cardinals are group-synchronous spawners, spawning a number of times during the spawning season. They base this conclusion on the recruitment of juveniles into the population. Another method for testing this is by examining the oocytes in the ovaries. According to Wallace and Selman (1981) fish can be grouped into three categories according to their spawning strategy. In *synchronous* spawners, oocytes grow and ovulate from the ovary in unison and further replenishment of one stage by an earlier stage does not take place. Such ovaries are found in fish that spawn once and then die, such as many species of salmonids. In *group-synchronous* spawners, at least two populations of oocytes can be distinguished at some time, a fairly synchronous population of larger oocytes (termed a "clutch") and a more heterogeneous population of smaller oocytes from which the clutch is recruited. Fish in this group spawn clutches of eggs at intervals varying from annually to every few weeks. Synchronous spawners and group-synchronous spawners generally have gonadosomatic indices (GSI) (GSI= gonad weight/(body weight - gonad weight)x100) of between 10 and 40, although European eels (*Anguilla anguilla*) implanted with carp pituitary extract may attain a GSI of 60 (Wallace & Selman 1981). In *asynchronous* spawners oocytes of all stages are present without dominant populations. These fish spawn relatively few eggs almost on a daily basis during the spawning season. Most fish falling into this group seldom have GSI's greater than 10 (Wallace & Selman 1981).

This experiment examined manually stripped oocytes to determine what spawning strategy cardinal tetras utilize, as well as to check for any possible defects in the oocytes. Further, this experiment investigated the effect of a hormone injection, and the effect of a live food diet against a pelleted feed diet during the conditioning period, on ovulation rate.

Materials and Methods

Forty mature females were randomly selected from the brood stock holding tanks and placed into four conditioning tanks, ten fish in each. Fish in two of the tanks were fed fine pelleted feed while the fish in the other two were fed live *Daphnia.* All fish were fed twice per day to satiation. The fish were conditioned for one week before hormone injections were administered. The fish in one tank of each diet regime were injected. These fish were anaesthetised in 0.3ml/L 2-phenoxyethanol and injected intramuscularly with 0.02ml of Aquaspawn^R diluted with saline such that the recommended dose of 0.5ml/kg was injected. The fish were then placed back in the conditioning tanks and fed as before for a further five days. Thereafter, four fish from each tank were randomly selected anaesthetised in 0.3ml/L 2- phenoxyethanol, weighed and measured and manually stripped under a dissecting microscope. The oocytes of each fish were photographed at a magnification of 25x.

The remaining six fish in each tank were anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed, measured and fixed in 5% formalin for two days. Thereafter, the ovaries of each fish were carefully removed and weighed to the nearest 0.001g. The eviscerated body of each fish was also weighed. These measurements were used to calculate a gonadosomatic index (GSI) according to the equation (Tyler & Sumpter 1996):

$$GSI = (\text{Ovary mass}/(\text{Eviscerated body mass} - \text{Ovary mass}))\text{x}100$$

Condition factors (CF) were then calculated according to the equation (Bolger & Connolly 1989):

$$CF = (W/L^3)\ 100\ 000$$

where W= weight (g)
and L= fork length (mm)

Multifactor ANOVA's were performed on the length, CF and GSI data with diet and injection being the main effects. Tukey's multiple range test was performed on data where significant differences were found in order to identify which treatment means differed significantly from each other. The ovulation data were transformed by the factor $\sqrt{x+1}$ and a Kruskal-Wallis test was performed on the transformed data.

Results

There was no significant difference in mean fork length of the fish between the four treatments (P=0.5401)(Table 3.7.1).

Table 3.7.1 The 95% Tukey confidence interval for the length data.

Mean	n	Lower limit	Upper limit
32.7mm	40	32.4mm	33.0mm

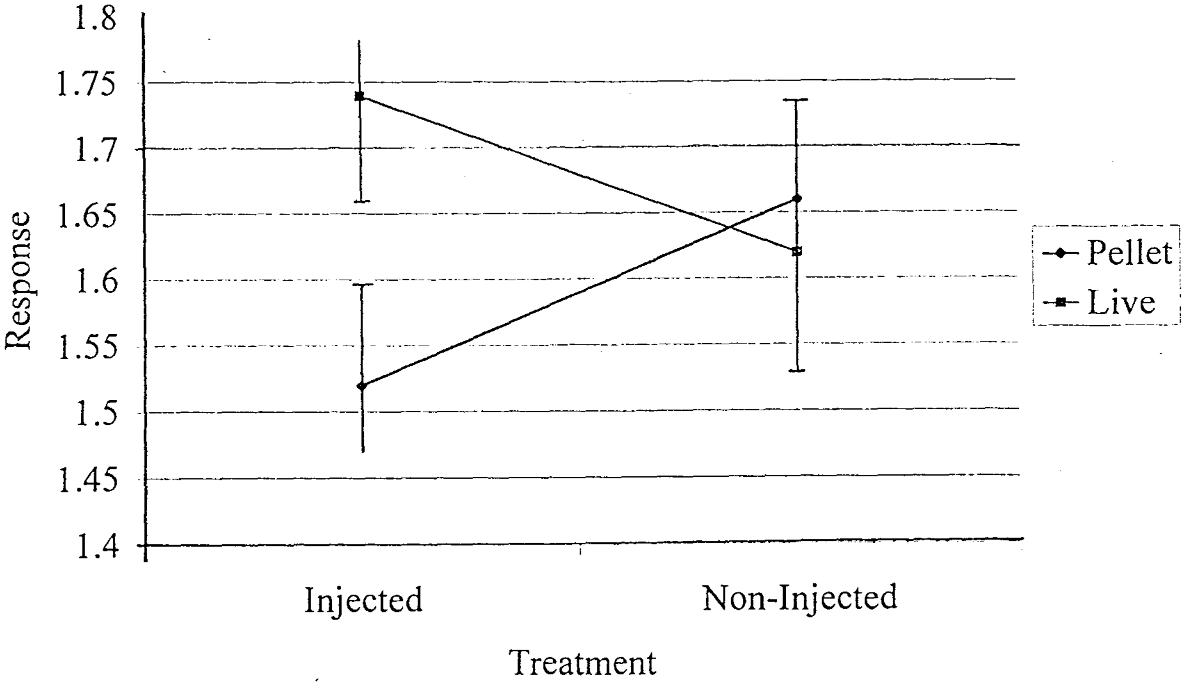
There was a significant interaction between diet and injection treatments for the CF data (P=0.0011). The CF of fish fed live food was higher for injected fish than non-injected fish while the CF of fish fed pelleted feed was lower for injected fish than for non-injected fish (Fig 3.7.1).

There was a significant interaction between diet and injection treatments for the GSI data (P=0.0236). The GSI for fish fed live food was higher for injected fish than non-injected fish. The reverse was true for fish fed pelleted feed (Fig 3.7.2).

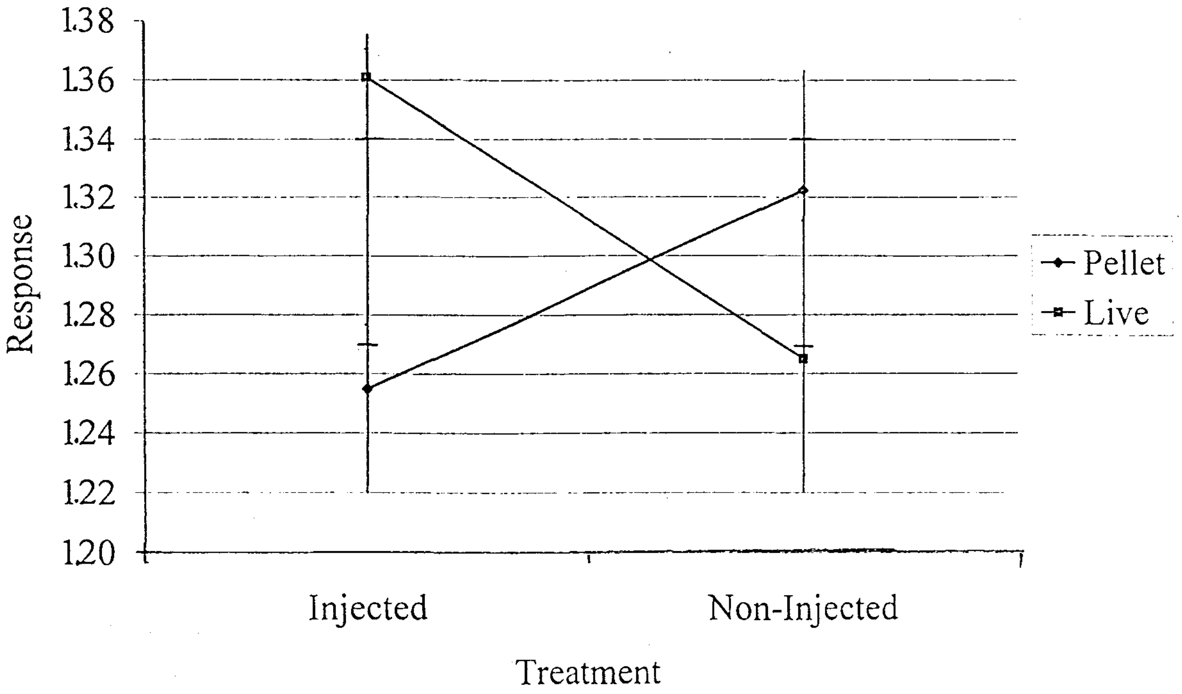
A Mann-Whitney U-test was performed on the GSI data for ovulated and non-ovulated fish (Table 3.7.2) and a significant difference was found for the GSI values between the two groups (P<0.05)

Table 3.7.2 Mean, minimum and maximum GSI values for ovulated and non-ovulated fish.

Ovary Development	n	Mean	Standard deviation	Min	Max
Ovulated	5	46.6	7.7	38.6	60.0
Non-Ovulated	19	26.3	6.2	14.6	36.3



3.7.1 Interaction plot displaying the significant interaction between diet and injection treatments for the CF data.



3.7.2 Interaction plot displaying the significant interaction between diet and injection treatments for the CF data.

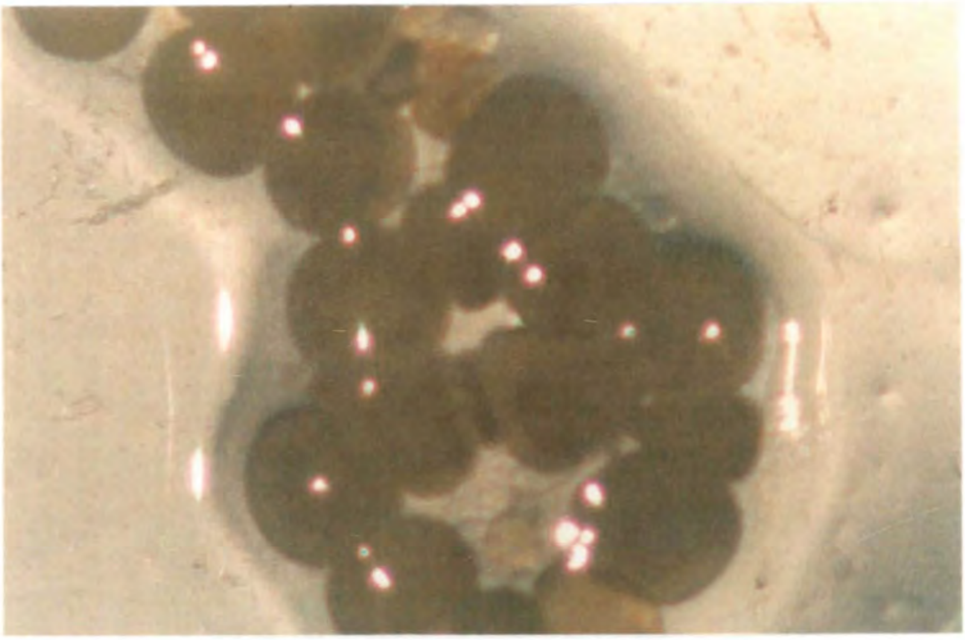
There were no significant differences in ovulation rate between the four treatments (P=0.3607).

Figs. 3.7.3 - 3.7.6 illustrate the oocytes of four of the manually stripped fish. These were considered to be representative of the rest of the photographs. Two of the fish were mature but had not ovulated (Fig. 3.7.3 & Fig. 3.7.4), one was in the process of ovulating (Fig 3.7.5) and the fourth had ovulated (Fig 3.7.6). It is evident that there was more than one cohort of oocytes present in the ovaries (Table 3.7.3). There is a dominant cohort of large oocytes which were either ready to be ovulated (Fig. 3.7.3; Fig 3.7.4), in the process of being ovulated (Fig 3.7.5) or ovulated (Fig 3.7.6). In all these cases this cohort of large oocytes would be the next clutch of eggs spawned. There is also a heterogeneous population of smaller oocytes which would recruit to replace the clutch in the event of spawning.

