REPRODUCTIVE BEHAVIOUR OF THE SKUNK CLOWNFISH, *Amphiprion akallopisos*, UNDER CAPTIVE CONDITIONS

Submitted in Fulfilment of the
Requirements for the Degree of
MASTER OF SCIENCE
of Rhodes University

by

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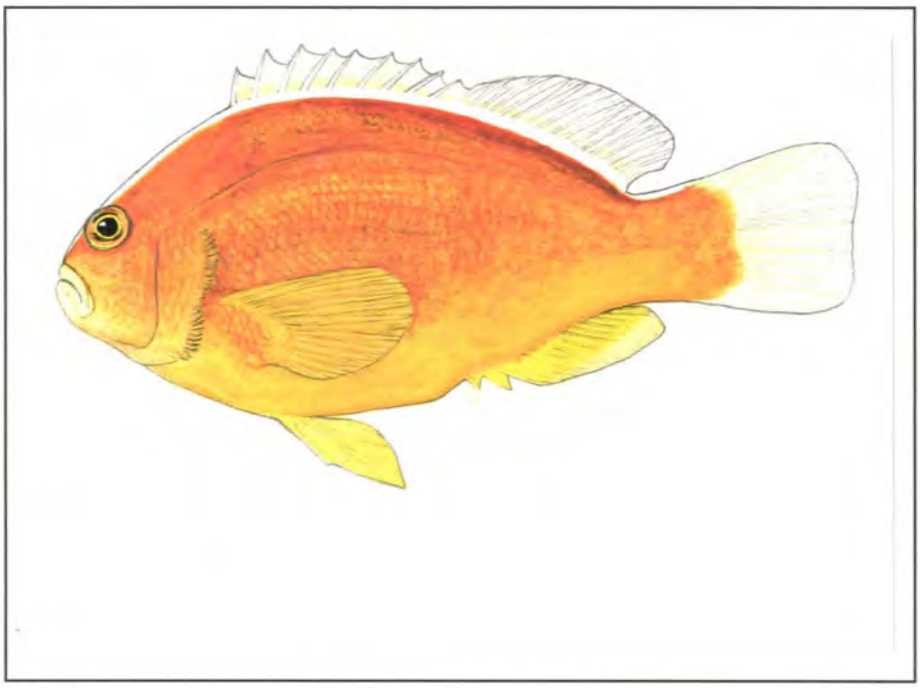
January 1998



Frontispiece: The Skunk Clownfish, *Amphiprion akallopisos*

This thesis is dedicated to my brother Kelvin, for his companionship
during my early ichthyological years and to my brother Justin,
for taking such good care of my home aquaria in my absence.

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ACKNOWLEDGEMENTS

Firstly, thanks go to my supervisors, Prof. Tom Hecht and Dr. Horst Kaiser for invaluable advice and constructive criticism throughout the course of the study

I wish to thank the following persons for help in various ways:
At Rhodes University: Larry Oellerman, Jackie Lockyear, Horst Kaiser and Andrew Gordon for feeding my fish while I was away, and for assistance in the field. Jack Atherfold for his efficiency in installing and modifying the electrics in my laboratory.

At the Oceanographic Research Institute and Sea World, Durban: Mike Schleyer, Pierre Pradervand, Derek Arthur and Tim Kay for help in collection of the broodstock. John Ballard, Darryl Harris and Paul Zammit, for making holding facilities available to me. Andre Bok for advice and essential information. A special word of thanks to John Ballard, Andre Bok and Mike Schleyer for help above and beyond the call of duty.

At Natal Parks Board: Mike Tyldesley, for his efficiency in organising collecting permits at short notice.

My parents, for support throughout the study and for giving me the opportunity to study in my chosen field. My girlfriend, Angela, for love and support during the write up period.

The study was funded by SANCOR, Liberty Life Education Foundation and the Foundation for Research Development.

ABSTRACT

The objectives of the study were to determine whether or not behaviour could be used to predict spawning in *Amphiprion akallopisos*, and to document the behaviour of this species under various environmental conditions in captivity.

The spawning behaviour of *A.akallopisos* was studied and quantified. Three behaviour patterns - belly touching, nest cleaning by the female and mutual nest cleaning (by the male and the female) were identified as predictors for spawning. The reproductive behaviour of *A.akallopisos* under three photoperiods was investigated. The photoperiods were: 14L:10D, 10hr15minL:13hr45minD and a natural photoperiod cycle condensed into three months. *A.akallopisos* maintained under 14L:10D exhibited a significantly higher frequency and duration of chasing, nest cleaning and total interaction compared to fish kept under 10hr15minL:13hr45minD. A photoperiod of 14L:10D was selected for further studies. As manipulation of photoperiod did not induce spawning, GnRHa was administered to the fish in the diet at levels of 10, 20, 40 and 80 μg/kg BW. Control groups were fed untreated food. None of the dosages were successful in inducing spawning, or spawning behaviour in *A.akallopisos*. It is possible that the method of hormone application was not suitable for this species. It is also possible that behaviour may regulate blood hormone levels as opposed to endocrine status influencing behaviour. In the third trial, *A.akallopisos* was maintained with, and without sea anemones in order to determine whether or not anemone hosts are necessary for spawning. The presence of anemones did not induce spawning and *A.akallopisos* kept without anemones exhibited significantly more interactive behaviour than fish kept with hosts. Spawning of

A.akallopisos at a later date without anemones suggests that anemones are not necessary for spawning in *A.akallopisos*. Light intensity was investigated as a cue for spawning. The reproductive behaviour of *A.akallopisos* was then studied under light intensities of 4.16×10^{15} quanta.sec^{-1}.cm^{-2}, 8.85×10^{15} quanta.sec^{-1}.cm^{-2} and this intensity plus natural light. Spawning occurred mainly under 8.85×10^{15} quanta.sec^{-1}.cm^{-2}. Fish maintained under this light intensity exhibited significantly more of nest cleaning behaviour in terms of frequency and duration than fish maintained under low light intensity. A minimum light intensity of 8.85×10^{15} quanta.sec^{-1}.cm^{-2} is recommended for conditioning of this species.

This study can be of practical relevance to hatchery managers who can use the methods developed and record predictors for spawning in *A.akallopisos* and other *Amphiprion* species. In this way imminence of spawning may be estimated. Most importantly, the study also has academic merit as little work has been undertaken in this field. Although the observational method used in this study was adequate for the purposes of the investigation, future work of this nature should incorporate other methods of documenting gonadal development such as gonadal staging and GSI in order to obtain more conclusive results.

CHAPTER 1

INTRODUCTION

The global value of the trade in marine ornamental fish and invertebrates in 1985 was estimated to be in the order of $US 20-40 million (Wood 1985). Not only is the marine sector of the international ornamental fish trade one of the most valuable, it is also one of the fastest growing. Andrews (1990) reports that while freshwater fish imports into the UK doubled from 1977 to 1989, marine imports trebled for the same period. In this time, the proportion of marine fish imported into the U.K. as a percentage of total ornamental fish imports rose from 11,4% to 17% (Wood 1992).

Almost all the marine ornamental fishes collected for the trade are harvested from the wild (Wood 1985, 1992). Many of these are collected using chemicals such as sodium cyanide which results in large-scale degradation of coral reefs (Wood 1985, Andrews 1990, Tongson and McAllister 1997). International concern about the destruction of coral reefs coupled with technological advancements in marine food fish culture has created a niche for the captive breeding of marine ornamental fishes. At present at least 14 *Amphiprion* species (clownfish), 5 *Pseudochromis* (dottybacks), 2 *Gramma* species (basslets), 5 *Gobiodon* species (gobies) and 3 *Hippocampus* species (seahorses) are produced on a commercial scale (Tucker and Jory 1991; 1997 sales lists from producing farms).

Two *Amphiprion* species, namely *A.allardi* and *A.akallopisos* occur off the South African coast (Smith and Heemstra 1986). *A.akallopisos* is the smaller and more abundant of the two species and

is also easier to maintain in captivity (Smith and Heemstra 1986, Dr. M.Schleyer, Oceanographic Research Institute, Durban, pers. com.). It was principally for these reasons that this species was chosen for the study.

A.akallopisos is a member of the sub-family Amphiprioninae (Family: Pomacentridae), commonly known as anemonefish or clownfish. The Amphiprioninae consists of 26 species. These particular damselfish species differ from the other pomacentrids in terms of their biology and taxonomy. The Amphiprioninae are best known for their symbiotic relationship with the large stichodactylid sea anemones. This dependence on these organisms is a characteristic which separates this group from the rest of the Pomacentridae. The only other pomacentrid species to associate with sea anemones is *Dascyllus trimaculatus*. However, this species is not dependant on the anemone for survival as the Amphiprioninae are (Mariscal 1970).

Three alpha taxonomical characteristics separate the Amphiprioninae from the other members of the family Pomacentridae. Firstly, the dorsal spines of the Amphiprioninae number VIII to XI while other pomacentrids have XII or more. The second difference lies in the bones of the opercular series. The suborbital, preopercle, opercle and interopercle bones all have serrated margins. All other pomacentrids only have one or two of these bones with serrated margins. Thirdly, transverse scale rows number in excess of 50 compared to 40 or less for other damselfish. The scales are also smaller in size (Allen 1972).

The Amphiprioninae consists of 25 species belonging to the genus *Amphiprion* and one to the genus

Premnas. The 25 *Amphiprion* species can be sub-divided further into four sub-genera. *A.akallopisos* falls within the sub-genus *Phalerebus*, a group which also contains the species *A.leucokranos, A.perideraion, A.nigripes* and *A.sandaracinos*. This group lacks the conspicuous transverse white bands, which is a characteristic feature of the rest of the Amphiprioninae, and has a higher number of soft-dorsal rays. The members of this sub-genus have a relatively elongate body compared to the other clownfishes. In shape they resemble the hawkfishes of the family Cirrhitidae. They also differ from the other Amphiprioninae in having scales in the interorbital region. Of the four species in the sub-genus, *A.akallopisos* is most closely related to *A.sandaracinos*. It differs from this species in having incisiform teeth as opposed to conical teeth. It also differs with respect to the number of soft-dorsal, anal and pectoral rays and gill rakers on the first arch (Allen 1972).

A.akallopisos is deep pink in colour with a white stripe running from the forehead through the dorsal fins to the caudal fin (see frontispiece). The rest of the fins are pale orange. The underside of the fish is bright yellow to orange in colour. Specimens cannot be sexed on the basis of colour. *A.akallopisos* reaches a maximum size of 110 mm total length (TL) for females. The males are considerably smaller and seldom exceed 70 mm TL (Smith and Heemstra 1986).

Like other *Amphiprion*, *A.akallopisos* is a protandrous hermaphrodite (Allen 1972). The fish form permanent pair bonds with a dominant female and male presiding over an anemone. A number of smaller males may also inhabit the same anemone. If the dominant male is removed the largest will become the dominant or beta male (Hattori 1994). If the alpha female is removed then the beta male will change sex, while the next largest male develops into the dominant male

(Fricke 1979). In spite of the presence of other males only one male ever mates with the female. It may be that the dominant male actually retards the growth and maturation of the other males (Fricke 1979).

A.akallopisos is host specific to the stichodactylid anemones *Heteractis magnifica* and *Stichodactyla mertensii* (Fautin 1991). It is found in symbiosis with these actinians in depths ranging from 3 to 25 metres throughout the Indian Ocean. *A.akallopisos* occurs from Aliwal Shoal (30°15'S) on the east coast of South Africa to Java, Sumatra, and the Andaman Islands. It does not extend to the Pacific Ocean (Smith and Heemstra 1986).

Due to the nature of the pair bond, *Amphiprion* have developed intricate behaviour patterns (Allen 1972, Fricke 1973, 1983). The major behaviour patterns of adult *Amphiprion* under natural conditions, as described by Allen (1972) include: foraging and feeding; nestling in the anemone; courtship and nesting activity; agonistic activity; spawning and brood care and miscellaneous activities. A brief summary of these activities and behaviours of adult clownfish in their natural environment is given in the following paragraphs.

Foraging and feeding

The greatest proportion of time is spent foraging and feeding. The duration of feeding and foraging is dependant on the species and on the locality. Territories and foraging trips were confined to the immediate water column around the host anemone for *A.chrysopterus* around an exposed pinnacle with a large number of predators present in the area. The same species living in a sheltered lagoon enjoyed a comparatively large territory of 20 square metres over which they

foraged freely. Foraging time is also determined by the diet of the species. Allen (1972) reported that a plankton feeder such as *A.chrysopterus* spent more time foraging than *A.perideraion*. Allen (1972) quantified these behaviours by expressing each type of behaviour as a percentage of the total time spent in observation of an *Amphiprion* species *A.perideraion* and *A.akallopisos* derive much of their nutrition from algae in the vicinity of the anemone and from the mucous secreted by the anemone which contains nematocysts and zooxanthellae (Mariscal 1970).

Nestling among the tentacles of the anemone.

In Allen's (1972) study of *Amphiprion* of the Eniwetok atoll, feeding expeditions were regularly interrupted by visits to the anemone. These activities consisted of either lying passively among the tentacles or actively scurrying about the oral disc. The fish also "snuggle" within the anemone, vibrating the body as if trying to effect deeper penetration into the tentacles. In the event of being threatened or chased, Fricke (1979) noted that the fish would disappear completely into the anemone, even entering the mouth. This behaviour was also observed when *A.akallopisos* were collected at Sodwana Bay, the fish often disappearing completely into the coelenteron (pers. obs).

Courtship and nest preparation

Clownfish generally form permanent monogamous pairs which is in contrast to the polygamous nature of most of the other pomacentrids (Allen 1980). Bigamy has been reported for *A.clarkii* and *A.tricinctus* (Allen 1972; Moyer and Bell 1976; Ochi 1989) but this behaviour is the exception rather than the rule. Fricke (1979) reported that although the potential for polygamy

was high in *A.akallopisos* where a dominant female presides over a number of smaller males, monogamy was still most common.

It may be that the dominant male actually prevents the other males from attaining sexual maturity, thereby preventing the sub-ordinate males from mating with the female (Fricke 1979). When the second largest or gamma male attempted courtship with the female, it was immediately attacked by the dominant beta male. Clownfish are protandrous hermaphrodites. Should a female be removed from the anemone the dominant or beta male will change sex to become the female and the next largest male will become the dominant male (Allen 1972; Fricke 1979).

Allen (1972) found that less than 5% of the time spent in the observation of three species was devoted to courtship during non-nesting periods. Although diverse in their other behaviours, all species exhibit similar courtship behaviours. Foraging and exploratory swimming were occasionally interrupted by displays between mates. Often these brief displays proved to be elements of courtship. This was evidenced by the fact that the pair would often visit the future nest site where substrate biting would ensue (Allen 1972).

Display patterns between mates consisted of either lateral or parallel posturing (Allen 1972). The most frequent lateral display consisted of a dipping of the dorsal fin toward the recipient and "shaking" of the head. Parallel movements consisted of either parallel swimming or leaning. Parallel swimming occurred when both male and female swam parallel to each other without any leaning or body quiver. When leaning, the fish would lean either their ventral or dorsal sides towards each other and swim parallel for short distances (Allen 1972). Substrate biting was found

to be an important interactive activity and was exhibited throughout the observation period. The frequency of substrate biting increased when a nest site was selected and as the time of spawning approached. The chosen nest site is cleared of algae and debris so that the eggs may be laid on a smooth surface. The male fish is responsible for most of the nest cleaning activity and is stimulated to begin nest cleaning when nudged gently in the belly by the female. The male fish would also exhibit nest cleaning behaviour without a cue from the female. The female would assist in the nest cleaning activity as the time of spawning approached (Allen 1972, Ross 1978). Moyer and Bell (1976) recorded nest cleaning activity by the female fish within one day of spawning The pair may also engage in rapid up and down swimming facing each other. This behaviour is known as signal jumping (Reese 1964, Allen 1972).

Agonistic behaviour

All *Amphiprion* species have evolved ritualised threat and submissive elements of agonistic behaviour. These may serve to prevent injury during intraspecific fighting under natural conditions (Allen 1972). Interspecific encounters are generally rare and are directed mainly at fishes such as juvenile *Dascyllus trimaculatus* which often inhabit anemones along with clownfishes. *Amphiprion* have also been observed chasing fish such as *Chaetodon* species away from the anemone so as to prevent them from picking at the host (Mariscal 1970).

Intraspecific agonistic encounters between *Amphiprion* are far more common than interspecific encounters. Although frequent, intraspecific encounters between fish in the wild have not been noted to result in physical damage (Allen 1972). The dominant pair in the anemone were always the main aggressors, chasing sub-adults and juveniles inhabiting the same anemone (Allen 1972;

Fricke 1979). The female, being the larger of the pair, often chased the dominant male who in turn directed its aggression at the other males (Allen 1972). In large groups of *A.akallopisos*, the greatest amount of aggression exhibited by the dominant male was directed at the second largest male (Fricke 1979) Most of the chasing was directed at the ventral surface or tail of the fish. Dorsal dipping, head shaking and vocalisation were noted as common submissive gestures and were all successful in breaking off the attack of the aggressor. Sometimes however, the fish being attacked simply fled from the aggressor and the chase was given up.