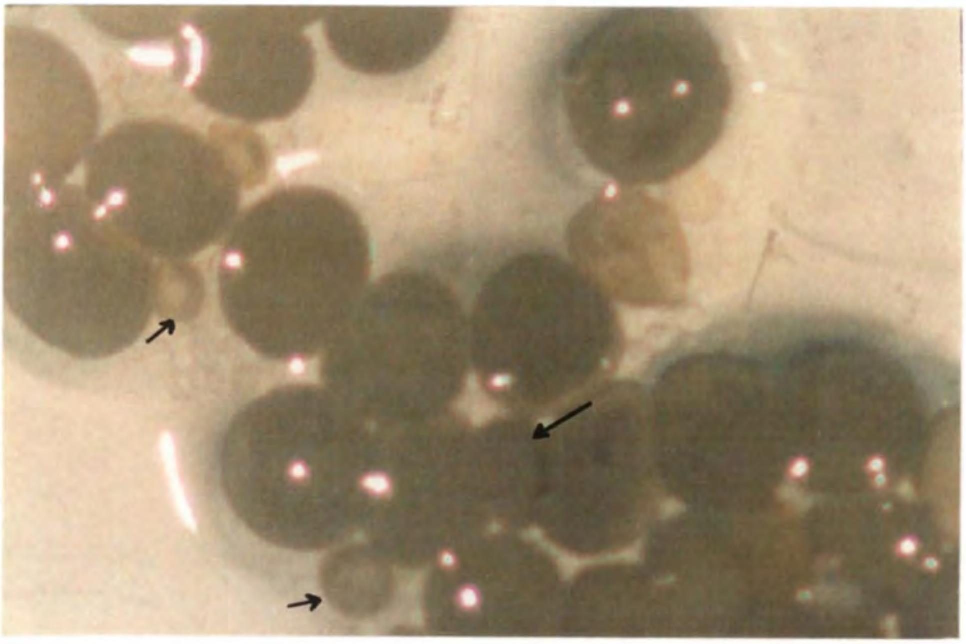
Table 3.7.3 Mean sizes of the three cohorts of oocytes observed in the ovaries.

Oocyte Stage	n	Mean Size	Std. Deviation
Ovulated	42	0.934mm	0.059
Mature	160	0.707mm	0.051
Small	51	0.298mm	0.052
TOTAL	253	-----	-----

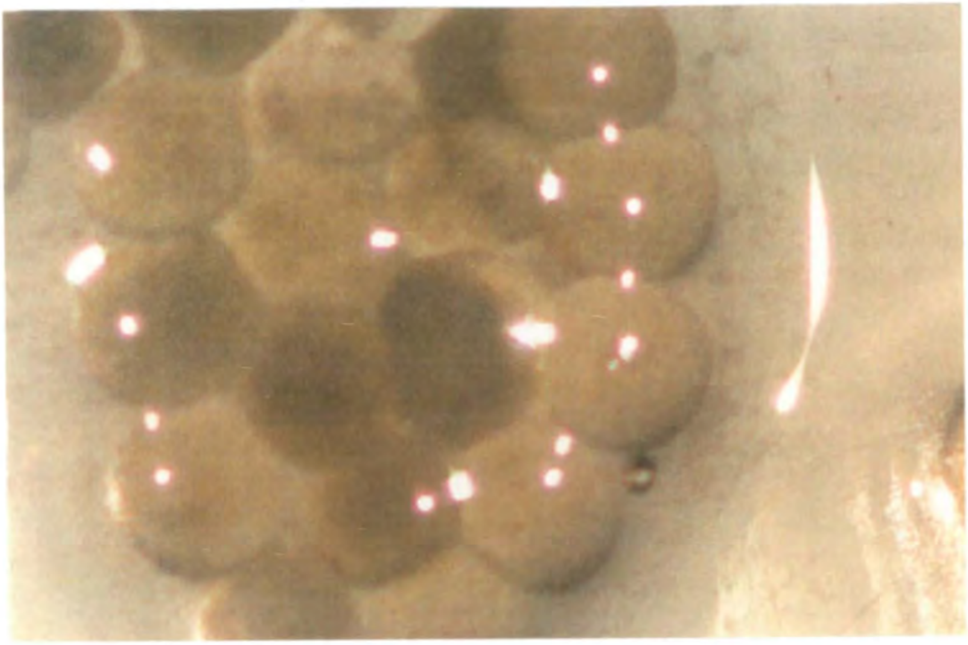
The mean size of the ovulated oocytes was compared to the mean size of mature oocytes by means of a t-test. The ovulated oocytes were significantly larger than the mature oocytes ($P\approx0.0001$).



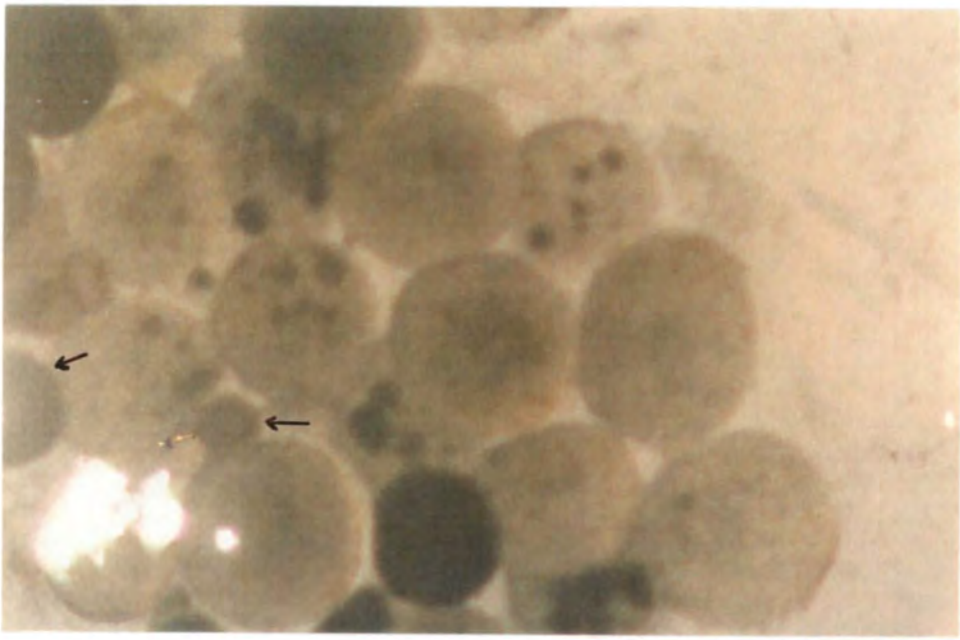
3.7.3 Photograph of mature oocytes from a non-ovulated female cardinal tetra (25X)



3.7.4 Photograph of mature oocytes from a non-ovulated female cardinal tetra (25X)
(Arrows indicate small, immature oocytes from the next cohort)



3.7.5 Photograph of oocytes from a female in the process of ovulating. Note some of the oocytes are not entirely transparent (25X).



3.7.6 Photograph of oocytes from a female that had completed ovulating, note the larger size of the ovulated oocytes (25X). (Arrows indicate small, immature oocytes from the next cohort)

Females that had ovulated were particularly easy to strip while females that had not ovulated only extruded oocytes under pressure.

Discussion

The interaction found in the CF data is almost exactly reflected by the GSI data. The interaction is difficult to interpret, however. Further studies will need to be performed in order to evaluate why injected fish fed live food should have higher CF's and GSI's than those fed live food and not injected, while injected fish fed pelleted feed should have lower CF'S and GSI's than those not injected. This could be investigated by testing different doses of hormone on the fish or by testing a number of different proportions of live food and pelleted feed in the diet.

There was no significant difference in ovulation rate between the four treatments. This could be due to the very low ovulation rate observed (seven out of 40 fish ovulating). Possible reasons for this were discussed in the previous experiment.

This experiment revealed a number of factors relating to the spawning strategy of cardinal tetras. Two cohorts of oocytes with mean diameters of 0.707mm and 0.298mm respectively, were present in the ovaries. This indicates that cardinals are group-synchronous spawners. It eliminates the possibility that cardinal tetras are true synchronous spawners since true synchronous spawners only ever recruit one cohort of oocytes, spawn and then die (Tyler & Sumpter 1996). Further evidence that cardinals are group-synchronous spawners is related to their GSI values. The GSI values for cardinals were in the range 14-60. According to Tyler & Sumpter (1996), asynchronous spawners very seldom have GSI values of more than 10 while the minimum GSI value for a cardinal tetra in this experiment was 14.6. This result matches that of Geisler & Annibal (1987) who regarded cardinal tetras as group-synchronous spawners, spawning a number of times during a spawning season.

Females that had ovulated had significantly higher GSI values (minimum 38.625) than those that had not ovulated (maximum 36.343), such that the GSI values of the two groups did not overlap. This could be due to the rapid increase in oocyte size during ovulation. Ovulated oocytes had a significantly larger mean diameter of 0.934mm than mature oocytes which had a mean diameter of 0.707 ($P \approx 0$), an increase in diameter of 32%. Further, the ovulated oocytes were transparent while mature oocytes were an opaque creamy-brown. These results closely parallel the results found by Rowland (1988) for the oocytes of Murray cod, *Maccullochella peeli.* Rowland (1988) related the increased transparency of ovulated oocytes with the coalescence of yolk globules associated with final maturation.

Most of the ovulated oocytes were almost totally transparent without any cloudy areas and appeared like the viable fertilised eggs, spawned in the first few experiments.

This study confirmed some of the features of the reproduction of cardinal tetras, as suggested by Geisler & Annibal (1987), i.e. they are group synchronous spawners.

CHAPTER 4: ENVIRONMENTAL STIMULI, CONDITIONING AND ENRICHMENT RE-VISITED.

4.1 THE EFFECT OF ENVIRONMENTAL STIMULI ON OVULATION RATE IN CARDINAL TETRAS.

Introduction

In the previous two experiments it was not attempted to spawn the fish and the results were interpreted according to ovulation rates. According to Hontel & Stacey (1990) ovulation is triggered by the same factors that trigger spawning since fish that ovulate should spawn. Hence, ovulation is a good indication of the reproductive effect of a treatment on the fish. This had the advantage that the number of treatments and replicates were not limited by the number of available males (38). However, in the first series of experiments, ovulation rates were not documented. This experiment was therefore designed to test the effect of environmental stimuli on ovulation rate (ovulation rate being the number of fish in each treatment that ovulated).

This experiment repeated the previous experiment on environmental stimuli (3.1) but tested the effect of environmental stimuli on ovulation rate rather than spawning success. Hence, the number of treatments tested was not limited by the number of males available or the number of spawning tanks available. Hence, in addition to the treatments previously tested; a rise in water temperature, a rise in water level, a fresh water exchange, and a control treatment, two additional treatments, namely, a drop in water temperature and a combination of a drop in water temperature, a rise in water level and a fresh water exchange, were tested. This last treatment was aimed to simulate a flood event under tank conditions.

This experiment tested the hypothesis that environmental stimuli had no effect on ovulation rate in cardinal tetras.

Materials and Methods

Ninety-six females were randomly selected from the brood stock holding tanks and eight fish were placed into each of twelve conditioning tanks. All tanks were then isolated from the re-circulating system and drained to a depth of 15cm. Heater-thermostats were placed in all tanks and the temperature was maintained at 25±0.2°C. The fish were fed twice per day to satiation on pelleted feed (Table 1.4.2) for a period of one week.

After the conditioning period, two randomly selected tanks were heated to 27±0.2°C to provide the stimuli of a rise in temperature. In a further two randomly selected tanks the temperature was allowed to drop to 23±0.2°C. Two randomly selected tanks were drained to a depth of 10cm and refilled to a depth of 15cm with fresh rain water to give a fresh rain water flush. Two randomly selected tanks were filled to a depth of 22cm with system water to provide a rise in water level. Two randomly selected tanks were filled to a depth of 22cm with fresh rain water and maintained at 23±0.2°C to simulate a flood event under tank conditions. The final two tanks were left as controls. The fish were left in the conditioning tanks for a further five days and then checked for evidence of ovulation.

Results

The pH and conductivity remained at 5.5 and 92-94µS/cm respectively in all tanks except those that were subjected to a fresh rain water change. In the two tanks where only the fresh rain water change occurred the pH rose to 5.8 and the conductivity dropped to 83µS/cm and 84µS/cm. In the two tanks where a flood event was simulated, the pH rose to 6.0 and the conductivity dropped to 77µS/cm and 79µS/cm. No fish ovulated.

Discussion

None of the environmental stimuli tested were effective in inducing ovulation in female cardinal tetras. This does not mean that environmental stimuli do not induce ovulation in this species, but that the above stimuli alone, or in combination, are not sufficient to trigger final maturation in captivity. Hence, the trigger is either a more complex suite of environmental factors, or one or more of the above factors may trigger ovulation in the wild but not in captivity, or that ovulation in cardinals is not influenced by environmental stimuli, but some other, possibly social, trigger.

If the trigger is a more complex suite of factors then it would be very difficult to identify all the factors involved and simulate them. If the environmental stimuli tested are not sufficient to induce ovulation in captivity, then some alternative method must be sought. No social cues have as yet been tested. Such social cues could include visual, chemical and behavioural stimuli from the males. Up until this point in the study the sexes had been conditioned separately. This decision was based on recommendations from the literature (Noznov 1986, Richter 1991). It was, therefore, decided to test the effect of conditioning the sexes together on spawning success and ovulation rate against conditioning the sexes separately.

4.2.1 DOES CONDITIONING FEMALE CARDINAL TETRAS TOGETHER WITH MALES AFFECT OVULATION RATE, CONDITION FACTOR AND SPAWNING SUCCESS? PART I.

Introduction

In the previous experiments, the males and females were conditioned separately. This was based on recommendations from the literature that the sexes should be conditioned separately (Noznov 1986; Richter 1991). This experiment was designed to test if conditioning the females together with the males would have any effect on ovulation rates or spawning success. According to Liley (1980), behavioural stimuli provided by conspecifics may influence endocrine activity and perhaps trigger ovulation and spermiation. Lambert and van Oordt (1974) and Eaton and Farley (1974), indicate that in *Brachydanio rerio*, the activity of the pituitary gonadotrophs may be related to the presence or absence of males. According to Stacey *et al.* (1979) and Hontela & Stacey (1990) the presence of males is necessary for spontaneous ovulation of female goldfish, *Carassius auratus*.

This experiment tested the hypothesis that conditioning the females for a period of one week with males had no effect on ovulation rate, condition factor (CF) or spawning success as compared to females conditioned separately from the males.

Materials and Methods

Eighteen males and 18 females were randomly selected from the brood stock holding tanks and placed into conditioning tanks. Six males and six females were placed into one conditioning tank, 12 males were placed into a second and 12 females into a third. Hence, stocking densities were the same in each tank. The fish were conditioned for one week and were fed twice per day to satiation on pelleted feed (Table 1.4.2).

After the conditioning period the six males and six females that were conditioned together were randomly paired up and placed into spawning tanks. Six of the separate males and six of the separate females were randomly selected, randomly paired up and placed into spawning tanks. All spawning tanks contained 5mm plastic mesh false bottoms and wool mops. The fish were left in the spawning tanks for four nights and removed on the morning of the fifth day. Thereafter they were all anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured and checked for evidence of ovulation or spermiation by manual stripping, before being returned to the brood stock holding tanks.

Condition factors were calculated from length weight data according to the equation:

$$CF = (W/L^3)x100\ 000$$

where W = weight (g)
L = fork length (mm)

Ovulation data was transformed by the factor $\sqrt{x+1}$ and a Kruskal-Wallis test was performed on the transformed data.

The system was maintained at 25±0.2°C and at a pH of 5.5 for the duration of the experiment.

Results

The conductivity in the system rose from 107µS/cm to 110µS/cm.

No fish spawned. All six females conditioned together with the males ovulated while none of the females conditioned separately ovulated. The ovulation rate was significantly higher for females conditioned together with the males than for females conditioned separately ($P=0.0009$). No milt could be stripped from any of the males.

The mean lengths of fish in each treatment were calculated and compared by means of a t- tests. There was no significant difference in mean lengths of males between the two treatments (P=0.4102).

The males conditioned with the females had significantly lower CF's than the males conditioned separately (P=0.0085) (Table 4.2.1.1).

Table 4.2.1.1 Mean CF values for male fish conditioned together with females and those conditioned separately.

Treatment	Mean	Standard Error
Separate	1.4027	±0.023
Together	1.2930	±0.019
TOTAL	1.3480	±0.021

There was no significant difference in mean female length between the two treatments (P=0.2094). The females conditioned together with the males had significantly higher CF's than the females conditioned separately (P=0.0042).

Table 4.2.1.2 Mean CF values of females conditioned together with males and females conditioned separately.

Treatment	Mean	Standard Error
Separate	1.4917	±0.026
Together	1.7352	±0.022
TOTAL	1.6134	±0.023

Discussion

These results indicate that conditioning females together with males affects ovulation rate and condition factor, but not spawning success. All females conditioned together with males ovulated while none of the females conditioned separately did. This indicates that the presence of males triggers final maturation in females with fully developed oocytes. This matches the results of Stacey *et al.* (1979) and Hontela & Stacey (1990) who found that the presence of males was necessary for spontaneous ovulation in female goldfish. This could be a response to visual, chemical (pheromonal) or behavioural stimuli. In goldfish, two other factors have been considered relevant for the occurrence of spontaneous ovulation; water temperature and the presence of aquatic vegetation (Stacey *et al.* 1979; Hontela & Stacey 1990). The presence of males is thought to cause the ovulation response in the females by means of a male pheromone (Hontela & Stacey 1990), since water from the males can induce the same response.

The results observed for the female cardinals were not reflected in the results for the males. The males conditioned together with the females had significantly lower CF's than those conditioned separately. To be able to interpret these results the experiment was repeated using a larger sample size and obtaining CF values before and after the experiment.

4.2.2 DOES CONDITIONING FEMALE CARDINAL TETRAS TOGETHER WITH MALES AFFECT OVULATION RATE, CONDITION FACTOR AND SPAWNING SUCCESS? PART II: LARGE SAMPLE.

Introduction

This experiment tested the hypothesis that conditioning the females together with males had no effect on ovulation rate, condition factor (CF) or spawning success compared to females conditioned separately. Experiment 4.2.1 was repeated with a larger sample size, 48 fish instead of 24. In addition lengths and weights were measured before the conditioning period and after the spawning period such that any changes in CF over the conditioning period can be quantified.

Materials and Methods

Twenty-four males and 24 females were randomly selected from the brood stock holding tanks, anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured, and placed into conditioning tanks. Two conditioning tanks were stocked each with six males and six females, another conditioning tank was stocked with 12 males and a final conditioning tank was stocked with 12 females. Hence, stocking densities were the same in each tank, at 12 fish per tank. The fish were conditioned for one week and were fed twice per day to satiation on pelleted feed (Table 1.4.2).

After the conditioning period the 12 males and 12 females that were conditioned together were randomly paired up and placed into spawning tanks. The 12 separate males and 12 separate females were randomly paired up and placed into spawning tanks. All spawning tanks contained 5mm plastic mesh false bottoms and wool mops. The fish were left in the spawning tanks for four nights and removed on the morning of the fifth day. Thereafter they were all anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured and checked for evidence of ovulation or spermiation by manual stripping, before being returned to the brood stock holding tanks.

Condition factors were calculated from length weight data according to the equation:

$$CF = (W/L^3) \times 100\,000$$

where W = weight (g)
L = fork length (mm)

The system was maintained at 25±0.2°C and at a pH of 5.5 for the duration of the experiment.

Results

The conductivity in the system rose from 97µS/cm to 105µS/cm.

No fish spawned. Two of the females conditioned together with the males ovulated while none of the females conditioned separately ovulated. This data was transformed by the factor $\sqrt{x+1}$ and a Kruskal-Wallis test was performed on the transformed data. There were no significant differences in the ovulation data ($P=0.1432$). No milt could be stripped from any of the males.

There were no significant differences or interactions in CF between females conditioned together with males and those conditioned separately ($P>0.05$). There was a significant interaction ($P<0.024$) in CF between males conditioned together with females and those conditioned separately (Fig 4.2.2). Males conditioned together with females lost condition over the experiment while those conditioned separately showed an increase in CF.

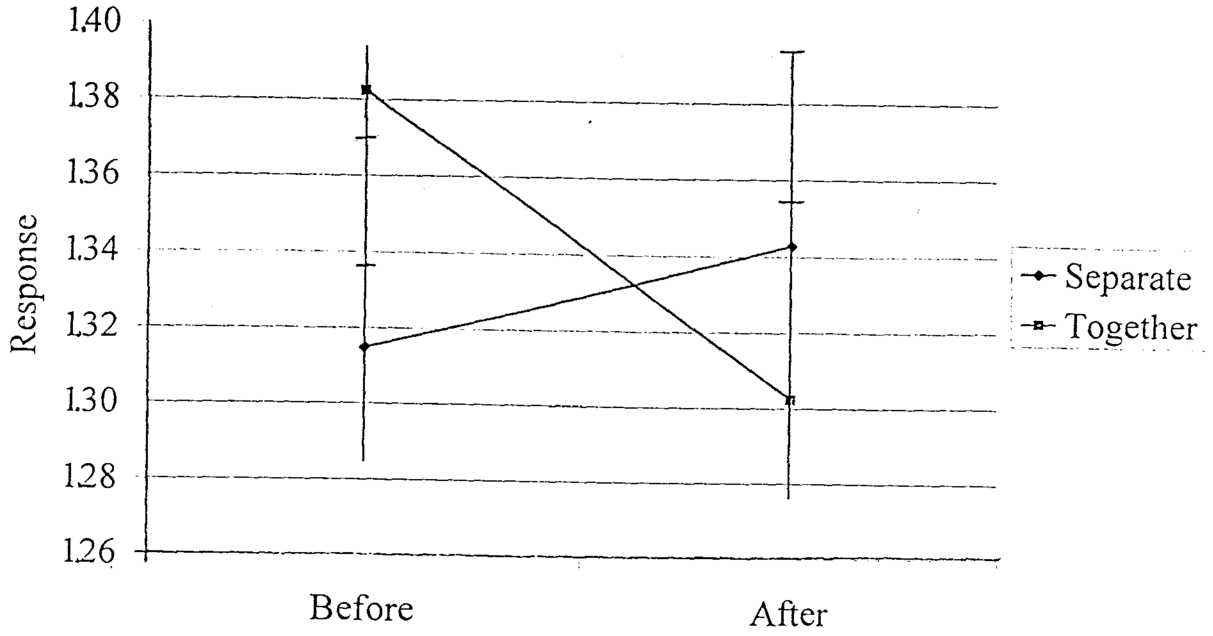


Fig.4.2.2 Interaction plot for males, displaying the significant interacion between conditioning males separately or together with females, and before and after conditioning, for the CF data.

Discussion

The ovulation results obtained in the previous experiment were not repeated in this experiment. There was no significant difference in ovulation rate between the two experimental groups even though the two females that ovulated were in the group conditioned together with the males. This low ovulation rate was probably influenced by the sample size. In the previous experiment the sample size was small and all fish used had fully developed oocytes visible in the ovaries. However, with the larger sample size used in this experiment, not all the females had fully developed ovaries and many had partially developed ovaries. It was hoped that these would develop over the conditioning period, since cardinals can spawn every 8-14 days (Geisler & Annibal 1987), and since the fish were randomly selected it was not considered to be a problem. However, the ovaries did not develop over the conditioning period and this resulted in the low ovulation rate. The low ovulation rate was reflected in the CF's, with no significant differences in female CF between the two treatments. This problem resulting from the state of ovarian development was investigated further in the following experiment.

The CF's of the males showed the same trends as in the previous experiment. The CF's of males conditioned together with the females decreased over the experimental period while the CF's of the males conditioned separately increased. This could be due to a number of reasons. The males may expend much energy chasing females in courtship behaviour or vice versa, although neither of these behaviours were observed. The fish remained fairly inactive while under observation, and given that courtship occurs at night (Geisler & Annibal 1987; Richter 1991; Benninger 1996) it was not likely to be observed. Richter (1991) filmed a pair of cardinals spawning late at night and reported that the female actively chased the male for two nights and only on the third night when the roles reversed and the male chased the female did the pair spawn. Hence, it is possible that the females chased the males in the conditioning tanks and could have been responsible for their decrease in CF.

4.2.3 DOES CONDITIONING FEMALE CARDINAL TETRAS TOGETHER WITH MALES AFFECT OVULATION RATE, CONDITION FACTOR AND SPAWNING SUCCESS? PART III: THE RELEVANCE OF THE STATE OF OVARIAN DEVELOPMENT.

Introduction

In this experiment the females were divided into two groups, a group that contained fully developed ovaries and a group that didn't. Both of these groups were then conditioned either with or separately from males. This experiment was of similar design to the previous experiment (4.2.2). However, spawning the fish was not attempted and only CF's and ovulation rates were investigated.

Materials and Methods

The female fish were separated into two groups according to ovarian development and then sixteen females were randomly selected from each group.16 males were randomly selected. The females were anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured, and placed into conditioning tanks. Four conditioning tanks were stocked each with four males and four females from either of the two groups. A further two conditioning tanks were stocked with eight females from either of the two groups. Hence, stocking densities were the same in each tank at eight fish per tank. The fish were conditioned for one week and were fed twice per day to satiation on pelleted feed (Table 1.2.4). Thereafter all the females were anaesthetised in 0.3ml/L 2-phenoxyethanol, weighed and measured and checked for evidence of ovulation, before being returned to the brood stock holding tanks.

Condition factors were calculated from length weight data according to the equation:

$$CF = (W/L^3)x100\ 000$$

where W = weight (g)
L = fork length (mm)

The system was maintained at 25±0.2°C and at a pH of 5.5 for the duration of the experiment.

Results

The conductivity in the system rose from 104μS/cm to 109μS/cm.

Females with fully developed ovaries had significantly higher mean CF's than those without fully developed ovaries ($P=0.0001$). Females conditioned together with males had significantly higher mean CF's than those conditioned separately ($P=0.006$)(Table 4.2.3.1). There were no interactions between any of the main effects.

Table 4.2.3.1 Mean CF values for females containing mature oocytes and those that do not, and for females conditioned together with males and those conditioned separately.

Treatment	n	Mean	Standard error
Grand Mean	64	1.4837	0.0274
No Oocytes	32	1.2929	0.0157
Oocytes	32	1.6744	0.0217
Separate	32	1.4472	0.0347
Together	32	1.5201	0.0413

Females that contained fully developed oocytes had a significantly higher ovulation rate than females without fully developed oocyes (Table 4.2.3.2). Females that contained fully developed oocytes and were conditioned with males had a significantly higher ovulation rate than females without fully developed oocytes and conditioned together with males. Females that contained fully developed oocytes and conditioned with males had a significantly higher ovulation rate than females without fully developed oocytes conditioned separately. There were no other significant differences.

Table 4.2.3.2 Output from a Kruskal-Wallis test performed on the ovulation data from the combinations of treatments.

Treatment	No. Ovulated	Test statistic	Significance Level
(S)eperate	1/16		
(T)ogether	4/16	2.06676	0.1506
(O)ocytes	5/16		
(N)o Oocytes	0/16	5.7407	0.0166*
SO	1/8		
TO	4/8	2.4346	0.1172
SN	0/8		
TN	0/8	----	1
SO	1/8		
SN	0/8	1	0.3173
TO	4/8		
TN	0/8	5	0.0254*
TO	4/8		
SN	0/8	5	0.0254*
TN	0/8		
SO	1/8	1	0.3173

* denotes a significant difference.

Discussion

These results confirm the observation of the previous experiment that the state of development of the ovaries affects ovulation rate. Hence, only fish with fully developed ovaries should be conditioned for spawning experiments.

Females conditioned together with males had significantly higher CF's than those conditioned separately. This reflects the results of experiment 4.2.1. Therefore, for spawning studies, females should be conditioned together with males before spawning experiments. This may not be true for males since it was discovered that males lose condition when conditioned with females in the previous experiment (4.2.2). Hence, for further spawning experiments it should be tested if females conditioned together with males should then be paired with males that have been conditioned separately.

Female cardinals may receive important social cues inducing final maturation and ovulation from the males in the conditioning tanks. However, these cues were not sufficient to induce spawning. The following experiment was designed to test if interspecific pheromones releases during spawning from another species of characin would induce spawning in cardinal tetras.