Moyer and Bell (1976) reported that in *A.clarkii* agonistic activity increased markedly with the onset of spawning. Where colonies of *A.clarkii* and anemones occur together, aggression was directed at sub-adult *A.clarkii.* However, in isolated locales where there were no such sub-adults, attacks were levelled at fish such as *Dascyllus trimaculatus* that lived close to the anemone.

Spawning and brood care

The reproductive behaviour of a number of *Amphiprion* species under natural conditions has been documented (Allen 1972; Fricke 1979; Moyer and Bell 1976; Ross 1978). The reproductive behaviour of all species studied is similar and can be summarised as follows:

Nest cleaning by the male is the first sign that spawning will take place. The belly of the female becomes noticeably distended with eggs and the frequency of chasing and belly touching increases. On the day of spawning the site is cleaned and the anemone is bitten to force it to draw away from the nesting site (Moyer and Bell 1976). The nesting site is normally situated adjacent to the anemone. The spawning process is initiated by the female. The whitish ovipositor

becomes visible and is pressed against the substrate as the female lays a row of eggs. The laying is accompanied by a quivering of the body and fluttering of the pectoral fins. The "body quiver" behaviour of the female initiates the mating response in the male (Fricke 1979). The male fish then follows the female and fertilises a row of eggs as they are laid down. The fish sometimes swim head to tail in slow circles as the eggs are deposited. The duration of spawning is normally 1-2 hours (Allen 1980; Ross 1978).

Spawning has been reported to occur at different times of the day. Ross (1978) noted that for *A.melanopus* spawning was always initiated between 08:00 and 09:00 hours (2-3 hours after sunrise), whereas Allen (1972) observed *A.perideraion* and *A.crysopterus* spawning during the mid-day hours (13:00 to 14:00 and 14:00 to 15:00 hours respectively).

The male assumes the responsibility for the care of the eggs, fanning them at frequent intervals, gently mouthing them and removing dead eggs. The female only rarely engages in caring for the eggs (Allen 1972; Bell 1976).

Spawning of *A.perideraion, A.chrysopterus* and *A.clarkii* showed peaks around 6 days before or after the full moon (Allen 1972; Ochi 1985). Allen (1972) suggested that the phenomenon could be due to increased currents that occur with spring tides and could aid in dispersal of the larvae.

Spawning in most *Amphiprion* species takes place throughout the year especially in populations within the tropics. In contrast, a temperate population of *A.clarkii* spawned only 6-8 times per

year but had clutches which ranged from 1100-2500 eggs with an estimated annual fecundity of 8000-17500 eggs (Ochi 1985). During the spawning period any number of eggs ranging from 100 to 1000 may be laid, with an average clutch size of 200-400 eggs (Allen 1980). Ross (1978) reported clutches of 200-400 eggs for *A.melanopus* (2 spawnings per lunar month) and an estimated annual fecundity of 7200 eggs per year for mature pairs. Allen (1975) estimated an annual fecundity of 3000-5000 eggs for *A.crysopterus* and only 2000-4000 eggs for *A.perideraion*. Allen (1972) also reported an estimated fecundity of 5000 eggs for *A.percula* in aquaria which spawned every two weeks with a two month non-spawning period from the end of September to the beginning of December. The ADAS hatchery in Norway reports spawning every two weeks with a break of only two weeks in the year in which the fish do not spawn. No details of clutch size are given and the popular literature is lacking in details apart from the fact that the average clutch size in aquaria ranges from 200-400 eggs (Allen 1980). At the marine hatchery of the Department of Ichthyology and Fisheries Science, Rhodes University, spawning in *A.ocellaris, A.percula, A.clarkii* and *Premnas biaculeatus* occurs every 14 days.

Miscellaneous activities

Rubbing of the body against a fixed object was observed only rarely in the wild but is common in captive *Amphiprion* which may harbour parasites (Allen 1972). Mariscal (1970) postulated that while the anemone may protect *Amphiprion* from fungal and skin diseases, it offered little protection from external parasites. This was evidenced by the fact that *A.akallopisos* were found to be more heavily infested with parasitic isopods than any other reef fish in the same area (Mariscal 1970).

Yawning is another behaviour observed frequently in *Amphiprion* (Allen 1972). This behaviour may be likened to stretching. The fish yawn by opening the mouth wide and extending the dorsal, anal and pelvic fins.

Sound production in *Amphiprion* species is common and is normally associated with agonistic activity (Allen 1972). Sounds are emitted by fish in conjunction with threat and submissive gestures and may comprise either "clicks" or "grunts." Clicks are emitted in volleys with an average duration of 35-45 ms for each click. The sound energy for these was concentrated below 1000 cycles per second (cps). Usually 3 to 15 clicks were delivered in succession. The clicks were emitted by both attacking and submissive fish. The second type of sound emitted, and only by *A.chrysopterus,* was a grunt of 100 ms duration with energy concentrated around 600 cps. This call was also emitted in association with threatening postures by resident fish faced with non-*Amphiprion* intruders.

Conditioning and spawning of broodstock is the first step of any aquaculture programme. Methods of documenting development in these conditioning stages are needed as is information about the species breeding habits and reproductive behaviour. *Amphiprion* species are too small to check egg development and imminent spawning by means of cannulation. For this reason the primary aim of the project was to determine whether or not behaviour could be used as a predictor for spawning in *A.akallopisos*. As the reproductive pattern of all *Amphiprion* species is similar (Allen 1972, Fricke 1979, Moyer and Bell 1976), the results of this study would be relevant to most, if not all other clownfish species. The second objective was to document the behaviour of *A.akallopisos* under various environmental conditions which are known to trigger

gonadal development and spawning in other marine species.

In order to determine whether behaviour can be used as a spawning predictor, a study on the reproductive behaviour of *A.akallopisos* was undertaken to provide a reference point for the behavioural data obtained in subsequent experiments (Chapter 3). Following the initial study, the effect of three photoperiods on the behaviour of *A.akallopisos* was investigated (Chapter 4). In addition, hormone treatment and its effect on the behaviour of *A.akallopisos* was explored (Chapter 5). As popular literature (Allen 1980, Hoff 1996) often recommends maintaining *Amphiprion* with their host anemones for breeding purposes, the behaviour of *A.akallopisos* kept with actinians was compared to those maintained without (Chapter 6). Conditions under which *A.akallopisos* were breeding were studied in order to determine if an environmental cue was missing and in the final experiment behaviour was studied under three light intensities (Chapter 7).

CHAPTER 2.

MATERIALS, METHODS AND RESEARCH APPROACH,

Collection and acclimation of broodstock

Fourteen adult pairs of *A.akallopisos* were collected using SCUBA and hand nets at Five Mile Reef, Sodwana Bay, South Africa (27°14'S). The fish were caught in depths ranging from 20-25m. On the reef *A.akallopisos* occurred predominantly in pairs and for this reason only pairs were collected. If one partner could not be captured the single fish was released. Although no information exists as to the size of *A.akallopisos* at sexual maturity, the male fish all measured over 30 mm TL and the females over 50 mm. *A.akallopisos* has been recorded spawning at these sizes (pers. obs). The divers also targeted large pairs within a similar size range. The pairs were decompressed at the rate specified by the dive computer and no barotrauma symptoms were noticed. On land, the pairs were packed in plastic bags containing 3 L of seawater, filled with pure oxygen and held at Sodwana Bay overnight. They were then repacked, given fresh water and oxygen and transported by road back to Durban. In Durban they were repacked before being transported to Grahamstown.

At Rhodes University the fish were held for a period of three weeks prior to the start of the first experiment to allow them to acclimatise to captivity. During this time the fish were held in the experimental system and in the aquaria that they were to be observed in. They were fed live adult *Artemia* at first and then weaned onto a fresh mixed diet (see Chapter 2) which was fed to them throughout the course of the study. The fish were fed to satiation twice daily. Temperature and salinity were kept constant at 26 $\pm 1^{0}$C and 34 ppt, respectively. Photoperiod was maintained

at 12L:12D for the acclimatisation period.

System design and management

A closed recirculating system with a total volume of 2900 litres was constructed for the study. Fish were housed in glass aquaria of two sizes. The sizes were 900x350x300 mm (94,5 L) and 600x500x380 mm (114 L) by volume. The filtration system consisted of a 350 L settlement tank and four 90 L flooded filter compartments, a 2,5 m x 200 mm diameter trickle tower and a foam fractionator. Two of the wet filter compartments were filled with oyster shells and two with shredded plastic and the trickle filter was filled with synthetic filter wool and coiled plastic. The settlement tank housed the foam fractionator and a 0,6 kW thermostatically controlled submersible heater. Temperature was kept constant at 26 $\pm 1^{0}$C. This temperature was chosen as it is the average summer water temperature for Sodwana Bay. It is also within the ideal temperature range of 26-28^{0}C for most *Amphiprion* species (Hoff 1996, Juhl 1992). Water was circulated through the system by means of a 0,3 kW pump which pumped at a rate of 4800 L/hr at a 2m head. Flow rate through the fish tanks was kept constant at a rate of 5 L/min. The water that was not fed to the aquaria flowed through the trickle tower and back into the filtration system at a rate of 90 L/hour. The system is illustrated in Figure 2.1.

Salinity was kept constant at 34 ± 1 ppt with the addition of rain water. Salinities lower than 35 ppt are recommended as they may reduce the chance of disease as well as providing a buffer against rising salinity due to evaporation (Hoff 1996, Allen 1980). Top-up water was provided through a ball valve attached to a 110 L drum.

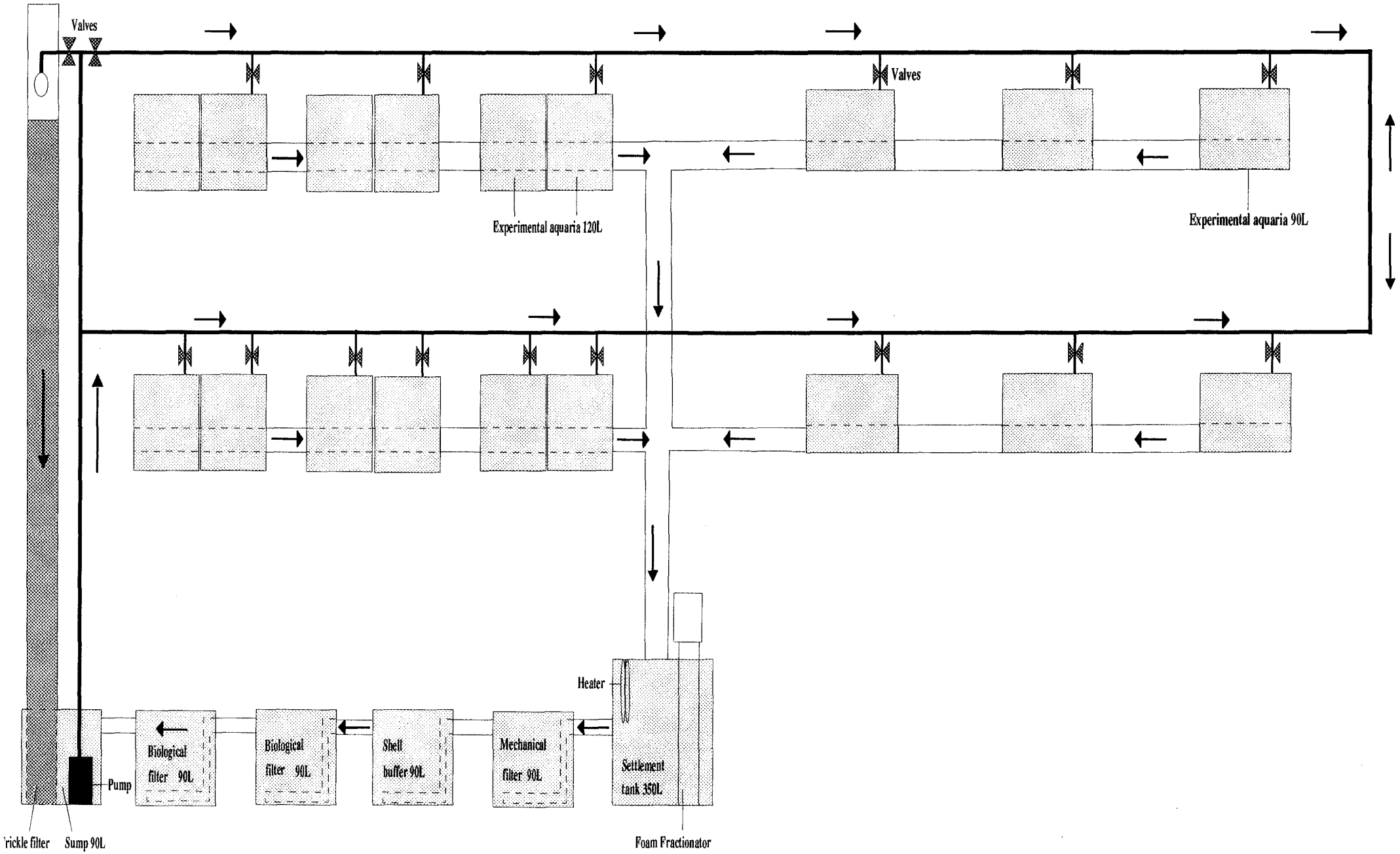

Figure 2.1: The Experimental System (arrows indicate direction of water flow). The system had a total volume of 2 900L and was situated in a room where temperature and photoperiod were controlled.

Ammonia-nitrogen (NH_4^+-N) was measured by means of the Salicylate method (Bower and Holm-Hansen 1980), nitrite-nitrogen (NO_2^--N) using the Diazotisation method (Boyd and Daniels 1988), and nitrate-nitrogen (NO_3^--N) by means of an aquarium test kit (Interpet Nitrate Test). NH_4^+-N values ranged from 0 - 0.01 mg/L; NO_2^--N ranged from 0.0 - 0.010 mg/L and NO_3^--N readings remained below 25 mg/L. pH and dissolved oxygen were measured weekly by means of electronic meters. pH ranged from 8,1 - 8,3 and dissolved oxygen from 6,7 - 7,1 mg/L.

The aquaria were isolated from one another by means of black plastic cardboard so that fish were not affected by different experimental photoperiods or by the fish in adjacent aquaria.

Shelter and substrata in all tanks were standardised. Substratum consisted of a thin layer of shale which could be vacuumed to remove debris. Shelter consisted of a 200 mm piece of 110mm diameter PVC pipe and a 200 mm concave piece of terra cotta tile to provide nesting sites. All tanks were covered with 1.5 mm clear acrilan plastic to minimise evaporation and to prevent the fish from jumping out of the tanks.

Aquaria were illuminated with single Biolux fluorescent tubes. These tubes have peaks in the 450 and 550 nm range of the spectrum. Light intensity was measured at the water surface and at 25 cm below the water surface. Mean intensities ± standard deviation were as follows:

Surface: 4.17×10^{15} quanta.sec^{-1}.cm^{-2}. ± 0.0103×10^{15}

Submerged: 1.25×10^{15} quanta.sec^{-1}.cm^{-2}. ± 0.0063×10^{15}

Diet and feeding regimen

The fish were fed a food consisting of blended sand-mussel (*Donax serra*), shrimp (*Penaeus indicus*), lettuce, (*Lactuca sativa*) and vitamin pre-mix and distilled water. Vitamins were added to the food at a ratio of 1:500 total weight as recommended by the manufacturer. The pre-mix used was developed and manufactured for marine ornamental fish by Vitamix (Pty) Ltd. The food was then frozen in ice-cube trays. A fresh batch of food was prepared every three weeks. The proximate composition of the diet (as % of dry matter) is shown in Table 2.1 Fish were fed to satiation twice daily after the food was allowed to thaw. The composition of the pre-mix is shown in Table 2.2

Table 2.1 Proximate analysis of diet fed to *A.akallopisos*. Values shown are in percentage of dry matter.

Component	Percentage composition
Protein	61,4 %
Lipid	4,8 %
Ash	13,7 %
Carbohydrate	20,1 %
Moisture	78 %
Energy value	19,526 MJ/Kg

Table 2.2 Composition of the vitamin pre-mix used in the diet.

Material	Amount per kilogram pre-mix
Biotin	0.75 g
Calpan	25.0 g
Choline	125.0 g
Folic acid	1.0 g
Inositol	50.0 g
Niasin	4.0 g
RSU	275.2 g
Vitamin A	5000 000 IU
Vitamin B1	5.0 g
Vitamin B12	0.005 g
Vitamin B2	7.5 g
Vitamin B6	7.5 g
Vitamin C	125.0 g
Vitamin D	750000 IU
Vitamin E	50000 IU
Vitamin K	9.0 g

Research approach and data collection

One of the aims of this project was to determine whether or not behaviour could be used to determine when spawning would take place. Under ideal conditions a control batch of fish would be sampled in order to correlate gonadal development and a gonado-somatic index (West 1990) with reproductive behaviour patterns. In a small fish species such as *A.akallopisos,* this technique would require the sacrifice of the fish. Sacrifice of broodstock *A.akallopisos* was not

possible due to the high costs of obtaining the fish and because of the restrictions placed on their collection. As *A.akallopisos* are kept in pairs and not in groups, large numbers of pairs would have to be maintained if individuals needed to be sacrificed. This would have required a major collecting effort and large holding facilities.