4.3 THE EFFECT OF INTERSPECIFIC PHEROMONES ON OVULATION RATE AND SPAWNING SUCCESS IN CARDINAL TETRAS.

Introduction

Pheromones have been defined by Stacey *et al.* (1993) as;"chemical(s) released by an individual that elicit reproductive response(s) in conspecifics, recognising that the term need not imply communication and can be used when referring to component(s) of a complex odor mixture." It is well documented that pheromones play fundamental roles in the reproductive physiology and behaviour of teleost fish (Liley 1982; Stacey *et al.* 1986; Stacey *et al.* 1993). More recent studies have shown that hormonal pheromones are widely used by teleosts, and have diverse chemical structures and functions (Stacey *et al.* 1993).

It has recently been shown in male goldfish, that exposure of males to periovulatory females or to female pheromones produced in the periovulatory period, affect the volume of milt that can be stripped (Hontela & Stacey 1990). These studies indicate that female goldfish produce two types of sex pheromone during the ovulatory process: a preovulatory pheromone released during final maturation of the oocytes and a post-ovulatory pheromone released after ovulation has occurred (Hontela & Stacey 1990; Stacey *et al.* 1993). These pheromones act as social cues triggering both endocrine and behavioural responses in the male (Liley 1980). The preovulatory pheromone is most likely 17α,20ß-dihydroxy-4-pregnen-3-one (17,20P) and triggers a GtH surge and rapid milt production in males (Hontela & Stacey 1990; Stacey *et al.* 1993; Sorenson *et al.* 1996; Stacey & Cardwell 1996). At ovulation, the surge of 17,20P synthesis and release collapses and prostaglandin F2α (PGF2α) levels increase (Stacey *et al.* 1993). Recent work indicates that PGF2α is an important component of the post-ovulatory pheromone (Hontela & Stacey 1990; Stacey *et al.* 1993). There is also considerable evidence that the close temporal synchrony between ovulation and spawning behaviour in females is mediated by plasma levels of ovarian PGF2α (Stacey *et al.* 1993). The release of PGF2α is thought to be a response to the stretching of the uterine muscle by a mass of freshly ovulated oocytes released into the ovary lumen (Liley

1980). It is not yet known whether ovulated females release PGF2α into the water and hence, the pheromone(s) released by ovulated females are referred to as prostaglandin-induced pheromone (PIP) (Hontela & Stacey 1990). Water-borne PIP is thought to trigger sex behaviour (courtship) in males and synchronise spawning behaviour. This process is illustrated in Fig. 4.3.1. In goldfish, response to 17,20P is not greatly influenced by gonadal maturity, indicating that central mechanisms block reproductive responses of gonadaly regressed individuals (Sorenson & Stacey 1987; Stacey *et al.* 1993).

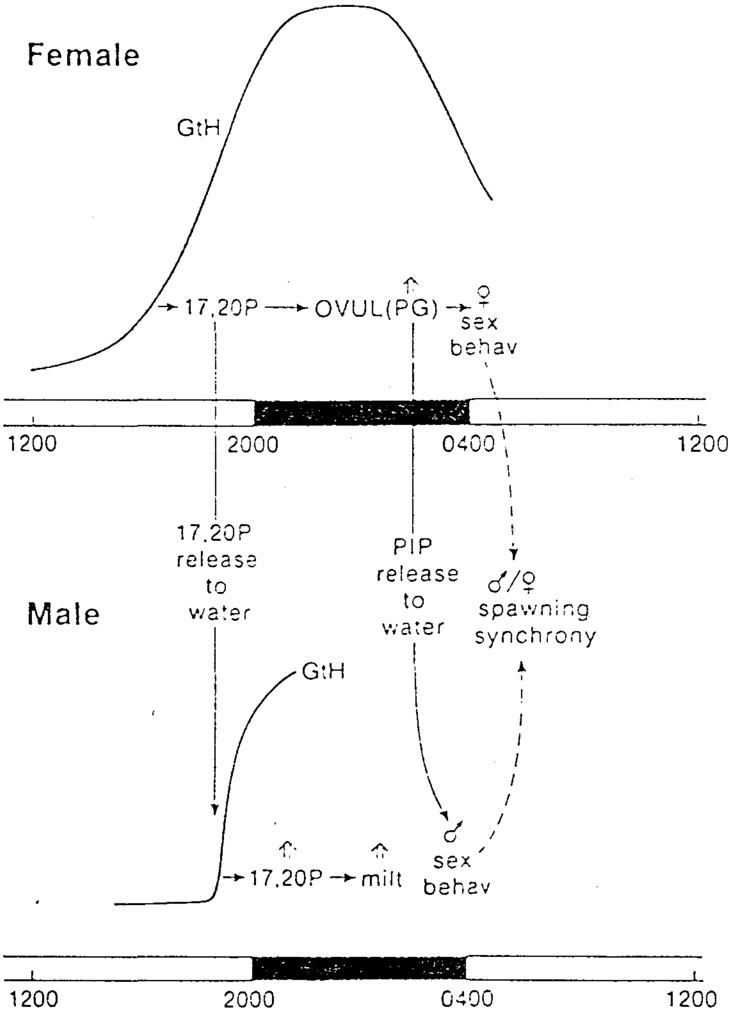


Fig.4.3.1. Model of hormonal and pheromonal factors proposed to synchronise gonadal maturation and spawning behaviour in goldfish. GtH; gonadotropin: 17,20P; 17α,20ß-dihydroxy-4-pregnen-3-one: ovul; ovulation: PG; prostaglandin $F_{2\alpha}$: PIP; prostaglandin-induced pheromone (From Hontela & Stacey 1990).

Although males are the target of most pheromonal effects, 17,20P has been shown to increase ovulation rate in goldfish (Sorenson & Stacey 1987) and in some cases male pheromones are used to stimulate females to ovulate, such as for *Gobio* spp. (Stacey & Cardwell 1996) though the exact species concerned was not mentioned. It is not known if male cardinal tetras release pheromones in order to stimulate females. If so, such pheromones could be cause for females ovulating in the presence of males in the conditioning tanks.

Since pheromones synchronize spawning behaviour between the sexes, a breakdown in this pheromonal system could explain why females that ovulate still don't spawn. According to Stacey *et al.* (1993) pheromones are relatively species specific. Further, closely related fish have very similar pheromones (Stacey *et al.* 1993). It has not been tested if pheromones from one species will stimulate another species but since 17,20P analogs have been proven effective it is quite possible that pheromones of closely related species will have a common effect.

This experiment tests if pheromones from spawning red serpae tetras (*Hyphessobrycon serpae*: Characidae) have any effect on ovulation rate and spawning success of cardinal tetras.

This experiment tests the hypothesis that pheromones from spawning red serpae tetras have no effect on ovulation rate or spawning success of cardinal tetras.

Materials and Methods

Sixteen males and 16 females were randomly selected from the brood stock holding tanks and conditioned for one week in four conditioning tanks, two with eight males in each and two with eight females in each. The fish were fed twice per day to satiation on fine pelleted feed (Table 1.2.4).

After the conditioning period eight spawning tanks were isolated from the recirculating system. These tanks and a further eight tanks still connected to the recirculating system were furnished with 5mm plastic mesh false bottoms and wool mops. Heater/thermostats were placed under the false bottoms in the isolated tanks. Pairs of fish were then randomly selected from the conditioning tanks and one pair was placed in each of the 16 spawning tanks. Six red serpae tetras, three males and three gravid females, were anaesthetised in 0.3ml/L 2- phenoxyethanol, injected with the recommended dose of Aquaspawn and placed into a 120 L tank connected to the recirculating system. All tanks were maintained at a temperature of 25±0.2°C and a pH of 5.5. The conductivity in all the tanks remained in the range 97- 99μS/cm.

The spawning tanks were checked for eggs each morning. The fish were left in the spawning tanks for five days and removed on the morning of the sixth day. On removal the females were checked for evidence of ovulation.

Results

The red serpae tetras spawned early on the first morning that they were in the spawning tank. No cardinal tetras spawned and no cardinal females ovulated.

Discussion

This form of pheromonal treatment was not effective in inducing ovulation or spawning in cardinal tetras. Hence, either the cardinals did not respond to the pheromones from the red serpae tetras (i.e.. the red serpae tetras' pheromones were species specific), or pheromones are not effective in inducing ovulation or spawning in cardinal tetras. This could be investigated by testing the effect of pheromone analogs on spawning success in cardinal tetras. Such pheromone analogs were not available for this study.

4.4 THE EFFECT OF FEEDING AQUASPAWN^R ENRICHED *ARTEMIA*, ON OVULATION RATE IN CARDINAL TETRAS.

Introduction

The previous bioencapsulation experiments (3.2; 3.3) were not effective in inducing the fish to ovulate or spawn. These experiments made use of a fine powdered GnRHa to enrich the *Artemia*. Garrett (1989) used a method of bioencapsulating water soluble hormones into *Artemia* without the use of liposomes. Garrett (1989) investigated the effect of various steroids either incorporated into pellet feed or bioencapsulated into *Artemia* on sex determination of larval largemouth bass. Garrett (1989) reported that bioencapsulated steroids were far more effective in determining sex (100% sex change for estradiol, estrone, testosterone and androsterone) than incorporating the hormone into pelleted feed (54% and 51% sex change for estradiol and estrone, respectively, and 90% and 93% sex change for testosterone and androsterone respectively). Garrett's (1989) procedure for enriching brine shrimp nauplii with steroids was used for enriching brine shrimp nauplii with Aquaspawn^R.

This series of experiments consisted of four trials testing the hypothesis that feeding cardinals *Artemia* bioencapsulated with Aquaspawn^R had no effect on ovulation rate in cardinal tetras.

Materials and Methods

The females were separated into two brood stock holding tanks according to ovarian development. In the first run of this experiment, 24 females with fully developed ovaries were randomly selected from the brood stock holding tanks and placed into four conditioning tanks, six in each. The fish were conditioned for one week and fed twice per day to satiation on pelleted feed (Table 1.2.4).

At the end of the conditioning period, 0.5g of one week old *Artemia* nauplii was placed into a conical flask containing 8ml sea water. The flask was heavily aerated and placed under a 100W incandescent bulb since *Artemia* enrichment is most effective under bright light. Thereafter, 0.07g was removed and fed to the fish in one randomly selected conditioning tank as a control. This figure of 0.07g was based on the calculation from experiment 3.2 where the fish were observed to eat a mean of 0.04g *Artemia*/g fish in a two minute period. The figure of 0.07g was calculated by multiplying the mean weight of the fish by six and again by 0.04. Four millilitres of Aquaspawn^R was then added to the *Artemia* solution. Since no research has investigated this method before, this concentration (33.3% Aquaspawn^R) was chosen to minimise the amount of Aquaspawn^R used, but still have a relatively high concentration of hormone in enough water to enrich the brine shrimps. After 30 minutes, 0.07g of *Artemia* was removed and fed to the fish in another randomly selected conditioning tank. The same procedure was repeated after 60 minutes and 120 minutes.

The fish were left in the conditioning tanks for four nights and removed on the morning of the fifth day. All females were checked for evidence of ovulation by manual stripping before being returned to the brood stock holding tanks.

In the second run, the procedure followed in the first run was repeated, except that in this instance eighty females were randomly selected from the brood stock holding tanks and placed into ten conditioning tanks, eight in each. At the end of the conditioning period, 1.5g of one week old *Artemia* nauplii were placed into a conical flask containing 16ml sea water. Thereafter, 0.1g was removed and fed to the fish in one randomly selected conditioning tank to serve as a control. This figure of 0.1g was based on the calculation from experiment 3.2 that the fish would eat 0.04g of *Artemia*/g fish in a two minute period. Eight millilitres of Aquaspawn^R was then added to the *Artemia* solution. After 15 minutes, 0.1g of *Artemia* was removed and fed to the fish in another randomly selected conditioning tank. The same procedure was repeated after 30, 45, 60, 75, 90, 105, 120 and 135 minutes.

In the third run, the procedure followed in the first run was repeated, except in this instance 40 females were randomly selected from the brood stock holding tanks and eight placed into each of five conditioning tanks. At the end of the conditioning period, 1g of one week old *Artemia* nauplii was placed into a conical flask containing 8ml sea water. Thereafter, 0.1g was removed and fed to the fish in one randomly selected conditioning tank as a control. Four millilitres of Aquaspawn^R was then added to the *Artemia* solution. After 20 minutes, 0.1g of *Artemia* was removed and fed to the fish in another randomly selected conditioning tank. The same procedure was repeated after 40, 60, and 80 minutes.

In the final run the procedure followed in the third run was repeated, except *Artemia* was removed and fed to the fish in randomly selected conditioning tanks after 0, 30, 40, 50, and 60 minutes.

Ovulation results were transformed by the factor $\sqrt{x}+1$ and a Kruskal-Wallis test was performed on the transformed data. Where a significant difference was found, a Student- Newman-Keuls multiple comparison procedure was performed to identify which of the treatments differed significantly.

Results

For the duration of the four runs the temperature of the system remained at 25±0.2°C and the pH at 5.5. The conductivity remained between 94 and 99µS/cm during the four runs.

There were no significant differences in ovulation rate between the treatments in the first run ($P>0.05$) (Table 4.3.1.1).

Table 4.3.1.1 Output from a Kruskal-Wallis test performed on the ovulation data observed in the first run.

Time (min)	No. Ovulated	Av. Rank	Test statistic	Significance level
0	0/6	9		
30	4/6	17		
60	2/6	13		
120	1/6	11	6.7647	0.0798

There was a significant difference in the ovulation rate between the different enrichment times in the second run (Table 4.3.1.2). These differences were between 45 minutes enrichment time and 0 minutes, 120 minutes and 135 minutes, respectively ($P<0.05$). Hence, enriching the *Artemia* for 45 minutes resulted in a significantly higher ovulation rate than in the control. The same was true between the 45 minute enrichment time and the 120 minute enrichment time, and the 45 minute enrichment time and the 135 minute enrichment time.

Table 4.3.1.2 Output from a Kruskal-Wallis test performed on the ovulation data observed in the second run.

Time min	Ovulation	Av. Rank	Test statistic	Significance level
0	0/8	30		
15	1/8	35		
30	4/8	50		
45	6/8	60		
60	4/8	50		
75	2/8	40		
90	3/8	45		
105	1/8	35		
120	0/8	30		
135	0/8	30	24.8031	0.00319*

* denotes a significant difference.

There was a significant difference in the ovulation rate between the different enrichment times in run three (Table 4.3.1.3). This difference was between 40 minutes enrichment time and 0 minutes enrichment time ($P<0.05$). Hence, enriching the *Artemia* for 40 minutes resulted in a significantly higher ovulation rate than the control.

Table 4.3.1.3 Output from a Kruskal-Wallis test performed on the ovulation data observed in the third run.

Time min	Ovulation	Av. Rank	Test statistic	Significance level
0	0/8	18.5		
20	1/8	21		
40	6/8	33.5		
60	5/8	31		
80	2/8	23.5	13.0278	0.01114*

* denotes a significant difference.

There were no significant differences in ovulation rate between the treatments in the fourth run ($P>0.05$) (Table 4.3.1.4).

Table 4.3.1.4 Output from a Kruskal-Wallis test performed on the ovulation data observed in the fourth run.

Time min	Ovulation	Av. Rank	Test statistic	Significance level
0	0	24.5		
30	0	24.5		
40	1	27		
50	0	24.5		
60	1	27	3.0625	0.5474

Discussion

Although there were no significant differences in the ovulation data for the first run, the results were promising. Firstly, none of the control fish ovulated while at least one fish in each of the other treatments ovulated. The highest ovulation rate occurred in the tank fed *Artemia* that were enriched for 30 minutes. These results seem to show a clear trend at first sight and the significance level was close to 0.05 ($P=0.0798$), but only seven out of 24 fish ovulated. This low ovulation rate, combined with the small sample size, could be the reason why there were no significant differences. If more fish were used and more of the fish ovulated then significant differences would be likely to appear. Further, there may be a trend appearing with regard to the enrichment time of the *Artemia*, with the most fish ovulating when fed Artemia enriched for 30 minutes. Ovulation rate appeared to decrease as enrichment time increased.

The results from the second run indicate that feeding female cardinals *Artemia* enriched with Aquaspawn^{R} is effective for inducing ovulation. Further, the effectiveness of the enriched

Artemia in inducing ovulation is influenced by the length of the enrichment period. The optimum enrichment period appears to be between 30 and 60 minutes. The third run was designed to investigate this enrichment period in greater detail.

The results from the third run further indicate that feeding female cardinals *Artemia* enriched with Aquaspawn^R is effective for inducing ovulation. This result reflects the result of the previous run where the effectiveness of the enriched *Artemia* in inducing ovulation was influenced by the length of the enrichment period. This result provides further evidence that the optimum enrichment period is between 30 and 60 minutes. The fourth run investigated this area in ten minute intervals.

There were no significant differences in ovulation rate between the different enrichment times in the fourth run. This is probably due to the fact that very few fish ovulated (two out of 40). The reasons for this low ovulation rate are not clear since the same hormone preparation and method of enrichment was used as was used for the previous three runs. This illustrates the need for further research into methods of enriching brine shrimp nauplii with hormones.

These bioencapsulation experiments revealed some promising results with regard to providing an alternative method for treating small fish with hormone preparations. However, the method is not reliable or repeatable enough and warrants further research. Bioassays should be performed on enriched brine shrimp nauplii in order to investigate how effectively and reliably the brine shrimps assimilate the hormone. Bioencapsulation of hormone preparations may have a number of practical applications for the propagation of small fish species.

CHAPTER 5: OVERALL DISCUSSION

Why did some fish spawn at the start of the study and none later?

All the spawnings observed in this study occurred at the beginning of the study, between 14 October 1996 and 2 December 1996. No spawnings were observed over the remainder of the study, which ended in early November 1997. This cannot be explained by the fish's natural spawning season since cardinals naturally spawn between April and June. Further, it cannot be explained by a change in photoperiod since the fish were quarantined for a period of one month and then acclimated in the system for another month at a photoperiod of 12L:12D prior to the study. A change in photoperiod is unlikely to have any effect on spawning in cardinals, since cardinals are equatorial and naturally experience very little change in photoperiod. Further, other tetras, such as emperor tetras, *Nematobrycon palmeri* (Nejmark 1987) and rummy-nosed tetras, *Hemigrammus rhodostomus* (Geisler & Annibal 1987; Palicka 1989) found in similar natural habitats have been recorded to spawn freely in captivity.

A possible explanation why the fish should have spawned at the beginning of the study and not later regards the water quality of the experimental system. For the purpose of this study a new 10 000L rainwater tank was installed and soon filled with fresh rain water. The first three experiments making use of this water resulted in successful spawnings. This tank continuously topped up every time it rained. Since each system water change only required 1000L, less than 10% of the water was exchanged for fresh rain water after each water change. Water changes were only performed when necessary, but this was seldom more than once per month. Hence most of the water in the tank aged and a rise in the conductivity was observed. All but one of the spawnings were observed when the conductivity was between 40 and 60μS/cm, with the last one occurring at 90μS/cm. Once the conductivity rose above this level, no spawnings were observed. This reflects the results of other authors summarised in Table 1.3.1. In the latter part of the study no rain water with a conductivity of less than 90μS/cm was available and this may have lead to the lack of spawning success. A solution to this problem would be to use deionised water, or a mixture of deionised and fresh rain water. It was not feasible to deionise the volumes of water needed for this study and hence this was not tested.

Another explanation that was considered was based on the age of the fish. According to Geisler & Annibal (1987), wild cardinal tetras mature and spawn at one year of age and few if any live more than 18 months. Although this conclusion was drawn from relatively few samples and based on the length frequency distributions of the fish at six month intervals, there was very little variance in the length frequency distributions of wild cardinals since only one size cohort was present in the population. The fish in this cohort were mature and ready to breed after one year. No cohort of larger fish was found in the samples, indicating that cardinals mature at one year and spawn, and few survive much longer than 18 months. Geisler & Annibal (1987) do not regard cardinals as annual fish since mortality levels are high throughout the year and not only after the spawning season. Further, it is unlikely that the larger fish move somewhere out of the sample area since the local fishermen extensively fish the entire basin and would know where such fish could be found. Therefore, cardinals naturally survive only one spawning season. Hence, it is possible that beyond the age of one year the reproductive ability of the fish deteriorates. Geisler & Annibal (1987) reported that wild fish are sexually mature at 17.5mm TL while in captivity they are ready to spawn at 33mm TL. They do not mention, however, how many fish were used to arrive at this figure or how sexual maturity was determined. Penrith (1987) recommends that neon tetras, *P. innesi*, of between 8-12 months of age should be used for spawning. The fish used for this study were originally wild caught but it is not known how long they were held in captivity since their capture. They were purchased because they were "broodstock size" at an average length of just over 30mm FL. Hence, the fish were already at least one year old and may have been too old for spawning. This would explain why a few fish spawned at the start of the study and not later. It would also explain why the fecundity and fertility rate of the fish that spawned was so low. However, this possibility was not tested and is therefore, speculative but should at least be tested. For further studies, young (less than one year old), wild caught fish should be obtained, acclimated to captivity and thereafter a spawning study should be performed when they reach sexual maturity. This was not possible in this study because of the availability and cost of the fish combined with time constraints.

Why did ovulating females not spawn?

The next question that needs to be addressed is why some females ovulated but failed to spawn. It is unlikely that the water quality was unsuitable, or that the relevant exogenous synchronising cue was lacking, since these factors inhibit ovulation. Once a female has ovulated, spawning should follow under pheromonal influence. According to Fig. 4.3.1 once a female ovulates, she releases 17,20P and PIP into the water column to stimulate the males and synchronise spawning behaviour. This was not the case in this study and it is possible therefore that there was a breakdown in this synchronising system. This could be a response to the fish being held in captivity, or a repercussion of handling stress. Stress has been shown to have a negative effect on fish, and particularly on their reproductive performance (Pankhurst & Van Der Kraak 1997; Wilson *et al.* 1995; Contreras-Sanchez *et al.* 1995). Rowland (1988) found that handling Murray cod, *Maccullochella peeli*, three months prior to the spawning season resulted in atresia and resorption of oocytes in most females. None of these females could be induced to spawn. In the case of cardinal tetras, the oocyte development phase is far shorter (8-14 days) than for Murray cod, and, hence, stress may cause the onset of atresia anywhere from two weeks to a few days prior to a spawning trial. Therefore, capturing the fish and placing them in the conditioning tanks one or two weeks prior to the spawning trials may have caused the onset of atresia. It was not possible to reduce handling stress since the minimum amount of netting and sorting was already being used, but fish had to be randomly selected and moved between tanks for each experiment.

According to Liley (1980), in goldfish, ovulation may occur with or without males present, but the presence of males and green plants is necessary for spawning. Hence, although the females ovulated and were paired up with males, some other (unknown) factor may have been necessary before spawning would commence.

The age of the fish was not considered to be a valid explanation as to why some females ovulated but failed to spawn. This conclusion was based on the observation that females (that were at least two years old at that time) ovulated naturally, without hormone treatment, when conditioned together with males. Some other factor was therefor inhibiting spawning.

Another factor is the role of the males. Partridge *et al.* (1976) found that unspermiated male goldfish failed to respond to female pheromones while spermiated males did respond. In this study no milt could be stripped from any of the males, even though relatively heavy pressure was often applied. Further, eggs were freely extruded from ovulating female cardinals when minimal pressure was applied. Therefore, this seems to indicate that the male cardinals failed to spermiate. However, histology would have been necessary in order to prove this hypothesis.

Why did hormone treatments not induce spawning?

Hormone treatments have been successfully used to induce spawning in a wide range of fish species, and particularly those that do not spawn freely in captivity (Liley 1980; Rothbard 1981; Hontela & Stacey 1990). However, Liley (1980), warns that unless appropriate stimuli from the genital tracts are present, such treatments are likely to yield negative results. Probably the most widely used method of hormone application is by way of injection. This method hardly had any effect on cardinal tetras, with few females being induced to ovulate and no fish being induced to spawn. This could be related to the size of the fish. Injecting a 0.5g female with 0.02ml of Aquaspawn^R equates to injecting 40ml/kg. This, along with the associated handling would be stressful to the fish (Pankhurst & van der Kraak 1997). Stress has been shown to have a detrimental effect on reproduction in fish (Pankhurst & van der Kraak 1997). Therefore, the method of bioencapsulating hormones into brine shrimp nauplii appears promising. Enriching brine shrimps with Aquaspawn^R gave the highest ovulation rates of all the methods tested in this study. Enriching brine shrimps with finely powdered GnRHa micropellets, however, was not effective in inducing cardinals to ovulate. This could be due to the properties of the respective hormones. The powdered hormone was taken up by the brine shrimps, but it was not calculated, by means of bioassay, how much was assimilated and available to the fish. Further, the brine shrimps may have partly digested or denatured the powdered hormones such that they were ineffective, since ongrown brine shrimps have been shown to have a wider spectrum of digestive hormones than newly hatched nauplii (Lavens & Sorgeloos 1996). This might not have been a problem with the Aquaspawn^R enriched brine shrimps because in this case newly hatched nauplii were enriched as compared with the ongrown brine shrimps enriched with the powdered hormone, and may not have had all the digestive enzymes present in the adults (Lavens & Sorgeloos 1996). Another possible explanation for the difference in the two methods is that the cardinals may have assimilated more hormone when feeding on nauplii as compared to fewer ongrown brine shrimps. This could be tested by performing a bioassay on brine shrimp nauplii and ongrown nauplii enriched in the same manner. Enriching brine shrimp nauplii with Aquaspawn^R appears to be an effective method for treating small fish with hormones. The advantages of such method are numerous. It does not harm the fish, they can be treated in the spawning tanks without undue stress, it is a practical process that does not require any extensive facilities or equipment, and brine shrimps are freely obtainable and easy to culture. A disadvantage is that it has been shown that assimilation rates of enrichment by brine shrimps varies according to a number of factors, such as temperature or light intensity, as well as varying between batches of brine shrimps (Sorgeloos *et al.* 1993; Lavens & Sorgeloos 1996). Hence, the procedure used must be carefully documented and repeated so as to eliminate any possible bias between researchers and batches. Another problem associated with this method, particularly in the case of cardinal tetras, is its effect on the conductivity of the system. In all cases this method caused a rise in the water conductivity (by as much as 16µS/cm in Experiment 3.3), probably due to small amounts of sea water being transferred with the brine shrimps. Although this is a problem for fish requiring such soft water for spawning, it would not be a problem for most fish species. Hence, this method of hormone treatment shows great promise with regard to treating small fish. However, the methods used require further research, particularly into the optimum enrichment times and doses, as well as the optimum conditions for enrichment, and should all include comprehensive bioassays.

According to Yamamoto & Yamazaki (1961) the few ovulated oocytes remaining in the ovaries of goldfish after spawning had taken place took over one month to be resorbed. These oocytes took up only a small proportion of the ovary. However, in the case of cardinal tetras that ovulated but did not spawn, the majority of the ovary would be taken up with atretic oocytes. These atretic oocytes were cloudy in appearance, were not as firm as newly ovulated oocytes and were fragile, rupturing easily (pers obs.). It is not known how efficient cardinals are at resorbing oocytes. Based on the results of Yamamoto & Yamazaki (1961) on goldfish, it may take the cardinals a considerable time periods to resorb this greater proportion of atretic oocytes. This would mean that the fish would be reproductively inactive for a few months after ovulating, and may have caused the low ovulation rates and the failure to spawn in the later stages of the study.

What is the value of a conditioning period?