In vivo sampling of oocytes by means of cannulae has been used for the grey mullet, *Mugil cephalus* (Kuo, Nash and Shehadeh 1974) and Florida pompano, *Trachinotus carolinus* (Hoff, Rowell and Pulver 1972; Hoff, Mountain, Frakes and Halscott 1978). Cannulation involves the insertion of a hollow cannula into the uro-genital tract and collecting oocytes by way of suction. These can then be staged and prepared for histological examination. This technique obviates the need to sacrifice the fish. Becaused *A.akallopisos* only grows to a maximum size of 110 mm TL it was decided that even if it were possible to sample oocytes by means of cannulation, the fish would be severely stressed which might have affected their behaviour. For this reason it was decided not to use this method.

The gondadal development of male fish may be assessed by means of blood steroid assay (Chang, Hu and Tang 1992) or histologically (Fraille, Saez, Vicentini, Gonzalez, de Miguel and Panigua 1994). Steroid assay involves plotting the profiles of the plasma hormones testosterone, estradiol-17b and 17-a hydroxyprogesterone over the experimental period. A GSI can be calculated to complement the results obtained from both of the above methods. All these methods involves killing of the male fish.

It has been documented that reproduction has a narrower tolerance to stress than any other life function (Gerking 1980) and that stress has inhibitory effects on reproduction (Pankhurst and Van Der Kraak 1997). Because of this, the methods described involving regular handling or injection could stress the fish to the point that gonadal development is impaired.

All *Amphiprion* species form monogamous pairs and exhibit a variety of interactive behaviours leading up to, and including spawning (Allen 1972, Moyer and Bell 1976, Fricke 1979, Allen 1980). It was tested if behaviour could be used as a means of assessing development of *A.akallopisos* due to the nature of pair formation and breeding that occurs in all *Amphiprion.* Behaviour patterns were also used to assess the effect of various environmental conditions. While behaviour observation alone could not serve as a means of documenting gonadal development, it could serve as an important point of reference for the aquaculturist, upon which to predict when spawning would take place.

Only one study has been undertaken on the behaviour of *A.akallopisos* in the wild (Fricke 1979). In this study, undertaken at Aldabra, Fricke reported that they occur in small groups of 3-5 individuals. On the coral reefs of Northern Kwa-Zulu Natal where the study animals were caught, *A.akallopisos* occur predominantly in pairs and it was therefore decided to keep the fish in pairs.

Behaviour was recorded by means of a lap-top computer using an integrated system for event recording and data analysis. The programme was designed for use in behavioural research. Ten activities were identified from the literature. These behaviour patterns are shown in Table 2.3.

Allen (1972) identified behaviour as being either interactive or non-interactive. For the purposes of this study only interactive behaviours were recorded and all non-interactive behaviours were included under the "exploratory swimming" behaviour. For each behaviour recorded it was noted whether it was exhibited by the male or female fish. The exceptions were parallel and exploratory swimming and dorsal and ventral leaning. These behaviours were exhibited by both fish simultaneously. Each observation period lasted for ten minutes. Frequency of occurrence and duration of each event (in seconds) were recorded. Observations were made at the same time each day (2 hours after feeding) and at a distance of 2 metres from the aquarium. At no stage did the fish appear to be affected by the presence of the observer.

Table 2.3 Behaviour patterns generally exhibited by *A.chrysopterus, A.perideraion, A.tricinctus* (Allen 1972); *Amphiprion bicinctus* (Fricke 1983), *A.akallopisos* (Fricke 1979) and Pomacentrids (Reese 1964).

Behaviour	**Description**
Head shaking	Fish hangs in the water column and shakes head rapidly from side to side.
Chasing	One partner chases the other and the fish being chased avoids contact with the other.
Signal jumps	Fish swims up and down in display to mate.
Belly touching	Fish nuzzles mate's belly with its head.
Parallel swimming	Fish swim side by side without leaning to any one side.
Vertical swimming	Fish ascend together in the water column.
Dorsal leaning	While swimming parallel to each other, fish lean dorsally towards each other.
Ventral leaning	While swimming parallel to each other, the fish lean ventrally towards each other.
Nest cleaning	Biting of the substratum by either partner.
Exploratory swimming	All non-interactive behaviours exhibited by the fish.

CHAPTER 3

SPAWNING BEHAVIOUR OF *A. akallopisos*

Introduction

The only work published on the reproductive behaviour of *A. akallopisos* was a study conducted by Fricke (1979) at Aldabra. He investigated the mating system, use of anemones and sex change of the species. The specific reproductive behaviour patterns of *A. akallopisos* were not investigated in the study but they were described in general terms and were noted as being similar to the behaviour patterns described for other *Amphiprion* species (Allen 1972, Fricke 1974, Moyer and Bell 1976).

In captivity, the behaviour of many fishes differs from that in the wild. This is a response to the confined space, resulting in a change in social structures (Okuno 1963). For example, Okuno (1963) noted that *Amphiprion melanotus, A. xanthurus, A. chrysogaster* and *A. perideraion* exhibited more chasing behaviour in captivity than in the wild and Allen (1972) noted that *Amphiprion* species in captivity exhibit far more agonistic behaviour (even killing each other) in captivity than in the wild. In both these studies, however, only the general behaviour was considered. No studies have been undertaken specifically on the reproductive behaviour of clownfish in captivity, although anecdotal comments seem to indicate that reproductive behaviour in captivity is similar to that in the wild (Allen 1972, 1980; Alava and Gomes 1989; Henningsen 1989; Hoff 1996). The experiments undertaken during the course of this study were designed to describe the effect of various environmental conditions on the reproductive behaviour of *A. akallopisos* i.e. the behaviour leading up spawning as well as the spawning behaviour. To be able to assess the state of "spawning readiness" of *A. akallopisos* pairs under the various environmental conditions tested in this study, a

reference point against which the reproductive behaviour could be compared was needed. It was for this reason that the pre-spawning and spawning behaviour of *A. akallopisos* pairs was studied and quantified.

The primary objectives of the study were to describe the pre-spawning and spawning behaviour of *A. akallopisos* and to identify the specific behaviours that predict the onset of spawning.

Materials and methods

The spawning behaviour of *A.akallopisos* was recorded using a VCR camera. Recordings of spawning were made at Sea World Aquarium, Durban. One recording was made on a pair of *A.akallopisos* maintained in a 2 m^3 display aquarium. Two recordings of spawning behaviour were made on another two *A. akallopisos* pairs. These pairs were maintained in separate aquaria which measured 900x500x480 mm and had a volume of 216 L. In all three recordings the pairs were maintained with their host sea anemone, *Heteractis magnifica.* These recording were analysed using an integrated system for event recording and data analysis. The programme was designed for use in behavioural research

Direct observations were also made on five additional pairs of *A. akallopisos*. These fish were maintained in 900x500x480 mm aquaria with a volume of 216 L. The tanks were illuminated by single fluorescent tubes with wavelength peaks in the 400 and 500 nm range and an intensity of $4{,}15x10^{15}$ quanta.sec^{-1}.cm^{-2}. The aquaria received natural lighting for most of the day. Photoperiod was maintained at 13L:11D. Temperature was maintained at 26 $\pm 1^{0}$C and 20% of the water was replaced twice weekly. Salinity was 34 ppt, the ammonia-nitrogen and nitrite-nitrogen levels were

0 mg/L, and the nitrate-nitrogen level was 6 mg/L.

Behaviour patterns that could be used as predictors for spawning were identified from the literature (Allen 1972, Ross 1978, Fricke 1979) as well as from the behaviour patterns exhibited by spawning pairs. Eighteen observations were made on 5 breeding pairs from 3 days before spawning up until the egg-laying process. Observations were made in a random fashion at each session starting at 9:30 am. The average duration (± standard deviation) and frequency (± standard deviation) of all the behaviour patterns exhibited by *A.akallopisos* in 18 observations was calculated. Regression analysis was used to test for differences (at $p < 0.05$) in frequency and duration of a given activity as a function of time.

Results

Behaviour patterns not recorded were ventral leaning, signal jumping and chasing by the male fish (cf. Table 2.3). The behaviours recorded during the 3 days prior to spawning and including the day when spawning took place are shown in Figures 3.1 and 3.2. No trends were evident for the following behaviour patterns: parallel swimming, exploratory swimming, vertical swimming, dorsal leaning, chasing by the female, head shaking by the female, head shaking by the male. Regression analysis also showed no significant change ($p > 0.05$) over time for any of these behaviour patterns.

However, two behaviours were clearly identified as predictors for spawning. These behaviours were belly touching (male and female fish) and nest cleaning (male and female fish). Belly touching was initiated by the female on 7 occasions and by the male on 5 occasions ($n = 12$). Belly touching by the male fish was only recorded on the day prior to spawning. Belly touching by the female fish was

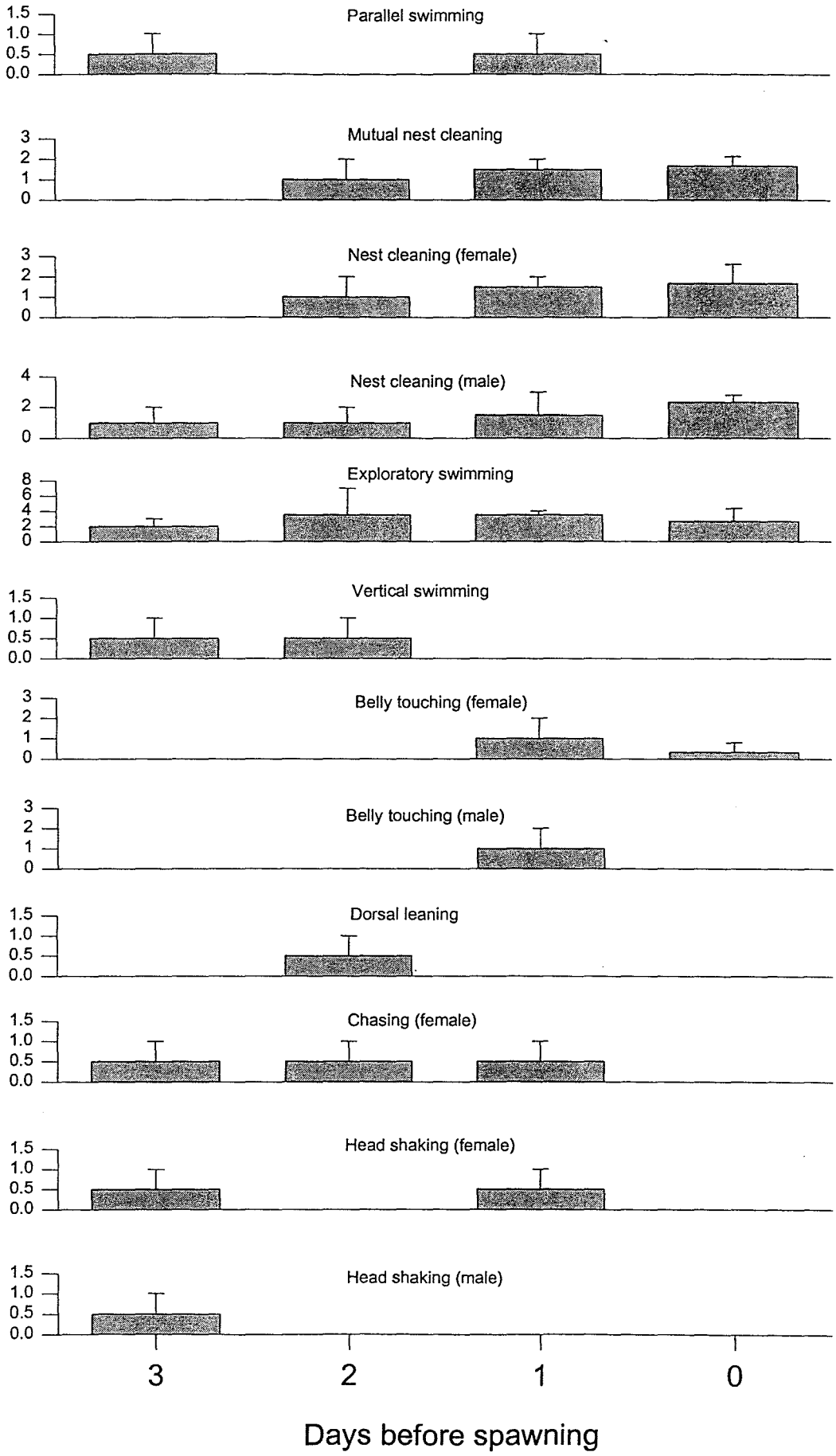

Figure 3.1. Time series graphs showing change in frequency of the behaviours exhibited of *A. akallopisos* in the days prior to spawning (n = 18). The bars show means and error bars indicate standard deviation for a ten-minute observation period.

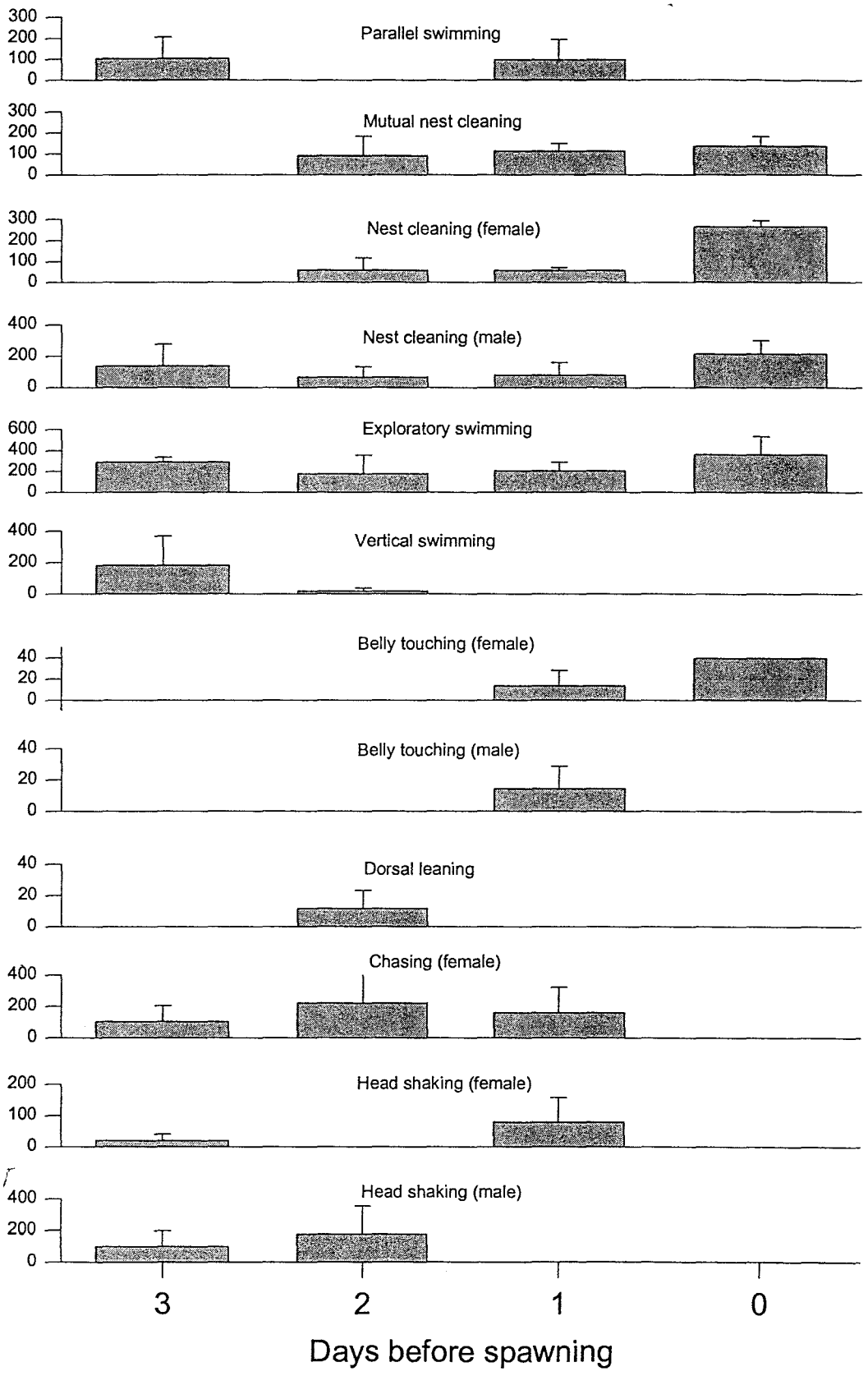

Figure 3.2 Time series graphs showing change in duration (in seconds) of the behaviours exhibited of *A. akallopisos* in the days prior to spawning (n = 18). The bars show means and error bars indicate standard deviation for a ten-minute observation period.

recorded on the day prior to, and on the day of spawning. Mutual nest cleaning was initiated by the male fish 12 times and by the female twice (n = 14). Mutual nest cleaning and nest cleaning by the

Table 3.1 The frequency and duration of those behaviour patterns that were identified as predictors for spawning in *A. akallopisos* (n = 18). Values shown are the means ± standard deviation for a ten minute observation period.