The first part of each experiment involved removing the fish from the brood stock holding tanks and placing them into conditioning tanks for a period lasting at least one week. For most of these experiments the sexes were conditioned separately. This was based on recommendations from the literature (Axelrod & Shaw 1967; Noznov 1986; Richter 1991) and was supposed to bring the fish into peak breeding condition and induce spawning when the sexes were united. It was shown, however, that the fish actually lost condition over the conditioning period. During this period the fish were fed twice per day to satiation on pelleted feed and sometimes with live food as well. During the initial acclimation period before the study commenced the fish were fed three times per day. However, the fish only seemed to feed twice per day and the food from one feeding (the midday feeding) would be wasted and would have to be syphoned out later. Further, cardinal tetras come from nutritionally poor waters and are adapted to survival on a limited diet (Geisler & Annibal 1987). Hence, this loss of condition during the conditioning period could not be attributed to insufficient feeding.

During the conditioning periods the fish formed tight shoals and moved around very little, except when feeding, while in the brood stock holding tanks the fish swam constantly and formed loose shoals. This indicates that the fish were stressed while in the conditioning tanks (Pankhurst & van der Kraak 1997).

Although it was shown that females could be induced to ovulate when conditioned together with males, males conditioned together with females did not follow this trend, but lost condition. Hence, the value of a conditioning period must be further investigated. Should the conditioning period be eliminated and fish from the brood stock holding tanks be placed directly into the spawning tanks? Should females be conditioned together with males prior to the spawning trial and then paired up with males taken directly from the brood stock holding tanks?

Other factors related to spawning that require further investigation.

There are a number of other factors that may have affected spawning in cardinal tetras that should be investigated. The peat used for the study, being South African in origin, may have contained compounds such as organic acids that the cardinals would not naturally encounter and may have rendered the water unsuitable for spawning. Hence, the effect of different types of peat on water quality and spawning success in cardinals should be tested. Oxygen levels in the experimental system may have been too high since they were maintained above 7.8mg.L^{-1} while the mean oxygen levels recorded for cardinal biotopes were between 2.5mg.L^{-1} and 5.7mg.L^{-1} (Geisler & Annibal 1987). Hence the effect of lower oxygen levels on spawning success could be tested. In goldfish, a strong stimulus for ovulation and spawning is the presence of green plants (Hontela & Stacey 1990). There are few if any green plants in cardinal biotopes (Geisler & Annibal 1987), but cardinals may require a particular spawning substrate, such as leaf litter or peat, for spawning. Hence, a number of natural spawning media should also be tested. Noznov (1986) suggests that cardinals should be spawned in larger tanks and in shoals. This recommendation was not based on scientific evidence but may be worthwhile testing.

The scientific approach to spawning experiments.

One feature of this study was that the experiments were performed in a scientific manner. In other words an initial set of variables was chosen, such as the water quality, temperature, photoperiod, sex ratio and conditioning period. These were based on variables encountered by the fish in the wild and recommendations from the literature pertaining to spawning cardinal tetras in captivity (Axelrod & Shaw 1967; Geisler 1972; Frank 1980; Tsilinszky, in Noznov 1986; Kuskow, in Noznov 1986; Noznov 1986; Hadrava, in Geisler & Annibal 1987; Zemanek, in Geisler & Annibal 1987; Geisler & Annibal 1987; Richter 1991; Benninger 1996). Thereafter, only one variable could be changed at a time such that the experiments could be compared to one another. There are a large number of variables involved in spawning experiments and for species that do not spawn freely in captivity, such as cardinal tetras, it is usually necessary to optimise all of these variables before the fish will spawn naturally while in captivity. The problem being that it is difficult and time consuming to determine all the necessary requirements by only changing one variable at a time, since there are almost endless combinations of variables. This is because until all the variables are optimal no results are going to be obtained and so individual variables cannot be tested. This is why many hobbyists report successful spawnings and scientists often do not. Hobbyists change a number of variables at a time until they, by chance, get it right. The use of scientific multivariate analysis would be ideal. However, this would necessitate much larger sample sizes and large scale systems. This is seldom practical or feasible in a scientific study of this nature.

For spawning experiments performed in a scientific manner, the fish have to be randomly selected and paired at random. A hobbyist, on the other hand, is likely to select fish that he “feels” are most likely to spawn together, pairing up the best male with the best female, or watching the fish to see if any pairs start to form in the holding tanks. Fish chosen in this manner may be more likely to spawn than those selected randomly.

A possible solution to these problems is to perform a non-scientific preliminary study. During this study a number of variables can be changed at a time and the fish can be selected in such a manner as to maximise the chances of the fish spawning. This would give a better starting point in terms of a better suite of initial variables and thereafter the study can continue scientifically.

CHAPTER 6: DEVELOPMENT OF A PROTOCOL FOR EVALUATING SPAWNING IN TROPICAL, FRESHWATER, EGG- LAYING FISH.

Before performing a study, such as a series of growth trials for some fish species on a particular set of diets for instance, one must investigate how other researchers have performed growth trials on similar species or on the same species using different diets. In short, for most experiments there are existing standards and procedures for achieving the desired results. This procedure may be formally written or may just be an accumulation of results from previous similar studies. These studies serve to elucidate the potential problems and downfalls of the procedure and each study contributes to the following studies until all or most of the problems have been isolated or solved.

However, there are still some areas that have not been as intensively studied. One such area is how to evaluate spawning in tropical freshwater egg laying fish. The spawning behavior of such fish has rarely been studied scientifically and given the wide diversity of spawning strategies and behaviors, few generalisations can and have been made. It would therefore, be very useful to have a set of guidelines to follow when performing a spawning study. This is where the current study can make a contribution. In this study the factors related to spawning a particularly difficult fish species in captivity was extensively studied and most of the problems and downfalls associated with evaluating spawning in fish were encountered. These problems and downfalls can be used as the basis for a set of guidelines. Other, researchers could be advised by being made aware of the pitfalls, traps and successes that were encountered during this study and could, therefore, save time in future studies.

Such a set of guidelines should include a general procedure that one should follow, as well as highlighting all the advantages, and particularly all the problems and downfalls one is likely to encounter. This can be called a protocol for evaluating spawning in fish. This study has provided the basis for developing such a protocol for facultative pair building, tropical, freshwater, egg laying fish.

There are several requirements for a protocol to evaluate spawning in fish. These include repeatability, reliability, feasibility, practicality, efficiency, logic and sequence, analysibility and documentability. These requirements are discussed in the following paragraphs.

The protocol should be repeatable in the sense that other researchers should be able to follow the protocol and be able to compare results between studies. Therefore a comprehensive list of environmental parameters, such as water quality, lighting etc., must be presented together with the results. Further, it should strive to achieve spawning in a reliable manner that is not biased by once off, chance successes that may not be repeated.

The protocol must be feasible with regard to the number of fish required and their availability, and the resources available in terms of facilities (tanks and space) and time. Further, it should not include procedures that are impractical in terms of effort and time.

The protocol should identify the most efficient method for achieving the desired results with regard to the effort invested. Experimental treatments should be chosen according to a factorial design so as to be able to test more than one variable per experiment and still obtain viable results.

The protocol must follow in a logical sequence of steps starting with the most efficient, feasible and practical method for achieving repeatable spawning results. More costly or labour intensive methods should only be adopted if no spawning success is observed.

The results must be statistically analysable and documentable.

The first factor to be considered when beginning a spawning study is the sample size, or the number of fish needed for the study. This decision depends on a number of factors and there are more than just scientific issues to be considered. These factors include the size of the fish, the cost of the fish, the availability of the fish, the fishes spawning behaviour (e.g. obligate or facultative pair builders, group spawners) and the resources available in terms of time and space. In all cases, however, one should use the greatest number of fish possible.

For spawning experiments at least six replicates of each treatment must be performed. The reason for this will be discussed under statistical analysis. Hence, sample sizes can be recommended in multiples of six. In other words, 18 pairs of fish would allow three treatments with six replicates of each. No spawning study should be started unless a sufficient number of fish are available from the start.

A typical spawning experiment can be divided into two phases: a conditioning period, followed by the spawning trial. Each of these periods contain a number of variables (Table 6.1).

Table 6.1 Variables relevant to a generalised spawning experiment.

	Spawning Experiment	
Phase	**Conditioning period**	**Spawning period**
Variables	Duration of conditioning period	Temperature
	Diet during conditioning	Water quality
	Water quality	Diet
	Temperature	Sex ratio
	Stocking density	Spawning medium
	Sex ratio	Number of fish
	Photoperiod	Photoperiod

At the start of the spawning study the researcher should review and draw information from the literature pertaining to spawning that species in captivity and spawning fish in general, as well as any information available on the spawning of that species and similar related species in the wild. It is then up to the researcher to decide on the initial suite of variables for the first spawning experiment. This can be based on previous work or the suite of variables that occur in the fishes (or a similar or related fishes) natural habitat during the spawning season. For example if the optimum temperature for spawning in a certain fish species X has not been determined then the researcher should start off by using the water temperature found in the fishes natural habitat during the spawning season. It is recommended that prior to the first spawning experiment, a non-scientific series of spawning trials should be performed in which a number of variables can be changed at a time, so as to identify the best suite of initial variables for the study. Once an initial suite of variables has been selected the first spawning experiment can be run and thereafter only one variable changed at a time. For example, if the optimum temperature for spawning is not known, the researcher must keep all other variables in the conditioning and spawning period constant. After the conditioning period the fish should be pooled and then randomly selected and placed into the spawning tanks. During the spawning part of the experiment, a number of different temperature treatments can be tested, with the control being the fishes natural spawning temperature. The number of treatments depends on the number of fish available and the number of spawning tanks available, remembering that at least six replicates of each treatment must be performed. If 24 pairs or 24 spawning tanks are available, then four different temperatures, including the control can be tested.

All the variables observed during a spawning experiment need to be accurately measured and documented in order to fulfill the requirements of repeatability.

Methods for analysing results of spawning experiments form an integral part of a protocol for evaluating spawning in fish. This can best be illustrated by means of a hypothetical example. The results of such a hypothetical example, an experiment testing the effect of different temperatures on spawning success, can be analysed as in Table 6.2 (note that a 1 indicates that the pair spawned while a 0 indicates that the pair failed to spawn).

Table 6.2 Results from a hypothetical spawning experiment investigating the effect of temperature on spawning success..

Replicate	Treatment 1 20°C	Treatment 2 22.5°C	Treatment 3 25°C	Treatment 4 27.5°C
1	1	1	1	0
2	0	1	1	0
3	1	1	0	1
4	0	0	1	1
5	1	1	1	0
6	0	0	1	0

In this case the null hypothesis would be that there is no difference in spawning rate between the four temperature treatments. One would then perform a transformation on the data to eliminate the zeros. This transformation would be $\sqrt{x+1}$, which would result in the data set in Table 6.3.

Table 6.3 Transformed results from Table 6.2.

Replicate	Treatment 1	Treatment 2	Treatment 3	Treatment 4
1	1.41	1.41	1.41	1
2	1	1.41	1.41	1
3	1.41	1.41	1	1.41
4	1	1	1.41	1.41
5	1.41	1.41	1.41	1
6	1	1	1.41	1

One can then perform a non-parametric Kruskal-Wallis test on the transformed data to test for significant differences between treatments. Where a significant difference is found, a Student-Newman-Keuls multiple comparison procedure can be performed to identify which of the treatments differed significantly. This method of statistical analysis is not valid for fewer than six observations for each treatment. In this case there are no significant differences in the data ($P>0.093$).

The condition of the fish will determine what the first set of experiments should investigate. If the fish do not contain fully developed oocytes, then the first set of experiments should investigate the effect of conditioning on oocyte development. Such a series of experiments should serve to induce oogenesis in the fish. However, most facultative pair building, tropical, freshwater, egg laying fish species, such as cardinal tetras, red serpae tetras or rosy barbs (Axelrod & Schultz 1990), freely develop oocytes which usually remain in a fully developed state until triggered into final maturation and spawning. If the fish lose condition in the conditioning tanks then conditioning should take place in the brood stock holding tanks. If the fish contain fully developed oocytes, the experiments should follow the sequence recommended in the following paragraphs until the fish can be induced to spawn reliably and repeatably.

The optimum temperature for spawning the respective species should be determined first. Razani *et al.* (1988) and Hontela & Stacey (1990) found that water temperature was one of the factors related to spontaneous ovulation of female goldfish. Hammon (1982) found that temperature greatly influences the survival of eggs and larvae of humpback chub and, therefore, that spawning at an appropriate temperature is highly advantageous. Temperature has been shown to be an important factor influencing fertilisation success, survival of larvae, growth of juvenile fish and probably other parameters as well (Hontela & Stacey 1990). Knowledge of optimum temperatures for particular species is, therefore, essential for successful aquaculture systems and propagating and rearing fish in captivity (Hontela & Stacey 1990). The optimum temperature for spawning in a particular species can be investigated by testing the effect of a number of different temperature regimes on spawning success. This should include the temperature of the fish's natural habitat during the spawning season, and some higher and lower temperatures.

A number of ornamental fish species have particular water quality requirements for spawning (Axelrod & Shaw 1967). This is true for many tropical, forest adapted fish species which require soft acidic water (Weitzman *et al.* 1996). If the fish being studied has particular water quality preferences for spawning, then the optimum water quality for spawning should be determined. In particular the optimum conductivity and pH for spawning should be determined. These can be determined in the same manner as temperature.

The introduction to Experiment 2.3 illustrated that the nutritional status of a fish affects its reproductive success. Hence, it is important to ensure that fish being used in a spawning study are in a peak nutritional status. This can be achieved by changing the composition of the staple feed, or by partially or completely substituting the staple feed with live food.

The introduction to Experiment 2.2 illustrated that sex ratio is relevant to the spawning success of a number of ornamental fish species. Hence, if at this stage reliable and repeatable spawnings have not been observed, then the optimum sex ratio for spawning should be determined. Sex ratios such as; 1♂:1♀, 2♂:1♀ and 1♂:2♀ should be tested and if necessary even higher sex ratios such as 4♂:1♀ and 1♂:4♀ can be tested. Spawning the fish in a shoal can also be tested.

Hontela & Stacey (1990) regard green plants as a powerful stimulus for spawning in goldfish, while peat is necessary for successful oviposition in killifish (Axelrod & Shaw 1967). Hence, it is worthwhile investigating whether the type of spawning medium offered has any effect on spawning success. Further, the use of live plants versus artificial media should also be tested. Artificial media are more practical because they are easy to sterilise and are usually freely available. Live plants are difficult to sterilise and must be cultured, but may be more readily accepted by the fish as a spawning medium.

If the above experiments have failed to induce a reliable procedure for achieving repeatable spawning, then the effect of changes in environmental conditions on spawning can be investigated. Relevant environmental stimuli include a rise or drop in temperature, a fresh rain water flush, a rise or drop in water level, changes in photoperiod, or combinations of above. These stimuli may be particularly relevant to flood dependent spawners (De Vlaming 1974; Bruton 1979; Hontela & Stacey 1990; Munro 1990b; Cornish & Smit 1995; Weitzman *et al.* 1996).

If environmental stimuli fail to induce spawning then hormonal applications can be tested. These can either be incorporated into the feed, either in artificial feeds or bioencapsulated into brine shrimp nauplii, or injected. Experiments investigating the effect of different inclusions into pelleted feed, different enrichment times and doses for brine shrimp nauplii, or injection types, intramuscular or intraperitoneal, and doses, can be performed. These should all be accompanied by comprehensive bioassays to determine what level of exogenous hormone the fish were exposed to.

Finally, histology should be performed to examine the state of the gonads and determine the spawning strategy of the fish. If sufficient fish are available, it is recommended that histology is performed early in the sequence of experiments.

There are a number of advantages to following such a protocol. All the experiments are similar and can be performed in the same system using the same resources. Fish that spawn easily or at least fairly easily will pass quickly and efficiently through this protocol. The protocol will yield reliable and repeatable results. It follows a logical sequence of experiments that provides the most feasible and practical method for achieving the desired results. The results are analysable and documentable. This protocol should be modified to apply to monogamous fish, obligate pair building fish or group spawning fish.

Possibly the most valuable aspect of such a protocol is that it highlights the disadvantages, traps and pitfalls that one is likely to encounter when performing such a study.
These disadvantages are that the results observed are seldom as clear cut as one would wish. This is particularly true for fish species that do not spawn freely in captivity. This poses a particular problem in terms of analysing the results. The method of analysing the results as proposed above, while perfectly suitable for analysing the results of fish that spawn freely, runs into difficulty when too few or no fish spawn. This yields results that are particularly difficult to document since the reasons for the low spawning success are not known and no inferences can be drawn. This point further illustrates the need for the largest possible sample size. Fish that do not spawn freely do not yield results that are reliable or repeatable. Another major problem with fish that do not spawn easily is the number of variables involved in spawning. The problem being that it is extremely difficult and time consuming to determine all the necessary environmental requirements by only changing one variable at a time. This problem was discussed earlier and may be avoided by performing a non-scientific preliminary study to identify the best initial suite of variables. Another disadvantage associated with such a protocol is that the feasibility and efficiency, particularly in terms of time, are reduced when the number of fish is limited. This is because fewer treatments can be tested during each experiment and experiments must run one after the other. Any such constraints should be avoided. Another problem that may be encountered in a spawning study of this nature occurs when the fish are wild caught, in which case they may be too stressed to spawn (Pankhurst & Van Der Kraak 1997; Contreras-Sanchez *et al.* 19956; Wilson *et al.* 1995) and may not feed properly in the conditioning tanks and may even lose condition over the conditioning period. Hence, if wild caught fish are used, they must be well acclimated to captivity long before the study is initiated.

It has been shown that there are a number of disadvantages and pitfalls associated with such a spawning study. However, the pitfalls experienced in this study form the basis of the guidelines of the protocol and are not the consequences thereof. Therefore the value of such a protocol is to warn potential researchers of the problems and demonstrate methods of avoiding such traps.

REFERENCES

AGUILAR-AGUILA, A.; TEJEDA MANSIR, A. & RUIZ MANRIQUEZ, A. 1994. Using brine shrimp as a drug carrier for therapeutic applications in aquaculture. *Aquacult. Eng.* 13:301-309.

AHSAN, S.N. & HOAR, W.S. 1963. Some effects of gonadotropic hormones on the threespined stickleback, *Gasterosteus aculeatus*. *Can. J. Zool.* 41:1045-1053.

ALOK, D.; KRISHNAN, T.; TALWAR, G.P. & GARG, L.C. 1993. Induced spawning of catfish, *Heteropneustes fossilis* (Bloch), using D-Lys^6 salmon gonadotropin-releasing hormone analog. *Aquaculture* 115:159-167.

ALOK, D.; KRISHNAN, T.; SALUNKE, D; GUPTA, H.; TALWAR, G.P. & GARG, L.C. 1994. Precocious ovarian recrudescence and induced off-season spawning of *Heteropneustes fossilis* by D-Lys^6 salmon gonadotropin releasing hormone analog (sGnRH-A). *J. Fish Biology* 45:909-915.

AXELROD, H.R. 1971. *Breeding Aquarium Fishes, Book 2*. TFH Publications Inc.

AXELROD, H.R. 1994 Should the importation of aquarium fishes be controlled? *Trop. Fish Hobbyist* 42(9)195-206.

AXELROD, H.R. & SHAW, J. 1967. *Breeding Aquarium Fishes, Book 1*. TFH Publications Inc.

AXELROD, H.R. & BURGESS, L. 1973. *Breeding Aquarium Fishes, Book 3*. TFH Publications Inc. Ltd.

AXELROD, H.R. & SCHULTZ, L.P. 1990. *Handbook of tropical aquarium fishes*. TFH Publications Inc.

BAGENAL, T.B. 1969. The relationship between food supply and fecundity in brown trout, *Salmo trutta* L. *J. Fish Biol.* 1:167-182.

BARAS, E. 1995. Thermal related variations of seasonal and daily spawning periodicity in *Barbus barbus*. *J. Fish Biol.* 46:915-917.

BéNECHE, V. & OUATTARA, S. 1990. Rôle des variations de conductivté de l'eau et d'autres facteurs externes dans la croissance ovarienne d'un poisson tropical, *Brycinus leuciscus* (Characidae). *Aquat. Living Resour.* 3:153-162.

BENNINGER, H. 1996. So züchtete ich den Roten Neon *Cheirodon axelrodi. Aquaria* 6:6-9.

BILLARD, R. & MARCEL, J. 1980. Stimulation of spermiation and induction of ovulation in pike (*Esox lucius*). *Aquaculture* 21:181-195.

BöHM, O. 1996. Keeping Copeland Tetra. *Trop. Fish Hobbyist.* 45(4):110-112.

BOLGER, T. & CONNOLLY, P.L. 1989. The selection of suitable indices for the measurement and analysis of fish condition. *J. Fish Biol.* 34:171-182.

BON, E.; NUNEZ RODRIGUEZ, J. & LE MENN, F. 1995. The effect of photoperiod on vitellogenin synthesis and oocyte endocytosis in rainbow trout (*Oncorhynchus mykiss*). *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium 95. Austin.

BROMAGE, N.R.; RANDALL, C.F.; PORTER, M.J.R. & DAVIES, B. 1995. How do photoperiod, the pineal gland, melatonin, and circannual rhythms interact to co-ordinate seasonal reproduction in salmonid fish? *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium 95. Austin.

BRUTON, M.N. 1979. The breeding biology and early development of *Clarias gariepinus* (Pisces:Clariidae) in Lake Sibaya, South Africa, with a review of breeding in species of the subgenus *Clarias*. *Trans. Zool. Soc. Lond.* 35:1-45.

BURTON, M.P.M. 1994. A critical period for control of early gametogenesis in female winter flounder, *Pleuronectes americanus*. *J. Zool. Lond.* 233:405-415.

BURTON, S.M. 1997. The Red Serpae Tetra. *S.A. Fishkeeping*. 2(5)9-10.

CANTIN, M. 1987. Oocyte development and induced spawning of small mouth bass (*Micropterus dolomieui*) in response to photoperiod and temperature manipulation: preliminary results. *J. World Aquacult. Soc.* 18(1):27-30.

CHAO, N.L. 1992. Diversity and conservation of ornamental fishes - the gems from flooded forests in Amazonia. *Canadian Biodiversity (Ottowa)* 2(2): 2-7.

CONTRERAS-SANCHEZ, W.M.; SCHRECK, C.B. & FITZPATRICK, M.S. 1995. Effect of stress on the reproductive physiology of rainbow trout, *Onchorhynchus mykiss*. *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*. Fish Symposium 95. Austin.

COOKE, A.F. & PETER, R.E. 1980. The effect of temperature on the clearance of intra-peritoneally injected gonadotropin in goldfish *Carassius auratus*. *Aquaculture* 19:275-285.

CORNISH, D.A. & SMIT, G.L. 1995. The correlation between environmental factors and the reproduction of *Oreochromis mossambicus*. *Water SA* 21(3):259-263.

DAVIES, P.R. & HANYU, I. 1986a. Effect of temperature and photoperiod on sexual maturation and spawning of the common carp I. Under conditions of high temperature. *Aquaculture* 51:277-288..

DAVIES, P.R.; HANYU, I.; FURUKAWA, K. & NOMURA, M. 1986b. Effect of temperature and photoperiod on sexual maturation and spawning of the common carp II. Under conditions of low temperature. *Aquaculture* 52:51-58.

DAVIES, B.; SWANSON, P. & BROMAGE, N.R. 1995. The effects of photoperiod and temperature on serum GtH I and GtH II and the timing of maturation in the female rainbow trout. *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium 95. Austin.

DeVLAMMING,V.L. 1974. Environmental and endocrine control of teleost reproduction. 18-83. *In* SCHRECK (ed.). *Control of sex in fishes.* Sea Grant Division, Virginia Polytechnic Institute and State University, Blacksburg, V.A.

DIXON, B.A.; VAN POUKE, S.O.; CHAIR, M.; DEHASQUE, M.; NELIS, H.J.; SORGELOOS, P. & DE LEENHEER, A.P. 1995. Bioencapsulation of the antibacterial drug sarafloxacin in the nauplii of the brine shrimp *Artemia franciscana. J. Aquat. Anim. Health* 7:42-45.

DONALDSON, E.M. & HUNTER, G.E. 1983. Induced final maturation, ovulation and spermiation in cultured fish. *In* Hoar, W.S.; Randall, D.J. & Donaldson, E.M. (eds.) *Fish Physiology, Vol. 9, Reproduction, (Part B), Behaviour and Fertility Control.* Academic Press, Orlando, FL.

EATON, R.C. & FARLEY, R.D. 1974. Spawning cycle and egg production of zebrafish, *Brachydanio rerio*, in the laboratory. *Copeia* 1974: 195-201.

FRANK, S. 1980. A propos de la réproduction du Neon rouge: *Cheirodon (Lamprocheirodon) axelrodi*. *Aquarama*, 72:11-12, 89.

GARRETT, G.P. 1989. Hormonal sex control of largemouth bass. *Prog. Fish Cult.* 51:146- 148.

GEISLER, R. 1972. Im land des Roten Neon - Biotop von *Cheirodon axelrodi*. *Aquarienmagazin* 1972:64-71.

GEISLER, R. & ANNIBAL, S.R. 1987. Ecology of the Cardinal Tetra, *Paracheirodon axelrodi* (Pisces, Characoidea), in the River Basin of the Rio Negro, Brazil, as well as breeding related factors. *Trop. Fish Hobbyist* 35(2): 66-87.

HAIDER, S. & RAO, N.V. 1994. Induced spawning of maturing Indian catfish, *Clarias batrachus* (L.), using low doses of steroid hormones and salmon gonadotropin. *Aquacult. Fish. Manage.* 25:401-408.

HALDER, S.; SEN, S.; BHATTACHARYA, S.; RAY, A.K.; GHOSH, A. & JHINGRAN, A.G. 1991. Induced spawning of Indian major carps and maturation of a perch and a catfish by murrel gonadotropin releasing hormone, pimozide and calcium. *Aquaculture* 97: 373-382.

HAMMON, R.L. 1982. Spawning and culture of the humpback chub. *Prog. Fish Cult.* 44:213-219.

HANSEN, T.; KJESBU, O.S.; HOLM, J.C. & KARLSEN, Ø. 1995. Growth, gonadal development and spawning time of Atlantic cod (*Gadus morhua*) reared under different photoperiods. *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*. Fish Symposium 95. Austin.

HARMIN, S.A. & CRIM, L.W. 1992. Gonadotropic hormone-releasing hormone analog (GnRH-A) induced ovulation and spawning in female winter flounder, *Pseudopleuronectes americanus* (Walbaum). *Aquaculture* 104:375-390.

HENDERSON-ARZAPALO, A. & COLURA, R.L. 1987. Laboratory maturation and induced spawning of striped bass. *Prog. Fish Cult.* 49:60-63.

HESTER, F.J. 1964. Effects of food supply on fecundity in the female guppy, *Lebistes reticulatus* (Petrs.). *J. Fish. Res. Board. Can.* 21:757-7764.

HISLOP, J.R.G., ROBB, A.P. & GAULD, J.A. 1978. Observations on effects of feeding level on growth and reproduction of haddock, *Melanogrammus aeglefinnus* (L) in captivity. *J. Fish Biol.* 13:85-115.

HONTELA, A, & STACEY, N.E. 1990. Cyprinidae. 53-77. *In* Munro, A.D.; Scott, A.P. & Lam, T.J. (eds) *Reproductive Seasonality in Teleosts: Environmental Influences.* CRC Press, Inc.