Behaviour	**Frequency**	**Duration (s)**
Belly touching (male)	1.11 ± 0.10	2.69 ± 1.52
Belly touching (female)	1.17 ± 0.12	9.51 ± 6.12
Nest cleaning (male)	2.56 ± 0.25	148.89 ± 33.51
Nest cleaning (female)	1.67 ± 0.22	95.26 ± 25.33
Nest cleaning (mutual)	0.77 ± 0.11	37.45 ± 9.36

female fish were recorded from the second day before spawning. Nest cleaning by the male fish occurred on all four days. All nest cleaning activities increased in frequency and duration up to spawning. On the day of spawning only three behaviours, viz. nest cleaning, belly touching and exploratory swimming were recorded. Regression analysis showed a significant increase ($p < 0.05$) over time for duration of nest cleaning by the female fish ($p \leq 0.0026$) and frequency of mutual nest cleaning ($p \leq 0.0037$) prior to spawning. No significant differences ($p > 0.05$) were found for any of the other predictors for spawning. For this reason the data from the 4 day experimental period

was pooled. The means are shown in Table 3.1. The values shown in Table 3.1 can be used as a reference point against which to compare behaviour patterns exhibited by non-spawning *A.akallopisos.*

Description of the spawning event

On the day of spawning nest cleaning was exhibited by both partners (see Figures 3.1 and 3.2 for mean frequency and duration of event). Belly touching, which involves nuzzling of the other fish's abdomen, was initiated by the female. The fish also chase each other in small circles above the nest site. The male fish exhibits a body quiver, a behaviour similar to head shaking. The fish hangs above the nest site, "shivers" and flaps its pectoral fins. This body quiver behaviour elicits a quivering response from the female. The white ovipositor of the female becomes visible 5 minutes before egg laying begins. The abdominal region of the female is often noticeably distended with eggs at this stage although with large females, gravid animals cannot always be distinguished by the size of the abdomen. In cases where spawning occurs adjacent to the host anemone, the fish bite the base and tentacles of the anemone causing it to shrink away from the area where the eggs are to be laid. In 15 attacks on the anemone, the tentacles were bitten 12 times and the base twice. Both partners attacked the host but on 12 occasions the male fish was responsible, compared to only 3 attacks by the female. In the community aquarium in Durban where one set of observations were made, other inmates of the aquarium were chased away from the spawning site by the female on three occasions and by the male once. Prior to egg-laying, the male and female fish swim over the surface of the nesting site as if making a "practise run". This activity is known as skimming. The spawning process begins with the female fish moving in slow circles over the nest site accompanied by fanning of the pectoral fins. The female lays a curved line of eggs and these are fertilised by the

male as he follows her in her circular movements. At the time of laying the eggs are colourless. The spawning process takes approximately thirty minutes ($\bar{x}$ = 28.12 minutes ± 11.88, n = 8). This appears to depend on the size of the clutch. Time of day of spawning was recorded for 12 spawnings. All occurred between 9h30 and 12h30. The male begins egg cleaning by mouthing and fanning the eggs almost immediately after fertilisation. The male spends more time tending the eggs (15% of a 10 minute observation period) than the female (0.66% of a ten minute observation period). The pair also engage in caring for the eggs simultaneously.

Incubation period

Out of 48 recorded hatchings, 32 occurred on the night of the eighth day (192 hours) and 16 on the night of the seventh day (168 hours). The average time taken was 184 hours ± 8 hours. No eggs were observed to hatch during daylight hours.

Discussion

Three behaviour patterns of *Amphiprion* species described in the literature (Reese 1964, Allen 1972, Moyer and Bell 1976, Fricke 1979) were not recorded in this study. These were; mutual ventral leaning, chasing by the male fish and signal jumping. Mutual ventral leaning is described as an interactive behaviour for three *Amphiprion* species by Allen (1972) but was not regarded as a precursor to spawning. Ventral leaning was also not identified as a pre-spawning behaviour for *A. melanopus* (Ross 1978), *A. clarkii* (Moyer and Bell 1976) or *A. akallopisos* (Fricke 1979). While ventral leaning was exhibited by *A. akallopisos* in experiments investigating the effect of

environmental factors on their behaviour, it was not a predictor of spawning activity.

Chasing by the male fish and signal jumping were included in the list of behaviour patterns for *A. akallopisos* because they were identified as important pre-spawning behaviours for pomacentrids in general (Reese 1964). In contrast to other pomacentrids however, the *Amphiprion* female is the dominant partner in the pair (Allen 1972), and therefore chasing behaviour by the male fish is unlikely to occur.

Signal jumping is considered a mate recognition behaviour in pomacentrids (Reese 1964). The effect of this behaviour pattern is to make the male visible to potential mates. In *Amphiprion* species, the pair bond is well developed and the species of this group are able to recognise their specific mates without this behaviour (Fricke 1973, Fricke 1974). This pair bond inhibits aggression between mates in *Amphiprion* species (Fricke 1973) and negates the need for elaborate displays such as signal jumping in order to attract spawning partners.

The behaviour patterns identified as predictors of spawning in this study, viz. belly touching and nest cleaning, were also recorded by Allen (1972), Fricke (1979), Ross (1978) and Moyer and Bell (1976) as definitive pre-spawning behaviour. Fricke (1979), regarded nest cleaning activities as the start of courtship. Moyer and Bell (1976), also found nest cleaning to be the major form of courtship, with the male initiating the behaviour and then joined in this activity by the female a few days prior to spawning. No details are given as to the timespan over which this occurs. Substratum

biting by the male fish can also be a displacement activity (i.e. an act of appeasement that occurs under conflict situations) brought about by agonistic displays by the female (Allen 1972, Moyer and Bell 1976). In experiments undertaken in this study investigating the effect of various environmental variables on the reproductive behaviour of *A. akallopisos*, males were recorded to bite the substrate in response to chasing by the females under conditions when spawning was not imminent. In these cases however, the immediate substratum was bitten and not a specific nesting site. Therefore, random substratum biting by the male fish should not be interpreted as a precursor to spawning. Repeated biting of a particular site by the male fish and particularly substratum biting by the female or by both partners are better indicators of pre-spawning behaviour. The frequency of nest cleaning activities in *Amphiprion* has been found to increase as spawning approaches (Allen 1972, Moyer and Bell 1976). In the studies by Allen (1972) and Moyer and Bell (1976), the frequency and duration of nest cleaning was not quantified. In the present study it was shown how the frequency and duration of nesting behaviours increases in the days prior to spawning. The values obtained can be used as a reference point for values obtained in experiments testing the effect of environmental variables on the reproductive behaviour *A. akallopisos*, in order to determine whether or not spawning is likely to occur. Since belly touching was only recorded in instances where spawning followed, the occurrence of this activity can be interpreted as a definitive pre-spawning behaviour of *A. akallopisos*. The fact that this activity occurs around the nesting site distinguishes it from other chasing activity.

The data presented in Figures 3.1 and 3.2 for the activities identified as predictors for spawning in *A.akallopisos* can be used to compare levels of these behaviours exhibited by this species in order to determine whether or not spawning is imminent. As not all the predictors showed a significant

change in levels of activity in the days preceding spawning, the means of the activities may also be used to compare general levels of activity in *A. akallopisos*. The frequency and duration of pre-spawning behaviour can also be used to show effects of different environmental conditions on behaviour patterns exhibited by *A.akallopisos*.

Agonistic activity has been shown to precede spawning behaviour in *A. chrysopterus*, *A. perideraion* and *A. melanopus* (Allen 1972, Moyer and Bell 1976). Agonistic activity includes aggressive chasing by the female, displacement behaviours such as head shaking and substrate biting by the male, and mutual behaviours such as ventral and dorsal leaning. Agonistic behaviour occurs more frequently among young or immature pairs and the extent of agonistic activity decreases as the pairs begin to breed (Moyer and Bell 1976). The interactive behaviours recorded in the study, but not identified as predictors for spawning, can be considered important indicators of the strength of the pair bond. The levels of agonistic activity obtained in this study did not change significantly in the days leading up to spawning. Although these activities cannot be used as predictors for spawning, the mean values obtained in this study can be used to compare the behaviour and the extent of agonistic activity exhibited by non-breeding *A. akallopisos*. The other two interactive behaviours recorded were vertical and parallel swimming. Vertical swimming was not a part of courtship as recorded for other pomacentrid species (Reese 1964). This activity seemed to be a natural form of swimming for *A. akallopisos* where the fish would rise into the water column above their shelter or anemone. Although the fish displayed this activity at the same time, no other interactive behaviours were noted in conjunction with this behaviour. Exploration of a prospective nesting site as well as normal side-by-side swimming were recorded as parallel swimming. Parallel swimming should be classed together with routine interactive events such as ventral and dorsal leaning i.e. as a definite

interaction between partners but not as a predictor for spawning.

The general pattern of spawning behaviour of *A. akallopisos* observed in this study did not differ from other accounts of *Amphiprion* spawning (Allen 1972, Moyer and Bell 1976, Fricke 1979). The only difference between our results and those of Fricke (1979) lay in brood care. In Fricke's (1979) study only male fish were observed to tend the eggs. In this study both partners cared for the brood, although the male was responsible for most of the care. This is consistent with the findings of Allen (1972), Reese (1964) and Moyer and Bell (1976). The incubation period of 7-8 days is identical to that recorded by Fricke (1979) for *A. akallopisos* in the wild.

The quantification of the spawning behaviour and of the behaviour in the days leading up to spawning has provided a reference point for comparison with the behaviour of *A. akallopisos* held under various environmental conditions.

CHAPTER 4.

EFFECT OF PHOTOPERIOD AND LUNAR PERIODICITY ON THE REPRODUCTIVE BEHAVIOUR OF *A. akallopisos*

Introduction

The reproductive cycle of most fishes is under the control of an endogenous physiological rhythm and an exogenous seasonal rhythm (Lam 1983). The refractory period in the reproductive cycle is the time when these two rhythms coincide and reinforce each other. As fish are exposed to changing environmental conditions the exogenous rhythm begins to dominate and its influences on the reproductive processes are transmitted by changes in the quantity of gonadotropin released from the pituitary gland (Lam 1983). Temperature and photoperiod are the two principal factors involved in regulating gonadal development (Lam 1983).

Only photoperiod was investigated in this study since the temperature at which most *Amphiprion* species breed is well defined. Although *Amphiprion* can breed at temperatures between 22 and 31°C (Hoff 1996), at least six commercial hatcheries maintain *Amphiprion* broodstock at temperature between 26 and 28°C (Henningsen 1989, Juhl 1992, Davis 1993, Hoff 1996). The experimental system used in this study was a recirculating system (described in Chapter 2). Temperature could only be controlled for the whole system and the number of broodstock pairs available was not sufficient to investigate the effect of two factors such as temperature and photoperiod simultaneously. Seasonal influence on breeding patterns in populations of *A. clarkii* have been documented in moderate (33-34°N) northern latitudes (Moyer and Sawyers 1973,

Moyer and Bell 1976, Ochi 1985, 1989). In the Northern hemisphere the breeding season lasts from late May/early June to early October, i.e. spring/summer. Changes in behaviour were not recorded. Photoperiod appeared to play a more important role than temperature as the temperature was higher at the end of the spawning season than at the onset, and remained high after spawning ceased while photoperiod decreased as winter approached (Moyer and Bell 1976, Ochi 1985). This indicates that photoperiod, rather than temperature should be investigated as a factor in conditioning *Amphiprion*.

There is no scientific literature concerning the effect of photoperiod on the gonadal development or breeding behaviour of *Amphiprion* species in captivity. The available literature is mostly of a popular nature which merely describes what photoperiod was used to achieve spawning. Goldstein (1989) advocates low-wattage, fluorescent lighting that is run continuously for 24 hours a day for conditioning of *A. ocellaris* and *A. melanopus*. Neither the spectrum nor the intensity used was mentioned despite the fact that Goldstein (1989) claims that intensity is more important than spectrum. The ADAS hatchery in Norway maintains a 12L:12D photoperiod with fluorescent lighting (Juhl 1992), but no details are given about spectrum, intensity or type of fluorescent tube used. Allen (1980) also advises maintaining a natural photoperiod similar to that of the tropics i.e. 12-14L:10-12D. This natural light regime is also employed by Dynasty Marine Hatchery in the U.S.A (Henningsen 1989). In addition to fluorescent tubes the Dynasty Marine Hatchery also makes use of metal halide lamps to provide a full spectrum of lighting at high intensities. Frakes and Hoff (1983) and Hoff (1996) advocate a 14L:10D photoperiod for conditioning marine tropical fish (such as clownfish) that spawn for extended periods.

In other fishes such as the ayu, *Plecoglossus altivelis*, a winter spawning fish, spermatogenesis and vitellogenesis are stimulated by low temperature (18°C) and short photoperiod (8L) (Chang *et al.* 1992). In the Mosquito fish, *Gambusia affinis holbrooki*, a fish which normally undergoes gonadal recrudescence in spring, spermatogenesis was stimulated by higher temperatures and long photoperiods (16L:8D) (Fraile *et al.* 1994). In rainbow trout, *Onchorhynchus mykiss,* early vitellogenesis is stimulated and advanced by long photoperiods, while later oocyte development and ovulation is best controlled by short photoperiods (Duston and Bromage 1986). In Atlantic salmon, *Salmo salar,* gonad development was enhanced by accelerating the natural seasonal light and temperature cycles from yearly to six- month-periods (Johnston *et al* 1992). In the Gulf croaker, *Bairdiella icistia,* gonad maturation was accelerated in female fish by means of maintaining higher temperatures (22 °C) and longer photoperiod (16L) in addition to hormonal manipulation (Haydock 1971).

Vitellogenesis and spawning has been achieved in red drum, *Sciaenops ocellata,* during the refractory period by condensing the natural light cycle by a factor of three experimental days to one natural day (Roberts *et al.* 1978). An abbreviated decelerating regime, condensed by a factor of 3:1 achieved the best results (83 days) as opposed to a regime of decelerating temperature and photoperiod after preconditioning with 16 and 10 hours light and 24°C, which induced spawning in 129 and 117 days respectively. This sciaenid species, like clownfish, also has the ability to spawn year round (Arnold 1991). Hoff *et al* (1978a) conditioned Florida pompano, *Trachinotus carolinus,* by condensing natural photothermal regimes by a factor of five and three natural days respectively, to one experimental day. Photoperiod and temperature data were collected from the natural habitat of the species and these regimes were manipulated in the laboratory. A

significantly greater amount of fish kept under the condensed photothermal regimes attained pre-spawning condition compared to the control fish, which were kept under ambient conditions. Kuo *et al* (1974) determined that vitellogenesis in the winter-spawning grey mullet, *Mugil cephalus* could be brought about by means of a short photoperiod regime (6L/18D), irrespective of conditioning photoperiod.

The aim of this experiment was to investigate the effects of photoperiod on the interaction of *A. akallopisos* pairs. Interaction was of importance in determining the strength of the pair bond as spawning could not take place in pairs where the pair bond is not well established. Photoperiod was investigated as an environmental factor that may influence the development of the pair bond in *A. akallopisos* and ultimately lead to spawning. The hypothesis for the experiment was as follows: Photoperiod would have no effect on frequency and duration of interactive behaviour patterns within *A. akallopisos* pairs.

In several *Amphiprion* species spawning events have been related to lunar cycles. Peaks in spawning activity around the full moon have been documented for *A. chrysopterus*, *A. perideraion* (Allen 1972) and *A. bicinctus* (Fricke 1974). In contrast, Ross (1978) found *A. melanopus* to have spawning peaks around the half moon phases. Ochi (1985) found spawning to be affected by lunar periodicity in *A.clarkii* for one year but could show no periodicity for the same population the following year. Since lunar periodicity has been shown to affect spawning in some *Amphiprion* species, and since *A. akallopisos* were observed to be spawning regularly in two separate systems under natural lighting, spawning dates of *A. akallopisos* maintained under these conditions were examined in order to determine if spawning followed a lunar pattern.

Given that the experiments involving the behaviour of *A. akallopisos* under various environmental conditions were undertaken in a laboratory where photoperiod was controlled and no external light entered the room, it is possible that the absence of a lunar cycle may have been a factor in preventing *A.akallopisos* from spawning. The objective of this investigation whether spawning of *A. akallopisos* followed a lunar cycle.

Materials and methods

Twelve pairs of *A. akallopisos* were housed in glass aquaria with one pair in each tank. The tanks were part of the recirculating system described in Chapter 2. The fish were conditioned for a period of three weeks prior to the start of the experiment. During the conditioning period they were maintained at a temperature of 26 ±1 °C and a photoperiod of 12L:12D. Six aquaria of two sizes each were used in the experiment with two tanks of each size held at each of the three photoperiods, resulting in a 3x2x2 factorial design (3 photoperiods x 2 tank sizes x 2 replicates). The behavioural programme described in Chapter 2 was used to make all observations in this experiment. Observations were made for 10 minutes/tank and the following behaviours were recorded; head shaking, chasing, nest cleaning, ventral leaning, dorsal leaning, parallel swimming, vertical swimming and exploratory swimming. All aquaria were observed every three days for a period of 66 days. On the day of observation the sequence of tanks to be checked was randomly chosen. A one-way Analysis of Variance was used to test for differences between treatment means. Tukey's multiple range test was used to identify where the differences between treatments occurred. Regression analysis was used to test if changes in frequency or duration of an activity were a function of time.

The two aquarium sizes were 900x350x300 mm (94,5 L) and 600x500x380 mm (114 L) by volume. Three photoperiods were tested in this study. The choice of photoperiod was based on the natural photoperiod for Sodwana Bay (27° 14'S) in the Northern Kwa-Zulu area, from which the fish were collected. Photoperiod data was obtained for the Sodwana Bay area from the S.A. Meteorological Office in Richards Bay and used in the experiment. The first photoperiod tested (P1) was the mid-summer photoperiod for Sodwana Bay - 14L:10D. The second photoperiod chosen was the and mid-winter photoperiod for Sodwana Bay - 10h,15minL:13h,45minD. The third photoperiod tested was an accelerated seasonal photoperiod, which consisted of a years photoperiod condensed into the three-month experimental period ie. 4 natural days = 1 experimental day.

The aquaria were isolated from one another with black plastic cardboard to avoid the fish from being affected by light from other photoperiods or by the activity of fish in adjacent aquaria. The front panes were covered by thick black sheeting before the start of the controlled dark period to avoid light from other photoperiods entering the aquarium. Tank decorations and the substratum in all tanks were standardised. The substratum consisted of a thin layer of shale, the surface of which could be vacuumed to remove debris. Decoration consisted of a single 200 mm piece of 110 mm diameter PVC pipe and a 200 mm section of a concave terra cotta tile to provide nesting sites. All tanks were covered with clear 1,5-mm acrilan plastic to prevent the fish from jumping out and to reduce evaporation. Flow rates were kept constant in all aquaria at 5 L/min which resulted in a turnover time of 23,8 minutes for the 114 L tanks and 18,9 minutes for the 94,5 L aquaria.

Water temperature was kept at 26 °C ± 1 °C for the duration of the experiment. Salinity was kept constant at 34 ppt ± 1 ppt with the addition of rain water. Ammonia-nitrogen (NH_4^+-N) levels ranged from 0 - 0.01 mg/L, nitrite-nitrogen (NO_2^--N) levels from 0.0 - 0.010 mg/L and nitrate-nitrogen (NO_3^--N) levels remained below 25 mg/L. pH ranged from 8,1 - 8,3 and dissolved oxygen from 6,7 - 7,1 mg/L.

Each aquarium was illuminated by means of a single Biolux fluorescent tube. These tubes have peaks in the 450 and 550 nm range of the spectrum. Light intensity was measured at the surface and at 25 cm below the water surface. The intensities measured are shown in Table 4.1.

Table 4.1. The light intensity (in quanta $sec.^{-1}$ $cm.^{-2}$) in the experimental aquaria measured at the surface and at 25 cm depth.

	Mean	Std. Deviation
Surface	$4{,}017 \times 10^{15}$	0.0103×10^{15}
Submerged (25-cm depth)	$1{,}85 \times 10^{15}$	0.0063×10^{15}

The study investigating the effect of lunar cycle on spawning was based on 66 spawnings of *A. akallopisos*. Fifty-six of these spawnings occurred in Durban and 10 in Grahamstown. None of the fish used in the photoperiod study were used in this study. Results of the lunar cycle study were analysed using chi-square statistics (Zar 1984) with the null hypothesis that spawning did not follow a random pattern.

Results

Analysis of variance showed no significant differences in frequency or duration of behaviour patterns between the two tank sizes for any of the activities recorded. Regression analysis also showed no significant ($p > 0.05$) increase or decrease in frequency or duration of any behaviour pattern over the experimental period. For these reasons the results obtained for both tank sizes were pooled before the effect of the photoperiods were analysed.

The following behaviours were not recorded during the observations: belly touching by either partner, cleaning of a nesting site by the female, mutual nest cleaning, chasing by the male fish or signal jumping. None of the pairs were therefore ready to spawn.

Different photoperiods did however have a significant effect on the frequency and duration of exploratory swimming, chasing by the female fish and nest cleaning by the male. These differences lay between treatments P1 (mid-summer photoperiod) and P3 (mid-winter photoperiod) for frequency and duration of chasing by female fish, frequency and duration of nest cleaning by the male fish, and frequency and duration of exploratory swimming. Mean values for frequency and duration of nest cleaning by the male fish and duration of chasing by the female fish under treatment P2 (accelerated seasonal photoperiod) were significantly different from those of P3 (mid-winter photoperiod) (Figures 4.1- 4.3).

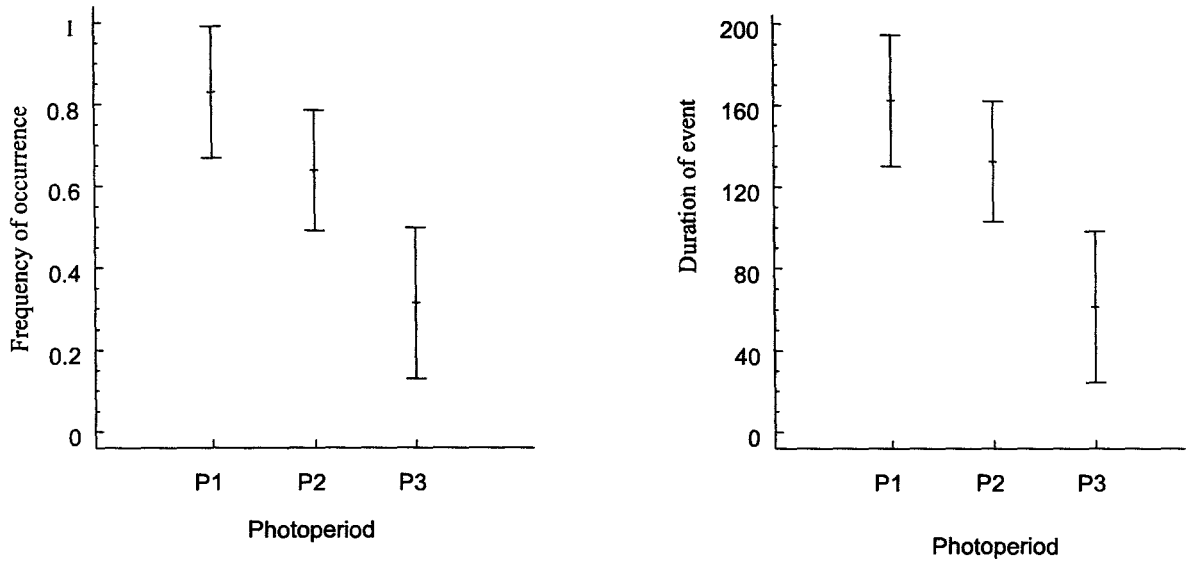

Figure 4.1. Frequency and duration (in seconds) of chasing by the female fish. Values shown are the means for a ten-minute observation period (n = 60). Error bars indicate 95 % confidence levels. (P1 = 10L:14D; P2 = accelerated seasonal photoperiod; P3 = 10h, 15 minL:13h,45minD).

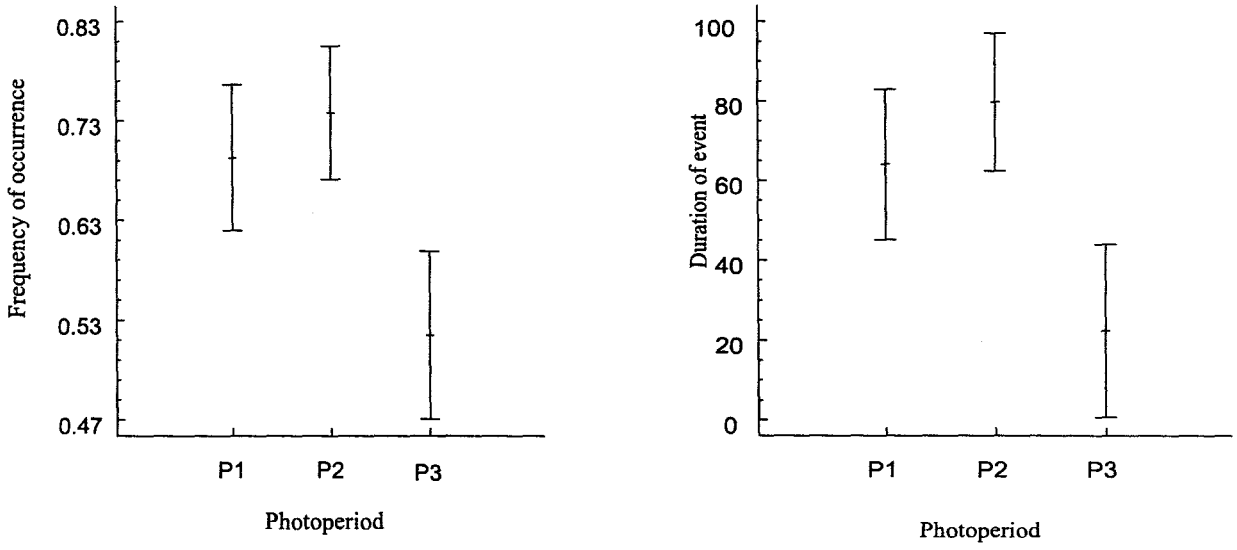

Figure 4.2. Frequency and duration (in seconds) of exploratory swimming. Values shown are the means for a ten-minute observation period (n = 60). Error bars indicate 95 % confidence levels. P1 = 10L:14D; P2 = accelerated seasonal photoperiod; P3 = 10h, 15 minL:13h,45minD).

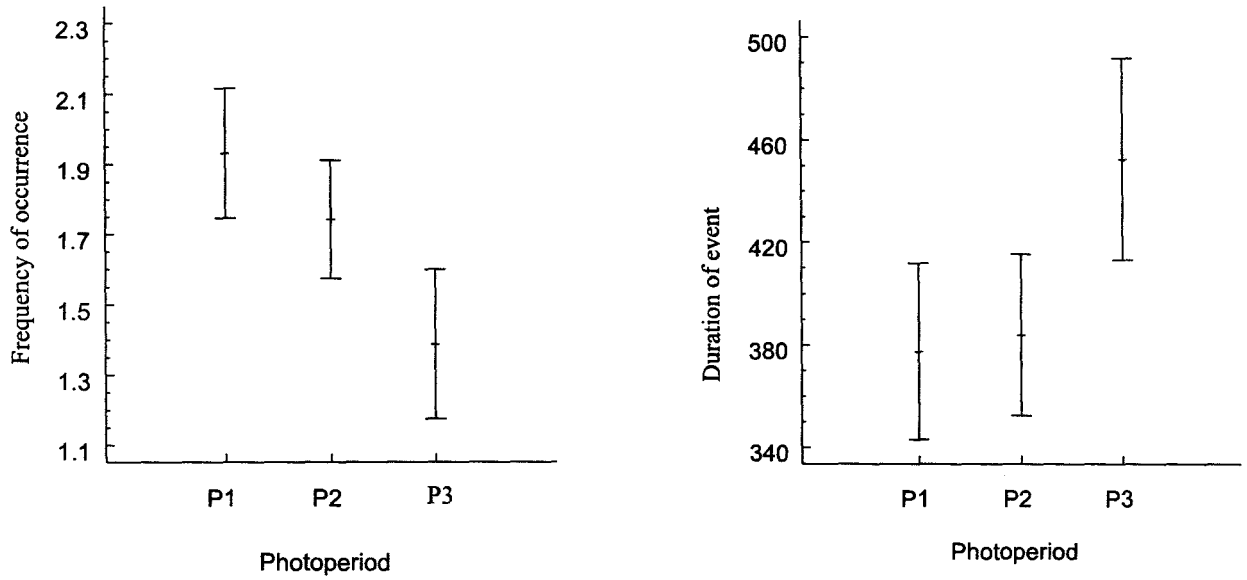

Figure 4.3 Frequency and duration (in seconds) of nest cleaning by the male fish. Values shown are the means for a ten-minute observation period (n = 60). Error bars indicate 95 % confidence levels (P1 = 10L:14D; P2 = accelerated seasonal photoperiod; P3 = 10h, 15 minL:13h,45minD).

Mean values for behaviours for which no significant differences were obtained are shown in Table 4.2. The results of the regression analyses are shown in Tables 4.3-4.5 A T-test showed the means obtained for frequency of nest cleaning (0.69 ± 0.05, 0.74 ± 0.47, 0.51 ± 0.02) and duration of nest cleaning (42.07 ± 11.79, 57.82 ± 13, 0.80 ± 0.8 sec) in this study to be significantly lower ($p < 0.05$) from levels of this activity exhibited by spawning pairs (2.56 ± 0.25, 148.89 ± 33.51 sec).

Effect of lunar phase on spawning of *A. akallopisos*.

The distribution of the total number of spawnings was not significantly different ($p < 0.05$; $\times^2 = 3.841$) from an even distribution (n = 66). No significant peaks in egg-laying occurred

Table 4.2. Mean values for frequency and duration of behaviour patterns recorded for which no significant differences were obtained between photoperiods. ($p > 0.05$; $n = 60$). Values shown are for a 10-minute observation period. P 1 = 10L:14D; P2 = accelerated seasonal photoperiod; P3 = 10h, 15 minL:13h,45minD.

Behaviour pattern	P1Frequency ± SD	P1 Duration (sec) ± SD	P2 Frequency ± SD	P2 Duration (sec) ± SD	P3 Frequency ± SD	P3 Duration (sec) ± SD
Head shaking (male)	0.69 ± 0.04	68.10 ±17.02	0.73 ±0.04	76.30 ±15.46	0.69 ±0.05	49.18 ±16.63
Head shaking (female)	0.52 ±0.06	13.03 ±8.82	0.54 ±0.09	13.74 ±7.51	0.54 ±0.02	11.95 ±8.69
Dorsal leaning	0.58 ±0.03	19.11 ±8.36	0.52 ±0.01	9.75 ±6.63	0.56 ±0.03	13.00 ±7.20
Ventral leaning	0.63 ±0.03	45.42 ±14.35	0.55 ±0.02	14.33 ±7.92	0.56 ±0.03	23.46 ±11.94
Vertical swimming	0.52 ±0.06	11.36 ±	0.48 ±0.01	1.55 ±0.55	0.53 ±0.02	16.38 ±11.23
Parallel swimming	0.68 ±0.03	20.23 ±8.68	0.65 ±0.04	36.75 ±10.12	0.65 ±0.05	63.04 ±20.32

Table 4.3. Results of regression analysis of Photoperiod 1 (14L:10D) for change in behaviour of *A.akallopisos* over a 75 day period. (n= 104).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.31	1.23
	duration	0.76	0.11
Head shaking (female)	frequency	0.80	0.07
	duration	0.83	0.05
Chasing by female	frequency	0.07	4.10
	duration	0.09	3.45
Dorsal leaning	frequency	0.42	0.75
	duration	0.77	0.11
Ventral leaning	frequency	0.56	0.39
	duration	0.61	0.30
Vertical swimming	frequency	0.35	1.01
	duration	0.31	1.18
Exploratory swimming	frequency	0.61	0.76
	duration	0.34	1.24
Nest cleaning (male)	frequency	0.99	0
	duration	0.61	0.31
Parallel swimming	frequency	0.71	0.16
	duration	0.92	0.01

Table 4.4. Results of regression analysis of Photoperiod 2 (accelerated seasonal regime) for change in behaviour of *A.akallopisos* over a 75 day period (n = 104).

Behaviour pattern		p value	r^2
Head shaking (male)	frequency	0.17	2.65
	duration	0.10	3.19
Head shaking (female)	frequency	0.63	0.27
	duration	0.19	2.02
Chasing by female	frequency	0.07	3.91
	duration	0.21	1.85
Dorsal leaning	frequency	0.07	3.73
	duration	0.10	3.16
Ventral leaning	frequency	0.11	3.32
	duration	0.06	4.33
Exploratory swimming	frequency	0.07	3.99
	duration	0.38	0.91
Nest cleaning (male)	frequency	0.15	2.45
	duration	0.67	0.22
Parallel swimming	frequency	0.12	2.72
	duration	0.26	1.50

Table 4.5. Results of regression analysis of Photoperiod 3 (10h15minL:13h45minD) for change in behaviour of *A.akallopisos* over a 75 day period (n = 104).