HONTORIA, F.; CROWE, J.H.; CROWE, L.M. & AMAT, F. 1994. Potential use of liposomes in larviculture as a delivery system through *Artemia* nauplii. *Aquaculture* 127:255- 264.

HUSSAIN, M.G. 1988. Development of induced spawning procedures for grass carp, *Ctenopharyngodon idella*, in Syria. *Asian Fish. Sci.* 21:115-119.

JOHNSON, D.C. 1995. Five secrets for building a herd of Congo Tetras. *Trop. Fish Hobbyist.* 44(12):110-116.

KAGAWA, H. & MORIYAMA, S. 1995. Effects of insulin-like growth factor-I on final maturation of oocytes of red seabream, *Pagras major, in vitro. In* Goetz, F.W. & Thomas, P.

(eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium '95, Austin.

KING, H. & YOUNG, G. 1995. Increased milt production by gonadotropin releasing hormone analog (GnRHa)- treated Atlantic salmon (*Salmo salar*) after injection of 17α-hydroxyprogesterone. *In* Goetz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium '95, Austin.

LAMBERT, J.G.D. & VAN OORDT, P.G.W.J. 1974. Ovarian hormones in teleosts. *Fortschr. Zool.* 22:340-349.

LAVENS, P. & SORGELOOS, P. 1996. Manual on the production and use of live food for aquaculture. FAO Technical Paper 1996. pp. 101-290.

LILEY, N.R. 1980. Patterns of hormonal control in the reproductive behaviour of fish, and their relevance to fish management and culture programs. 210-246. *In* Bardach, J.E.; Magnuson, J.J.; May, R.C. & Reinhart J.M. (eds) *Fish behaviour and its use in the capture and culture of fishes.* ICLARM Conference Proceedings 5.

LILEY, N.R. 1982. Chemical communication in fish. *Can. J. Fish. Aquat. Sci.* 39:22-35.

LIN, H.; ZHOU, X.; VAN DER KRAAK, G. & PETER, R.E. 1991. Effects of gonadotropin-releasing hormone agonists and dopamine antagonists on gonadotropin secretion and ovulation in Chinese loach, *Paramisgurnus dabryanus. Aquaculture* 95:139-147.

LINHART, O.; PROD'HOMME, M.; BILLARD, R.; KOURIL, J. & HAMACKOVA, J. 1995. Extended spermiation by repeated injection with carp pituitary gland in the European catfish (*Silurus glanis* L.) *In* Goetz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium '95, Austin.

LOCKYEAR, J. 1994. A comparison of different hormone application methods in ornamental fish. Unpublished Honours Thesis. JLB Smith Institue for Ichthyology Library. Grahamstown. South Africa.

LUQUET, P. & WATANABE, T. 1986. Interaction "nutrition-reproduction" in fish. *Fish Physiol. Biochem.* 2(1-4)121-129.

MUNRO, A.D. 1990a. General introduction. *In* Munro, A.D.; Scott, A.P. & Lam, T.J. (eds) *Reproductive Seasonality in Teleosts: Environmental Influences.* CRC Press, Inc.

MUNRO, A.D. 1990b. Tropical Freshwater Fishes. *In* Munro, A.D.; Scott, A.P. & Lam, T.J. (eds) *Reproductive Seasonality in Teleosts: Environmental Influences.* CRC Press, Inc.

NEJMARK, L. 1987. Spawning the emperor tetra, *Nematobrycon palmeri. Trop. Fish Hobbyist.* 35(10):46-52.

NGAMVONGCHON, S.; PAWAPUTANON, O.; LEELAPATRA, W. & JOHNSON, W.E. 1988. Effectiveness of an LHRH analogue for the induced spawning of carp and catfish in Northeast Thailand. *Aquaculture* 74(1-2):35-40.

NORBERG, B.; THRANDUR BJORNSSON, B. & HAUX, C. 1995. Photoperiod controls the timing of reproduction in Atlantic cod (*Gadus morhua*). *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium 95. Austin.

NOZNOV, A. 1986. Spawning the Cardinal Tetra. *Trop. Fish Hobbyist* 34(5): 40-48.

PALICKA, J. 1989. The successful care and breeding of the rummy-nosed tetra. *Trop. Fish Hobbyist*. 37(9):84-89.

PANKHURST, N.W. & VAN DER KRAAK, G. 1997. Effects of stress on reproduction and growth of fish. *In*: Iwama, G.K.; Pickering, A.D.; Sumpter, J.P. & Schreck, C.B. (eds) Fish *Stress and health in aquaculture*. Cambridge University press. UK.

PARTRIDGE, B.L., LILEY, N.R. & STACEY, N.E. 1976. The role of pheromones in the sexual behaviour of the goldfish. *Anim. Behav*. 24:291-299.

PAVLADIS, M.; PASCHOS, J.; THEOCHARI, V. & DESSYPRIS, A. 1992. Photoperiod and the control of rainbow trout spawning time endocrinological aspects. *Aquaculture* 100(1- 3):327.

PENRITH, M. 1987. Neons. Why the neon tetra is always in the top five. SA Fishkeeping 2(3)3-5.

PETER, R.E. 1981. Gonadotropin secretion during reproductive cycles in teleosts: Influences of environmental factors. *Gen. Comp. Endocrinol*. 45:249.

PETER, R.E. 1982. Nature, localisation and actions of neurohormones regulating gonadotropin secretion in teleosts. *In* Richter, C.J.J & Goos, H.J.Th (eds.) *Proceedings of the International Symposium on the Reproductive Physiology of Fish*. Pudoc. Wageningen, Netherlands.

PETERSON, D.A. 1972. Barometric pressure and its effect on spawning activities of rainbow trout. *Prog. Fish Cult*. 34:110-112.

PONCIN, P. 1989. Effects of different photoperiods on the reproduction of the barbel, *Barbus barbus* (L.), reared at constant temperature. *J. Fish. Biol*. 35:395-400.

RAZANI, H.; HANYU, I. & AIDA, K. 1988. Environmental influences on ovarian activity and related changes in yearling goldfish. *Nippon Suisan Gakkaishi* 54(9):1505-1511.

RICHTER, H. 1991. Keeping and breeding the cardinal tetra. *Trop. Fish Hobbyist* 39(10):26- 30.

ROTHBARD, S. 1981. Induced reproduction in cultivated cyprinids, the common carp and the group of Chinese carps: I. The technique of induction, spawning and hatching. *Bamidgeh* 33(4)103-121.

ROUHANI, Q. 1997. The breeding and rearing of *Synodontis petricola.* Department of Ichthyology and Fisheries Science Research Report Series 10 pp 114-119, Rhodes University, RSA.

ROWLAND, S.J. 1988. Hormone induced spawning of the Australian freshwater fish Murray cod, *Maccullochella peeli* (Mitchell) (Percichthyidae). *Aquaculture* 70:371-389.

SCHIFF, S.J. 1994. Aquarium set-up. *Freshwater and Marine Aquarium* 17(10):136-142.

SCHULTZ, L.P. 1956. The amazing new fish called the Scarlet Characin (*Cheirodon axelrodi*, a new species). *Trop. Fish Hobbyist* 4: 42-43.

SCOTT, D.P. 1962. Effect of food quantity on fecundity of rainbow trout, *Salmo gairdneri. J. Fish. Res. Board Can.* 19:715-730.

SHIMIZU, A. 1995. Effect of photoperiod and temperature on reproductive activity of the mummichog, *Fundulus heteroclitus,* during various seasons. *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International.Symposium on the Reproductive Physiology of Fish.* Fish Symposium 95. Austin.

SHIREMAN, J.V. & GILDEA, J.A. 1989. Induced spawning of rainbow sharks (*Labeo erythrurus*) and redtail black sharks (*L. bicolor*). *Prog. Fish Cult.* 51:104-108.

SIOLI, H. 1968. Hydrochemistry and geology in the Brazilian Amazon region. *Amazonia* 1:74-83.

SORENSON, P.W. & STACEY, N.E. 1987. 17α,20ß-dihydroxy-4-pregnen-3-one functions as a bisexual priming pheromone in goldfish. *Amer. Zool.* 27:abs 412.

SORENSON, P.W.; BRASH, A.R.; GOETZ, F.W.; KELLNER, R.G.; BOWDIN, L. & VRIEZE, L.A. 1996. Origins and functions of F-prostaglandins as hormones and pheromones in the goldfish. *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium 95. Austin.

SORGELOOS, P.; LAVENS, P.; LEGER, P. & TACKAERT, W. 1993. The use of *Artemia* in marine fish larviculture. *TML Conference proceedings* 3:73-86.

STACEY, N.E. 1984. Control of timing of ovulation by exogenous and endogenous factors. *In* Potts, G.W. & Wooton, R.J. (eds.) *Fish Reproduction, Strategies and Tactics.* Academic Press, Orlando, FL.

STACEY, N.E.; SORENSON, P.W. & CARDWELL, J.R. 1993. Hormonal pheromones: Recent developments and potential applications in aquaculture. *In* Lahlou, B. & Vitello, P. (eds) *Aquaculture: Fundamental and Applied Research.* Coastal and American Geophysical Union, Washington DC.

STACEY, N.E.; COOK, A.F. & PETER, R.E. 1979. Spontaneous and gonotropin-induced ovulation in the goldfish, *Carassius auratus* L.: Effects of external factors. *J. Fish Biol.* 15:349.

STACEY, N.E.; KYLE, A.L. & LILEY, N.R. 1986 Fish reproductive pheromones. *In*: Duvall, D.; Muller-Schwarze, D. & Silverstein, R.M. (eds) *Chemical signals in vertebrates IV*. Plenum Press. New York. pp 117-133.

STACEY, N.E. & CARDWELL, J.R. 1996. Hormones as sex pheromones in fish: widespread distribution among freshwater species. *In* Geotz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*. Fish Symposium 95. Austin.

SUMPTER, J.P. 1990. General concepts of seasonal reproduction. 13-31. *In* Bardach, J.E.; Magnuson, J.J.; May, R.C. & Reinhart J.M. (eds) *Fish behaviour and its use in the capture and culture of fishes*. ICLARM Conference Proceedings 5.

TAN-FERMIN, J.D. & EMATA, A. 1993. Induced spawning by LHRHa and pimozide in the Asian catfish *Clarias macrocephalus* (Gunther). *J. Appl. Ichthyol.* 9:89-96.

TOURAKI, M.; RIGAS, P. & KASTRITSIS, C. 1995. Liposome mediated delivery of water soluble antibiotics to the larvae of aquatic animals. *Aquaculture* 136:1-10.

TOURAKI, M.; MOURELATOS, S.; KARAMANLIDOU, G.; KALAITZOPOULOU, S. & KASTRITSIS, C. 1996. Bioencapsulation of chemotherapeutics in *Artemia* as a means of prevention and treatment of infectious diseases of marine fish fry. *Aquacult. Eng.* 15(2):133-147.

TRIPATHI, V. & SINGH, T.P. 1995. Effect of some steroids and prostaglandins on GVBD and ovulation in catfish, *Heteropneustes fossilis*. *In* Goetz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*. Fish Symposium '95, Austin.

TYLER,C.R. & SUMPTER, J.P. 1996. Oocyte growth and development in teleosts. *Rev. Fish Biol. Fish.* 6:287-318.

WALLACE, R.A. & SELMAN, K. 1981. Cellular and dynamic aspects of oocyte growth in teleosts. *Amer. Zool.* 21:325-343.

WANG, D.S.; LIN, H.R. & GOOS, H.J.TH. 1995. The neuroendocrine regulation of gonadotropin secretion and ovulation in the bagrid catfish, *Mystus macropterus*. *In* Goetz, F.W. & Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Fish Symposium '95, Austin.

WATANABE, T., ARAKAWA, T., KITAJIMA, C. & FUJITA, S. 1984a. Effect of nutritional quality of brood stock diets on reproduction in Red Sea bream. *Bull. Jap. Soc. Sci. Fish.* 50: 503-515.

WATANABE, T., ITOH, A., KITAJIMA, C., & FUJITA, S. 1984b. Effect of dietary protein levels on reproduction of Red Sea bream. *Bull. Jap. Soc. Sci. Fish.* 1015-1022.

WEITZMAN, S.H. & FINK, W.L. 1983. Relationships of the Neon Tetras, a group of South American fresh-water fishes (Teleostei: Characidae) with comments on the phylogeny of the New World Characiforms. *Bull. Mus. Comp. Zool. Harvard University* 150(6):339-395.

WEITZMAN, S.H.; PALMER, L.; MENEZES, N.A. & BURNS, J.R. 1996. Maintaining tropical and subtropical forest-adapted fishes, especially the species of *Mimagoniates* (Part1). *Trop. Fish Hobbyist* 44(12): 184-194.

WILSON, C.E.; CRIM, L.W. & MORGAN, M.J. 1996. The effect of stress on spawning performance and larval development in the Atlantic cod, *Gadus morhua* L. *In* Goetz, F.W. &

Thomas, P. (eds.) *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*. Fish Symposium '95, Austin.

WOODHEAD, A.D. 1960. Nutrition and reproductive capacity in fish. *Prog. Nutr. Soc.* 19:23-28.

WOOTON, R.J. 1973. The effect of size of food ration on egg production in the female three-spined stickleback, *Gasterosteus aculeatus* L. *J. Fish Biol.* 5:89-96.

YAMAMOTO, K. & YAMAZAKI, F. 1961. Rhythm of development in the oocyte of the goldfish, *Carassius auratus*. *Bull. Fac. Fish., Hokkaido Univ.* 12(2):93-114.

YAMAMOTO, K.; NAGAHAMA, Y. & YAMAZAKI, F. 1966. A method to induce artificial spawning of goldfish all through the year. *Bull. Jap. Soc. Sci. Fish.* 32:977-982.

ZONNEVELD, N.; RUSTIDJA, V.; VIVEEN, W.J.A.R & MUDANA, W. 1988. Induced spawning and egg incubation of the Asian catfish, *Clarias batrachus*. *Aquaculture* 74:41-47.