Behaviour pattern		**p value**	**r^2**
Head shaking (male)	frequency	0.26	1.5
	duration	0.07	3.83
Head shaking (female)	frequency	0.86	0.04
	duration	0.28	1.33
Chasing by female	frequency	0.07	3.82
	duration	0.18	2.03
Dorsal leaning	frequency	0.33	1.09
	duration	0.26	1.52
Vertical swimming	frequency	0.09	3.29
	duration	0.11	2.91
Exploratory swimming	frequency	0.76	0.15
	duration	0.06	4.23
Nest cleaning (male)	frequency	0.73	0.14
	duration	0.54	0.45
Parallel swimming	frequency	0.08	3.50
	duration	0.06	4.20

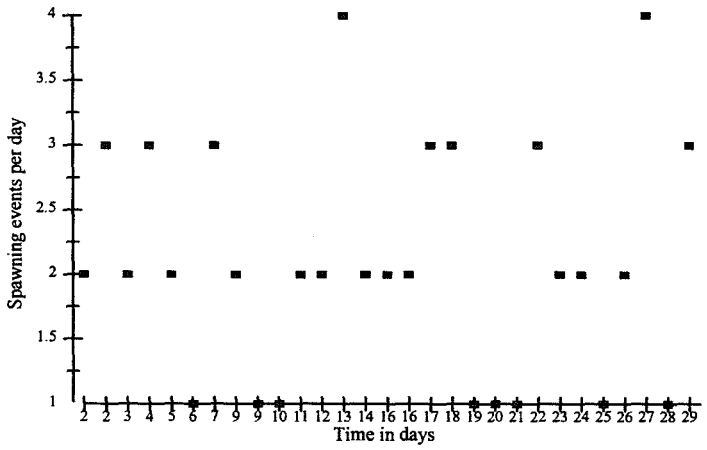

Figure 4.4 The number of spawning events of 6 pairs of *A. akallopisos* during a lunar month. New moon occurred on day 2, first quarter on day 9, full moon on day 16 and last quarter on day 23. (N = 66).

between 1 and 6 days before or after either full moon or new moon. No peaks occurred around half-moon as opposed to full or new moon for 1, 2 and 3 days before and after each phase. Figure 4.4 shows the distribution of spawns over a lunar month. The results show that the spawning of *A. akallopisos* did not follow a lunar cycle.

Discussion

The reasons for the absence of chasing by the male fish and the absence of signal jumping are discussed in Chapter 3. Chasing by the male fish is unlikely to occur as the female is the dominant partner in the relationship. Signal jumping is a display used by male pomacentrids to attract mates but the monogamous pair bond in *Amphiprion* negates the need for this activity.

The other behaviours not recorded (mutual nest cleaning, nest cleaning by the female, belly touching) are all behaviours identified as predictors of spawning in Chapter 3, and are associated with the final stages of courtship prior to spawning (Allen 1972, Fricke 1979, Moyer and Bell 1976). The absence of these behaviour patterns suggests that spawning was not imminent in any of the pairs under any of the three photoperiods. The fact that none of these behaviours were recorded under any of the three photoperiods indicated that none of the photoperiods used served as a key factor in inducing spawning. This is further evidenced by the fact that no significant trends in levels of any activity were recorded over the experimental period.

Nest cleaning by the male fish is both a displacement activity and a courtship behaviour (Allen 1972, Moyer and Bell 1976). Fricke (1979), recorded nest cleaning by the male as the start of

courtship in *A. akallopisos*. Moyer and Bell (1976), also found nest cleaning to be the major form of courtship in *A.clarkii*, with the male initiating the behaviour and only being joined in the activity by the female within a few days of spawning. The fact that nest cleaning in this study was not accompanied by nest cleaning activity by the female or mutual nest cleaning indicates that "nest cleaning" was more likely to be random substratum biting rather than actual nest cleaning.

The results of this study show differences in mean frequency and mean duration of interactive behaviours between the mid-summer and accelerated regimes, and the mid-winter photoperiod. The activities for which significant differences were recorded viz. chasing by the female and nest cleaning by the male, are regular behaviours of established *Amphiprion* pairs (Allen 1972, Fricke 1979) and can be considered as indicators of interaction and pair bonding. The total extent of non-interactive behaviour can be obtained from the frequency and duration of exploratory swimming. Any time not spent in exploratory swimming was spent in various interactive behaviours. Fish maintained under the mid-summer photoperiod exhibited significantly more overall interaction than those maintained under the mid-winter photoperiod. Although the frequency and duration of total interactive behaviour between the accelerated photoperiod and the mid-winter photoperiod was not significantly different, the fish maintained under the mid-summer photoperiod exhibited more interactive behaviour than the fish kept under the accelerated photoperiod. Seasonal influence on breeding patterns in populations of *A.clarkii* have been documented in moderate (33-34°N) northern latitudes (Moyer and Sawyers 1973, Moyer and Bell 1976, Ochi 1985, 1989). Like *A.clarkii*, *A. akallopisos* may have a condensed breeding season at moderate latitudes in contrast to tropical populations that spawn

throughout the year. If so, a constant mid-summer photoperiod or accelerated photoperiod is equally likely to condition *A. akallopisos* if compared to a mid-winter photoperiod. It is also possible that a short photoperiod approximating the winter photoperiod regime may have inhibited behavioural interaction and thus gonadal development.

Accelerated seasonal photoperiods have proved to be successful in conditioning other marine sub-tropical species for spawning, such as red drum, *Sciaenops ocellatus* (Roberts 1978, Arnold 1991), pompano, *Trachinotus carolinus* (Hoff *et al.* 1972), black sea bass, *Centropristes melanus* (Hoff *et al.* 1973), and sea bass, *Dicentrarchus labrax* (Zanuy *et al.* 1986). These species are seasonal spawners except for the red drum which has the ability to spawn year round. An accelerated seasonal photoperiod has the effect of inducing gonadal development out of season or conditioning the species in a time period shorter than their annual breeding cycle. If *A. akallopisos* has a condensed breeding season at moderate latitudes (e.g. Sodwana Bay), then an accelerated seasonal photoperiod could prove successful in conditioning this species for spawning. Although the pairs maintained under the accelerated photoperiod exhibited significantly more interaction than those maintained under the mid-winter photoperiod, there were no significant differences in frequency or duration of any behaviours between the condensed photoperiod and the mid-summer regime. As spawning of *A.akallopisos* is desired on a continual basis a constant photoperiod is more suitable than a condensed photoperiod after spawning has been achieved. For day-to-day hatchery management a constant photoperiod is also more practical and the available literature indicates that all commercial *Amphiprion* hatcheries employ constant photoperiods (Henningsen 1989, Davis 1993, Hoff 1996).

Constant long photoperiods have been successful in advancing spawning in both warm and cold-water fish. For example, spawning was advanced by three months in the common carp, *Cyprinus carpio* (Davies and Hanyu 1986), by two months in the rainbow trout, *O.mykiss* (Duston and Bromage 1986) and advanced by three months in the gulf croaker, *Bairdiella icistia* (Haydock 1971). Although the fish did not spawn under the mid-summer photoperiod in this study, the fish maintained under this regime exhibited significantly more interaction than those maintained under the mid-winter photoperiod. The greater amount of interaction exhibited by *A. akallopisos* in this study support Frakes and Hoff's (1983) and Hoff's (1996) recommendation of a 14L:10D regime for conditioning of *Amphiprion* species. In contrast to studies using accelerated seasonal regimes where the objective is to delay or advance the spawning process, the objective in conditioning fish with a highly protracted spawning season such as *Amphiprion* species, is to induce spawning on a continuous basis. For this reason a constant photoperiod is more suitable than a changing regime. If *A.akallopisos* has a seasonal spawning period at moderate lattitudes, it is likely to occur during the spring-summer season. Because a long photoperiod favours gonadal development in species which spawn in spring or summer (Baggerman 1980), and because of the levels of interaction exhibited by *A. akallopisos* in this study, the 14L:10D regime was considered to be the most suitable photoperiod for conditioning this species.

Several factors may have been responsible for the absence of spawning in *A. akallopisos*. It is possible that either some other environmental cues were missing or that the pairs had not settled down sufficiently in captivity to proceed to spawn. Stress has inhibitory effects on reproduction in every species in which the relationship has been examined (Pankhurst and Van

Der Kraak 1997). Reproduction also has a narrower tolerance to stress than any other life function (Gerking 1980, Schreck, Olla and Davis 1997). The stress of collection, handling, transport and conditions in a captive environment may have caused inhibition of spawning during the experimental period. Anecdotal information seems to indicate that adult *Amphiprion* species also take longer to settle down than juveniles, and that the presence of an anemone may also help the fish to acclimatise to captivity (Davis 1993). The fact that the *A. akallopisos* pairs used in this study were adults and were not maintained with anemones could have lengthened the time needed to acclimatise and to begin breeding.

Although the influence of lunar phases on spawning has been documented in various *Amphiprion* species (Allen 1972, Fricke 1974, Ross 1978, Ochi 1985), patterns vary between species. Lunar periodicity of spawning in *A. akallopisos* has not been investigated in the wild, and this study has shown that there is no lunar synchronicity between lunar periodicity and spawning in captivity. Lunar influence on the spawning of *A. akallopisos* can therefore be discounted as a possible reason for inhibiting spawning in *A. akallopisos* kept under controlled light conditions.

CHAPTER 5.

EFFECT OF GONADOTROPIN HORMONE RELEASING HORMONE ON THE REPRODUCTIVE BEHAVIOUR OF *A.akallopisos.*

Introduction

The environmental control of gametogenesis involves both long-term effects on the gametogenetic process and acute effects on the processes of spermiation, ovulation and spawning (Zohar 1989). Fish kept in captivity often fail to spawn because some environmental cue is missing from the conditions under which they are held. By manipulating the long term environmental cues gametogenesis may be induced but the final stages of spermiation or ovulation and spawning may still not occur. In these cases hormonal manipulation has been widely used to provide the short-term trigger for the spawning process (Zohar 1989).

One of the most successful hormone groups employed in aquaculture are the synthetic analogues of the gonadotropin hormone releasing hormone - GnRHa (Zohar 1989). GnRHa stimulates the hypothalamus to release gonadotropin (GtH), the hormone responsible for final spermiation, ovulation and spawning. Although injection of GtH has proved successful in inducing spawning, GnRHa has several advantages over GtH . If the treatment is not effective gonadotropin is not released from the pituitary gland and no accumulation of GtH occurs in the blood stream. The oocytes also remain intact and atresia is avoided. GnRHa analogues are also non-immunogenic and have a low degree of biological specificity. To increase the potency of GnRHa, it is combined with a dopamine receptor antagonist such as pimozide or domperidone (Zohar 1989).

There are various methods of applying GnRHa. The analogue solution has been effectively applied by injection (Mylonas, Hinshaw and Sullivan 1992; Glubokov, Kouril, Mikodina and Barth 1994; Pankhurst and Carragher 1995). To reduce broodstock loss due to handling stress caused by repeated injections, methods using implantation of micropellets were developed. Micropellets may be implanted intramuscularly (Marte, Sherwood, Crim and Harvey 1987; Harmin and Crim 1992; Linhart and Billard 1994; Mylonas, Zohar, Richardson and Minkkinen 1995), or intraperitoneally (Marte *et al.* 1988; Harmin and Crim 1992; Hodson and Sullivan 1993). Implantation of micro-pellets also allows for a constant release of GnRHa to be administered over a period of time (Mylonas *et al.* 1995a).

The micro-capsules used in this study were water insoluble pellets developed by the Laboratory for Ecology and Aquaculture of the Catholic University of Leuven, Belgium, for incorporation in the diet. Oral administration of GnRHa has been successfully applied in spotted seatrout, *Cynoscion nebulosus* (Thomas and Boyd 1989) and Thai carp, *Puntius gonionotus* (Sukumasavin. Leelapatra, McLean and Donaldson 1989). This application method is likely to be more suitable than injection or pellet implants for small fish such as *A. akallopisos* which attains only 110 mm TL for females and 70 mm TL for males (Smith and Heemstra 1986).

There is a high degree of variation in the amounts of GnRHa applied in the various studies. Mylonas *et al.* 1992 induced spawning in *Salmo trutta* with two injections of 10 μg GnRHa /kg three days apart. Peter *et al.* 1988 obtained spawning success in 6 species of carp with injections of 100 μg GnRHa /kg. Spermiation was induced in the European catfish with GnRHa pellets implanted intramuscularly at doses of 13 and 25 μg/kg (Linhart and Billard 1994). Mylonas *et al.*

1995b used doses of 117 μg/kg for males and 77 μg/kg for female American shad *Alosa sapidissima* in order to induce spermiation and ovulation. Glubokov *et al.* (1994) induced gonadal maturation in Pacific mullet with 25 and 50 μg/kg injections 4 and 28 hours respectively after injection of dopamine antagonists. Gonadal maturation, ovulation and spawning was induced in the flounder *Pseudopleuronectes americanus* by intramuscular implants of slow release pellets at a level 55 μg GnRHa /kg and by intramuscular implants of 20 μg GnRHa /kg fast release pellets. Injections of 20 μg GnRHa /kg three times a week for the six week period also proved effective but increased mortalities due to handling stress (Harmin and Crim 1992). Marte *et al.* (1987) induced spawning in the milkfish, *Chanos chanos* by means of injection and intraperitoneal pellet implants. The intraperitoneal pellets at a concentration of 22 μg GnRHa/kg, proved to be the most effective treatment for milkfish. Mylonas *et al.* (1995a) used micropellets for GnRHa administration over a period of 8 weeks. Striped bass were implanted at 50 μg/kg, 75 μg and 150 μg and the micropellets were effective in maintaining elevated GnRHa levels for eight weeks with 60 - 70% of the analogue released in the first four weeks.

The effect of hormone treatment on reproductive behaviour has been investigated for *Puntius goniotus* (Liley and Tan 1985) and *Chromis dispillus* (Pankhurst 1995, Pankhurst and Carragher 1995). Liley and Tan, (1985) induced spawning behaviour in *Puntius goniotus* by treatment with prostaglandin. In *C. dispillus,* behaviour was found to influence endocrine status as opposed to hormone levels influencing behaviour (Pankhurst 1995, Pankhurst and Carragher 1995). Plasma hormone levels of testosterone, 11 ketotestosterone and 17α20β-dihydroxy-4-pregen-3-one were elevated by the intensity or frequency of behavioural interaction between fish, with fish from high density aggregations having higher plasma levels of hormones than those from sparsely populated

areas. No differences in plasma levels of hormones were found between the two groups of fish in non-breeding times. Hormone levels were also found to be higher in males that hold a territory. Plasma hormone levels fell at night as territorial behaviour levelled off and rose again with an increase in territorial behaviour the next morning. Injecting the fish with hormones failed to produce the behaviours associated with spawning behaviour, despite the fact that the levels of hormones in the blood were now the same as spawning fish. The possibility that endocrine factors higher in the hypothalamic-pituitary-gonadal axis might regulate behaviour was investigated by injecting fish with the gonadal hormones HCG, GnRHa and LHRHa. No significant differences in behaviour were recorded.

Manipulation of photoperiod failed to induce spawning in *A. akallopisos* (Chapter 4). Hormone treatment was therefore investigated as a method of conditioning this species for spawning. The objectives of this experiment were to document the behaviour of *A.akallopisos* subjected to several dosages of GnRHa administered orally. The hypothesis was as follows: Treatment with GnRHa would have no effect on the reproductive behaviour of *A. akallopisos*

Materials and methods

Twelve pairs of *A. akallopisos* were housed in glass aquaria in the recirculating system described in Chapter 2. The two aquarium sizes were 900x350x300 mm (94,5 L) and 600x500x380 mm (114 L) by volume. The aquaria were isolated from one another by means of black plastic cardboard to avoid the fish from being affected by the activity of fish in adjacent tanks. Decorations and the substratum in all tanks were standardised. Substrate consisted of a thin layer of shale, the surface of which could be vacuumed to remove debris. Housing consisted of a 200

mm piece of 110 mm PVC pipe and a 200 mm section of a concave ceramic tile to provide nesting sites. All tanks were covered with clear 1,5 mm acrilan plastic to prevent the fish from jumping out and to reduce evaporation. Flow rates were kept constant in all aquaria at 5 L/min which resulted in a turnover time of 23,8 minutes for the 114 L tanks and 18,9 minutes for the 94,5 L aquaria.

Water temperature was kept at 26°C ± 1°C for the duration of the experiment. Salinity was kept constant at 34 ppt ± 1 ppt with the addition of rain water. Ammonia-nitrogen (NH_4^+-N) levels ranged from 0 - 0.01 mg/L, nitrite-nitrogen (NO_2^--N) levels from 0.0 - 0.010 mg/L and nitrate-nitrogen (NO_3^--N) levels remained below 25 mg/L. pH ranged from 8,1 - 8,3 and dissolved oxygen from 6,7 - 7,1 mg/L.

Each aquarium was illuminated by means of a single Biolux fluorescent tube which have peaks in the 450 and 550 nm range of the spectrum. Light intensity was 4,017 x 10^{16} quanta $sec.^{-1}$ $cm.^{-2}$ at the surface and 1,85 x 10^{16} quanta $sec.^{-1}$ $cm.^{-2}$ at 25 cm below the water surface. Photoperiod was kept constant in all aquaria at the mid-summer photoperiod of Sodwana Bay, ie. 14L:10D.

A preliminary experiment involved the injection of a pair of *A. akallopisos* with a GnRHa/ Domperidone mixture (Aquaspawn®, manufactured by Spawnrite Ltd.) at a dosage of 1 ml/kg fish. This pair was held separately from the main system and injected during the course of the first (photoperiod) experiment. They were injected weekly for a period of three weeks at a dosage of 1 ml/kg. This pair was not used in the oral micro-pellet experiment.

Micro-pellet experiment

Three pairs of clowns were anaesthetised with 2-phenoxyethanol and weighed in order to determine mean body weight. The mean weight for females was 12,5g ± 2.6g. Mean weight for males was 6,6g ± 1.02g. The pairs were then fed untreated food that was pre-weighed in order to determine how much each male and female fish consume on a daily basis. From this information it was determined how much food, treated with GnRHa, should be fed to the animals. The fish were fed to satiation twice daily but were only fed the treated food once a day. They were otherwise fed the diet described in Chapter 2.

The microcapsules used in the study were developed by the Laboratory for Ecology and Aquaculture of the Catholic University of Leuven, Belgium. Each microcapsule contained 50 μg GnRHa per gram bound in a starch base. The recommended dosage given by the manufacturers is 40 μg per kg of fish for carp and 80 μg/kg for trout. The treatments were as follows (each aquarium contained a single pair of *A.akallopisos*:

Treatment 0: 2 aquaria of 114 L fed untreated food (control).

Treatment 1: 2 aquaria of 114 L fed 10μg GnRHa/kg body weight.

Treatment 2: 2 aquaria of 94,5 L fed 20μg GnRHa/kg body weight.

Treatment 3: 2 aquaria of 114 L fed 40μg GnRHa/kg body weight.

Treatment 4: 2 aquaria of 94,5 L fed 80μg GnRHa/kg body weight

Treatment 5: 2 aquaria of 94,5 L fed untreated food (control).

The behavioural programme described in Chapter 2 was used for all observations in this experiment. Observations were made for 10 minutes/tank and the observations were made on the basis of a random design. All aquaria were observed for the behaviours which were outlined in

Table 2.3. Recordings of behaviour were made every three days for a period of six weeks. Statistical analysis was by means of a one-way Analysis of Variance. Tukey's multiple range test was used to identify differences between treatment means at $p < 0.05$. Regression analysis was used to test for the significance of trends in behaviour as a function of time over the experimental period.

Results

None of the fish spawned during the course of the experiment. Consequently, none of the predictors for spawning were recorded. The following behaviours were also not recorded in any of the treatments; chasing by the male fish and signal jumps.

No significant differences ($p > 0.05$) were obtained between the two aquarium sizes for any activity. Significant differences over time ($p < 0.05$) were obtained for duration of exploratory swimming in treatment 0 (control) , duration of head shaking in treatment 1 (10 μg/kg bw), and frequency of chasing in treatment 3 (40 μg/kg bw). The statistics of the regression models are shown in Tables 5.1 to 5.6.

Significant differences ($p < 0.05$) were recorded between treatment 1 (10 μg/kg bw) and the control treatments for frequency of head shaking by the male fish. Elevated levels of this activity were exhibited by fish under all four hormone treatments when compared to the controls. A similar trend was apparent for duration of this activity although there were no significant differences between treatments ($p > 0.05$). This result is shown in Figure 5.1. Significant differences were also obtained between treatment 2 (20 μg/kg bw) and both controls for

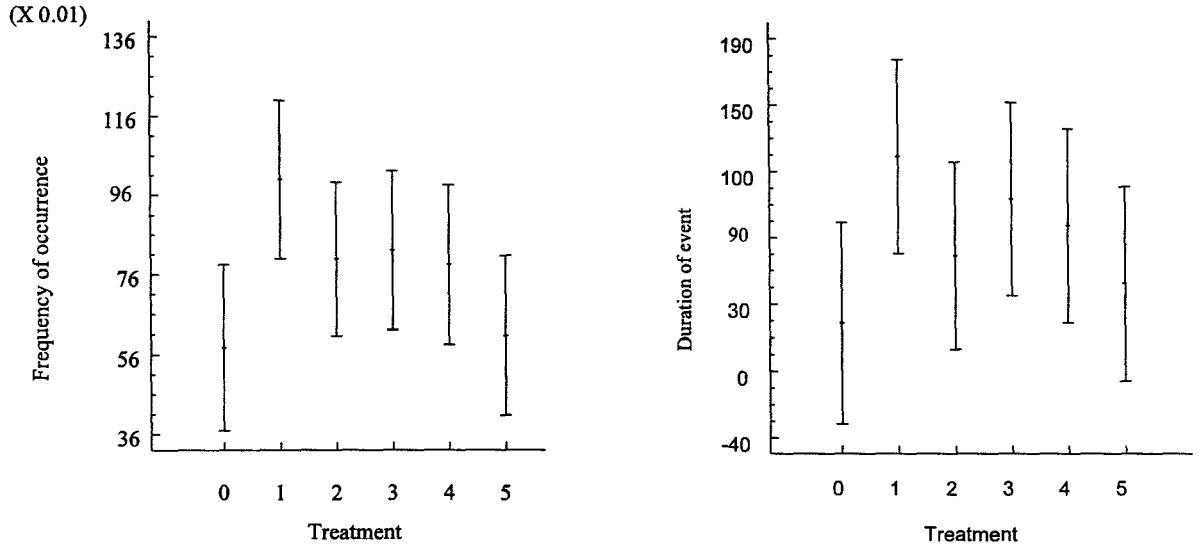

Figure 5.1. Mean frequency and duration (in seconds) of head shaking exhibited by male *A. akallopisos* maintained under four dosages of GnRHa. Error bars indicate 95 % confidence levels. Values shown are for a ten-minute period. Controls are indicated by treatments 0 and 5. Treatments 1 to 4 show GnRHa levels of 10, 20, 40 and 80 μg/kg bw respectively.

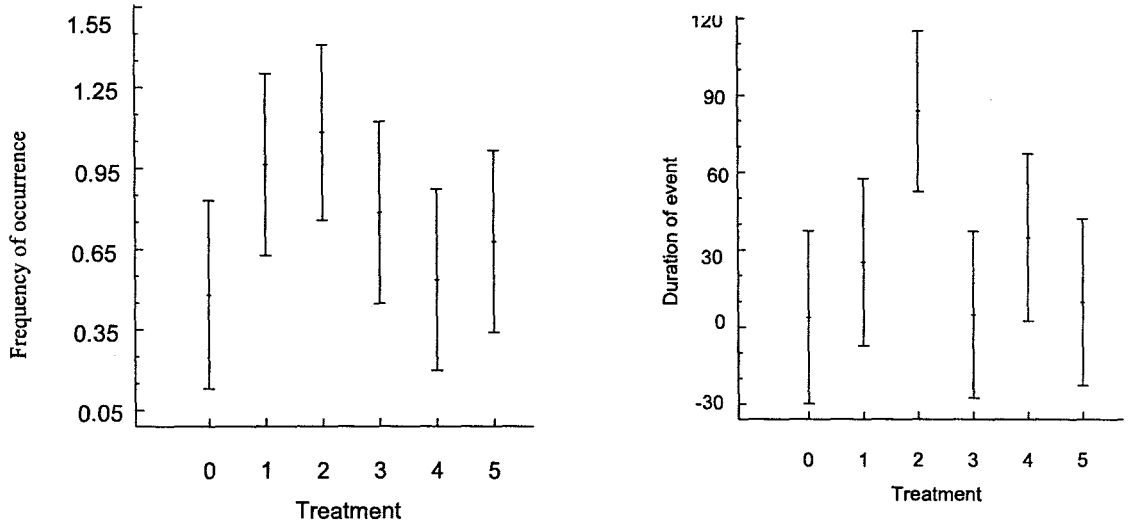

Figure 5.2. Mean frequency and duration (in seconds) of nest cleaning exhibited by male A. akallopisos maintained under four dosages of GnRHa. Error bars indicate 95 % confidence levels. Values shown are for a ten minute period. Controls are indicated by treatments 0 and 5. Treatments

Table 5.1. Results of regression analysis of Control treatment 0 (0 μg/kg bw) for change in behaviour over the 54 day experimental period (n = 36).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.17	7.78
	duration	0.08	12.10
Chasing by female	frequency	0.06	13.67
	duration	0.15	8.47
Exploratory swimming	frequency	0.52	1.72
	duration	0.02	21.1
Nest cleaning (male)	frequency	0.40	2.95
	duration	0.40	2.95
Parallel swimming	frequency	0.40	2.95
	duration	0.40	2.95

Table 5.2. Results of regression analysis of Treatment 1 (10 μg/kg bw) for change in behaviour over the 54 day experimental period (n = 36).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.08	11.34
	duration	0.02	19.13
Chasing by female	frequency	0.91	0.05
	duration	0.50	1.73
Dorsal leaning	frequency	0.39	2.76
	duration	0.39	2.76
Ventral leaning	frequency	0.63	0.87
	duration	0.69	0.62
Exploratory swimming	frequency	0.52	1.58
	duration	0.40	2.69
Nest cleaning (male)	frequency	0.35	3.28
	duration	0.46	2.11
Parallel swimming	frequency	0.48	1.93
	duration	0.48	1.93

Table 5.3. Results of regression analysis of Treatment 2 (20 μg/kg bw) for change in behaviour over the 54 day experimental period (n = 36).

Behaviour pattern		p value	r^2 (%)
Head shaking (male)	frequency	0.06	11.92
	duration	0.08	10.70
Head shaking (female)	frequency	0.46	1.99
	duration	0.26	4.40
Chasing by female	frequency	0.67	0.65
	duration	0.71	0.49
Dorsal leaning	frequency	0.85	0.12
	duration	0.87	0.08
Ventral leaning	frequency	0.13	7.89
	duration	0.09	9.47
Exploratory swimming	frequency	0.80	0.23
	duration	0.78	0.29
Nest cleaning (male)	frequency	0.84	0.15
	duration	0.48	1.78
Nest cleaning (female)	frequency	0.14	7.45
	duration	0.14	7.45
Parallel swimming	frequency	0.45	1.99
	duration	0.61	0.91

Table 5.4. Results of regression analysis of Treatment (40 μg/kg bw) for change in behaviour over the 54 day experimental period (n = 36).

Behaviour pattern		p value	r^2 (%)
Head shaking (male)	frequency	0.17	7.78
	duration	0.25	4.96
Head shaking (female)	frequency	0.10	10.34
	duration	0.08	11.52
Chasing by female	frequency	0.04	15.44
	duration	0.52	1.6
Ventral leaning	frequency	0.10	10.34
	duration	0.08	11.56
Exploratory swimming	frequency	0.98	0
	duration	0.32	3.84
Nest cleaning (male)	frequency	0.10	10.14
	duration	0.10	10.14

Table 5.5. Results of regression analysis of Treatment 4 (80 μg/kg bw) for change in behaviour over the 54 day experimental period (n = 36).

Behaviour pattern		p value	r^2 (%)
Head shaking (male)	frequency	0.38	3.0
	duration	0.75	0.40
Chasing by female	frequency	0.23	5.33
	duration	0.37	3.03
Dorsal leaning	frequency	0.90	0.06
	duration	0.90	0.06
Exploratory swimming	frequency	0.91	0.05
	duration	0.91	0.05
Nest cleaning (male)	frequency	0.11	9.47
	duration	0.24	5.23
Parallel swimming	frequency	0.35	3.28
	duration	0.54	1.39

Table 5.6. Results of regression analysis of Control treatment 5 (0 μg/kg bw) for change in behaviour over the 54 day experimental period (n = 36).

Behaviour pattern		p value	r^2 (%)
Head shaking (male)	frequency	0.49	1.84
	duration	0.55	1.38
Chasing by female	frequency	0.98	0.05
	duration	0.18	6.62
Ventral leaning	frequency	0.10	10.14
	duration	0.10	10.14
Exploratory swimming	frequency	0.61	1.02
	duration	0.50	1.80
Nest cleaning (male)	frequency	0.75	0.39
	duration	0.75	0.39

frequency and duration of nest cleaning activity by the male fish. This concentration was also significantly different from treatment 3 (40 µg/kg bw) for this activity. These results are shown in Figure 5.2. The means and standard deviations for frequencies of all recorded activities are summarised in Table 5.7. Means and standard deviations for duration of all activities recorded are shown in Table 5.8.

Table 5.7. Mean frequencies ± Standard deviations for a ten-minute period for the activities exhibited by *A. akallopisos* maintained under four concentrations of GnRHa (n = 36).

Behaviour	**Control ±SD**	**10 μg/kg bw ±SD**	**20 μg/kg bw ±SD**	**40 μg/kg bw ±SD**	**80 μg/kg bw ±SD**	**Control ±SD**
Head shaking (male)	0.58 ±0.05	1.11 ± 0.11	0.81 ± 0.11	0.82 ± 0.12	0.79 ± 0.10	0.78 ± 0.06
Head shaking(female)	0	0	0.57 ± 0.04	0.57 ± 0.05	0	0
Nest cleaning (male)	0.49 ± 0.02	0.61 ± 0.06	0.90 ± 0.14	0.53 ± 0.05	0.71 ±0.07	0.54 ±0.03
Nest cleaning (female)	0	0	0.33 ± 0.02	0	0	
Dorsal leaning	0	0.53 ± 0.02	0.56 ± 0.05	0	0.54 ± 0.03	0
Ventral leaning	0	0.60 ± 0.06	0.64 ± 0.07	0.61 ± 0.09	0	0
Chasing (female)	1.04 ± 0.14	1.28 ± 0.17	1.03 ± 0.12	1.15 ± 0.14	0.78 ± 0.10	1.18 ± 0.19
Parallel swimming	0.43 ± 0.04	0.53 ± 0.04	0.51 ± 0.03	0	0	0
Exploratory swimming	1.73 ± 0.10	2.21 ± 0.15	2.33 ± 0.24	2.04 ± 0.15	1.78 ± 0.24	1.92 ± 0.16

Table 5.8. Mean durations in seconds ± Standard deviations for a ten-minute period for the activities exhibited by *A. akallopisos* maintained under four concentrations of GnRHa (n = 36).

Behaviour	**Control ±SD**	**10 μg/kg bw ±SD**	**20 μg/kg bw ±SD**	**40 μg/kg bw ±SD**	**80 μg/kg bw ±SD**	**Control ±SD**
Head shaking (male)	19.33 ±16.88	119.54 ±2.85	59 88 ±24.42	93.75 ±36.36	77.88 ±31.81	43.01 ± 23.62
Head shaking(female)	0	0	28.93 ±20.17	15.74 ±2.11	0	0
Nest cleaning (male)	4.17 ±3.67	26.68 ±14.78	84.27 ±28.88	5.32 ±4.73	35.32 ±14.34	10.15 ±9.65
Nest cleaning (female)	0	0	12.13 ±9.36	0	0	0
Dorsal leaning	0	16.97 ±6.47	13.62 ±9.15	0	10.26 ± 9.11	0
Ventral leaning	0	40.43 ±27.22	46.91 ±31.11	34.23 ±23.78	0	6.39 ±5.89
Chasing (female)	109.70 ±30.42	148.64 ±37.13	73.75 ±21.01	132.74 ±36.95	91.93 ±33.38	16.43 ±32.60
Parallel swimming	5.82 ±5.82	5.57 ±5.57	30.66 ±21.82	0	33.72 ±21.11	0
Exploratory swimming	379.61 ±36.65	340.93 ±34.68	433.10 ±30.64	369.64 ±36.74	413.75 ±35.01	381.92 35.48

Discussion

The absence of behaviours associated with spawning (belly touching, mutual nest cleaning and nest cleaning by the female) indicate that the final stages of courtship that precede spawning were not reached in any of the pairs. The absence of predictors in the hormone treatments compared to the controls suggests that administration of GnRHa at concentrations of 10, 20, 40 or 80 μg /kg bw was not the critical factor in inducing spawning behaviour in the experimental fish used in this trial. The lack of significant changes in behaviour as a function of time suggests that administration of varying levels of GnRHa did not alter behaviour over the study period.

Although nest cleaning by a female was recorded, it was only observed in one replicate of a treatment and the frequency and duration exhibited (see Table 5.7 and 5.8) did not approach that of the levels recorded of spawning pairs (1.67 ± 0.22 and 95.26 ± 25.33 sec.). Similarly, the levels of nest cleaning by the male fish exhibited in this study did not approach that shown by pairs in spawning condition (2.56 ± 0.25 and 148.89 ± 33.51 sec).

The reason why the fish failed to spawn is not clear but it is possible that stress caused by parasites or medication may have affected their development. An infestation of the gills with monogenean trematodes necessitated treatment with Praziquantel HCL for a period of ten days. This problem occurred six weeks prior to the start of the experiment. Although the pairs were maintained for a full month after recovery, before the start of the experiment, it is possible that the stress effects caused by the infection or the medication may have had long-term effects that inhibited spawning.

Frequency and duration of nest cleaning was one of only two activities showing significant differences between treatments. A similar pattern was obtained by Smith (1969, 1970) for the sunfish, *Lepomis gibbosus* and *L. megalotis.* In that study treatment with both gonadotropin (HCG) and androgen (Testosterone) significantly increased the level of nest cleaning but did not significantly affect aggressive behaviour. Smith (1970) used nest cleaning by male fish as an indicator of gonadal activity. Although it was conceded that a number of factors may influence nest cleaning, the fact that androgens restored this activity in castrated fish was taken as a sign that this behaviour was controlled by hormone action. Although a similar experiment has not been performed on *A. akallopisos*, if nest cleaning is taken as an indicator of gonadal activity, the treatment of 20 μg/kg bw induced a significantly higher level of gonadal activity in *A. akallopisos*. This treatment was also the only one in which nest cleaning by the female fish was recorded, indicating that this treatment was possibly more instrumental in inducing nest cleaning behaviour than the other treatments.

The significant differences in behaviours recorded between the means of the hormone treatments and the controls, indicate that GnRHa does have an effect on the behaviour of *A. akallopisos*. From the available literature on the effect of hormone treatment on pomacentrid behaviour (Pankhurst 1990, Pankhurst 1995, Pankhurst and Carragher 1995), it seems likely that behaviour modifies endocrine status in damselfishes rather than the other way round. In *Chromis dispillus* plasma hormone levels of testosterone, 11 ketotestosterone and 17α20β-dihydroxy-4-pregen-3-one were elevated by the intensity or frequency of behavioural interaction between fish, with fish from high density aggregations having higher plasma levels of hormones than those from sparsely populated areas. Plasma levels of hormones in the two groups of fish were not different when

sampled during non-breeding periods. Hormone levels were also found to be higher in males that hold a territory. Plasma hormone levels fell at night as territorial behaviour levelled off and rose again with an increase in territorial behaviour the next morning. Injecting the fish with hormones failed to produce the behaviours associated with spawning, despite the fact that the levels of hormones in the blood were now the same as spawning fish. The possibility that endocrine factors higher in the hypothalamic-pituitary-gonadal axis might regulate behaviour, was investigated by injecting fish with the gonadal hormones HCG, GnRHa and LHRHa. No significant differences in behaviour were recorded although occasionally a change in behaviour was noted. Although elevated levels of some interactive behaviour compared to the controls were recorded, a clear pattern was not evident. In *A. akallopisos* and other *Amphiprion* species behaviour is an important part of the life history because of the monogamous pair relationship (Allen 1972, Fricke 1974). The results indicate that while GnRHa treatment has an effect on behaviour in *A. akallopisos*, behaviour is more likely to influence endocrine status as it does in other fish species. Polder (1971) supported the idea that behaviour modifies endocrine status in *Aequidens portalegrensis*, a monogamous cichlid. In *A. akallopisos,* measurement of plasma hormones to compare with behaviour would have been beneficial in order to corroborate this assumption as none of the fish could be sacrificed, this was not possible. Although plasma hormone levels were not measured, it seems likely that behaviour may modify hormone levels in the blood. Hormonal changes may regulate behaviour only in the appropriate social context. For instance, in male rainbow trout, *O. mykiss,* gonadotropin levels increased markedly when males were placed with nesting females (Liley *et al.* 1986). In a later study, gonadotropin and milt production were found to only increase in fish maintained in the presence of ovulated females (Rouger and Liley 1992).

Investigations into endocrine mechanisms involved in aquaculture has not been accompanied by a comparable interest in the behavioural link between fish and its environment, especially as regards reproductive behaviour. Behaviour plays a major role in regulating the endocrine system (Liley 1980), and for this reason behaviour needs to be taken into account in hormonal manipulation studies. Species of the genus *Amphiprion* provide an ideal animal for this sort of study due to its monogamous lifestyle. However, if such an experiment was to be run, large numbers of pairs at different stages of gonadal maturation would be needed. Although different methods of applying GnRHa were not tested in this study, alternative means of administering GnRHa should be investigated. For instance, Burton (1998) found that while GnRHa was not taken up by *Artemia* in a crushed micropellet form, *Artemia* were able to bioenscapulate GnRHa in a solution of Aquaspawn®.

Injection of *A. akallopisos* proved unsuccessful in inducing spawning. Injection also appeared to stress the fish to such an extent that observation after injection was not possible as the fish hid away for a number of days. For this reason injection is not suitable as a means of applying *GnRHa* in *A. akallopisos*, especially if behavioural studies are to accompany investigations of blood hormone levels.

CHAPTER 6

EFFECT OF HOST ANEMONES ON REPRODUCTIVE BEHAVIOUR OF *A. akallopisos*

Introduction

The symbiotic relationship between *Amphiprion* species and stichodactylid anemones was first recorded by the British naturalist Collingwood in 1868 when he noticed "Gigantic sea anemones" in the Sea of Batavia, "containing within them quasi-parasitic fish". Since then, the association has been the subject of several studies dealing with subjects such as behaviour, physiology and ethology and has been reviewed by Fautin 1991.

Anemone tentacles contain nematocysts which are fired upon contact with a prey item such as a small fish. *Amphiprion* are able to exist among these tentacles of the anemone because they possess a mucous covering which lowers the threshold value of the nematocyst discharge and so prevents the cells from firing (Mariscal 1970, Miyagama 1989).

In the wild, *Amphiprion* are obligate symbionts in this relationship - they need an anemone in order to survive. This is evidenced by the fact that *Amphiprion* are never found away from their hosts under natural circumstances (Allen 1972, 1980). Furthermore, *Amphiprion* released into the wild away from their hosts quickly fall prey to other fish (Allen 1972, 1980).

In the wild, the presence of an anemone appears to be essential for successful reproduction (Allen 1972, Moyer and Bell 1976, Fricke 1979). These authors found that eggs are always laid under

the protective mantle of the host where the parent fish can care for the batch without being exposed to predators. *Amphiprion* may also derive nutritive benefits from the anemone as tentacles and nematocysts have been found in the stomachs of *A.akallopisos* and ingestion of tentacles has been observed for five other species in aquaria (Mariscal 1970). Other unsubstantiated benefits that *Amphiprion* species may derive from the relationship include tactile stimulation and protection from fungoid and protozoan diseases (Mariscal 1970).

The dependence on anemones is behavioural and not physiological. This is evidenced by the fact that *Amphiprion* can survive and reproduce in captivity without an anemone (Mariscal 1970, Allen 1972). No evidence exists to suggest that anemones are necessary for *Amphiprion* to breed in captivity. Furthermore, keeping of anemones necessitates high light intensities and precludes the possibility that several disease treatments may be administered through the water (Fautin and Allen 1992). Anemones are also intolerant of nitrate levels that have no effect on *Amphiprion* species (Fautin and Allen 1992). Other disadvantages of anemones include their high price (R120-200 each) and the possibility that they may move over egg batches and kill them (pers. obs.). Commercial operations such as Instant Ocean Hatcheries (Hoff 1996) and the Rhodes University Marine Hatchery have also had success in breeding clownfishes without the use of anemones.

Nonetheless, many operations employ anemones in their breeding programmes including ADAS Hatchery (Davis 1993), Dynasty Marine Hatchery (Henningson 1989), Sea World Hatchery (Hoff 1996) and Aqualife Research Hatchery (Hoff 1996). Anemones may be especially useful in settling wild-caught broodstock (Davis 1993).

No behavioural comparisons have been made between *Amphiprion* maintained with or without anemones. This fact, together with the possibility that *A.akallopisos* may require an anemone host for spawning, prompted the experiment described in this chapter.

A.akallopisos is host specific for *Heteractis magnifica* and *Stichodactyla mertensii* (Fautin and Allen 1992). All *A.akallopisos* collected at Sodwana were found in association with *H.magnifica* and therefore this actinian was used as host in this study. The hypothesis was as follows: The presence of a host anemone would have no effect on the behaviour of *A.akallopisos*.

Materials and methods

Six pairs of *A. akallopisos* were housed in 600x500x380 mm (114 L) glass aquaria in the recirculating system described in Chapter 2. Three pairs were maintained with one *H.magnifica* per pair and three pairs were maintained without anemones. The behavioural programme described in Chapter 2 was used to make all observation in this experiment. The behaviours recorded are outlined in Table 2.5. All pairs were observed every three days for a period of 81 days. Observations were made for 10 minutes/pair and the tanks were randomly chosen on each day of observation. A T-test was used to test for differences in means between treatments. A Regression analysis was used to test for changes in frequency or duration of an activity as a function of time over the experimental period.

The aquaria were isolated from one another by means of black plastic cardboard to avoid the fish from being affected by the activity of fish in adjacent aquaria. Decorations and the substratum in all tanks were standardised. Substrate consisted of a thin layer of shale, the surface of which

could be vacuumed to remove debris. Housing consisted of a 200 mm piece of 110 mm PVC pipe and a 200 mm section of a concave ceramic tile to provide nesting sites. All tanks were covered with clear 1,5 mm acrilan plastic to prevent the fish from jumping out and to reduce evaporation. Flow rates were kept constant in all aquaria at 5 L/min which resulted in a turnover time of 23,8 minutes.

Water temperature was kept at 26 °C ± 1 °C for the duration of the experiment. Salinity was kept constant at 34 ppt ± 1 ppt with the addition of rain water. Ammonia-nitrogen (NH_4^+-N) levels ranged from 0 - 0.01 mg/L, nitrite-nitrogen (NO_2^--N) levels from 0.0 - 0.010 mg/L and nitrate-nitrogen (NO_3^--N) levels remained below 25 mg/L. pH ranged from 8,1 - 8,3 and dissolved oxygen from 6,7 - 7,1 mg/L.

Each aquarium was illuminated by means of a single Biolux fluorescent tube which have peaks in the 450 and 550 nm range of the spectrum. Light intensity was 4,017 x 10^{15} quanta $sec.^{-1}$ $cm.^{-2}$ at the surface and 1,85 x 10^{15} quanta $sec.^{-1}$ $cm.^{-2}$ at 25 cm below the water surface. Photoperiod was kept constant in all aquaria at the mid-summer photoperiod of Sodwana Bay (14L:10D).

Results

None of the pairs spawned during the course of the experiment. Head dipping, mutual substrate biting, biting of the anemone away from the nesting site and belly touching behaviours were not recorded. Regression analysis showed significant changes ($p < 0.05$) in frequency of exploratory swimming as a function of time over the experimental period in *A. akallopisos* maintained without anemones. A significant change was also obtained for duration of exploratory swimming

exhibited by *A. akallopisos* kept with anemones. In both cases a decrease in activity occurred over time. Exploratory swimming for fish maintained with anemones included time spent in the anemone during which no interaction occurred. No significant changes in any individual behaviour were recorded over the experimental period ($p > 0.05$). The results of the regression analysis are summarised in Tables 6.1 and 6.2.

Since no significant changes in any individual activity were found, the results were pooled and the means calculated. No significant differences ($p > 0.05$) were recorded for any of the interactive behaviours recorded. The mean values ± standard deviation are shown in Table 6.3.

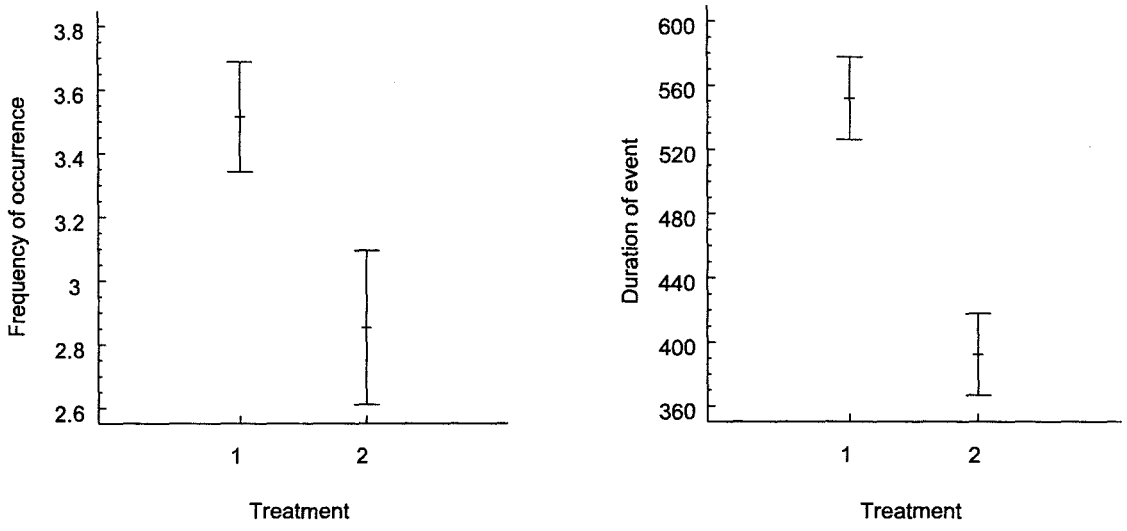

Figure 6.1. Mean frequency and duration (in seconds) of non-interactive behaviour exhibited by *A.akallopisos* maintained with anemones (Treatment 1) and without anemones (Treatment 2). Values shown are the means for a ten minute observation period. Error bars indicate 95 % confidence levels.

Table 6.1. Regression analysis of behaviours recorded for *A.akallopisos* maintained with host anemones. (n = 86).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.25	1.69
	duration	0.41	0.88
Head shaking (female)	frequency	0.11	3.08
	duration	0.11	3.08
Chasing by female	frequency	0.20	2.12
	duration	0.52	0.51
Dorsal leaning	frequency	0.20	2.11
	duration	0.20	2.11
Vertical swimming	frequency	0.09	3.57
	duration	0.09	3.57
Anemone nestling	frequency	0.89	0.02
	duration	0.35	1.09
Exploratory swimming	frequency	0.22	1.91
	duration	0.04	5.41
Nest cleaning (male)	frequency	0.34	1.16
	duration	0.32	1.27
Nest cleaning (female)	frequency	0.27	1.53
	duration	0.27	1.53
Parallel swimming	frequency	0.08	3.88
	duration	0.09	3.75

Table 6.2. Regression analysis of behaviours recorded for *A.akallopisos* maintained without host anemones. (n = 86).

Behaviour pattern		**p value**	**r^2**
Head shaking (male)	frequency	0.07	4.09
	duration	0.06	4.42
Head shaking (female)	frequency	0.06	4.32
	duration	0.07	4.02
Chasing by female	frequency	0.34	1.11
	duration	0.62	0.29
Dorsal leaning	frequency	0.56	0.43
	duration	0.45	0.71
Ventral leaning	frequency	0.46	0.67
	duration	0.46	0.67
Exploratory swimming	frequency	0.01	8.52
	duration	0.65	0.25
Nest cleaning (male)	frequency	0.25	1.63
	duration	0.35	1.08
Nest cleaning (female)	frequency	0.26	1.57
	duration	0.26	1.57
Parallel swimming	frequency	0.14	2.69
	duration	0.39	0.91

A difference was obtained for the total amount of non-interactive behaviour, with *A. akallopisos* maintained without anemones exhibiting significantly more interactive behaviour ($p < 0.05$) than those maintained with host anemones (Figure 6.1).

Table 6.3. Mean values ± Standard deviation for activities exhibited by *A. akallopisos* maintained with host anemones over a 90 day period (n = 86). Values shown are the means for a ten-minute observation period.

Behaviour pattern	**Frequency ± SD**	**Duration (sec) ± SD**
Head shaking (male)	0.18 ± 0.04	59.53 ± 16.33
Head shaking (female)	0.01 ± 0.01	6.24 ± 6.24
Chasing (female)	0.59 ± 0.07	136.28 ± 19.88
Dorsal leaning	0.01 ± 0.01	6.94 ± 6.94
Exploratory swimming	3.55 ± 0.91	552.12 ± 22.36
Nest cleaning (male)	0.23 ± 0.05	45.36 ± 13.46
Nest cleaning (female)	0.01 ± 0.01	5.90 ± 5.90
Parallel swimming	0.04 ± 0.02	11.23 ± 7.44

Table 6.4. Mean values ± Standard deviation for activities exhibited by *A. akallopisos* maintained without host anemones over a 90 day period (n = 86). Values shown are the means for a ten-minute observation period.

Behaviour pattern	**Frequency ± SD**	**Duration (sec) ±SD**
Head shaking (male)	0.24 ± 0.05	83.40 ± 18.26
Head shaking (female)	0.02 ± 0.01	6.54 ± 4.71
Chasing (female)	0.61 ± 0.07	158.33 ± 21.92
Dorsal leaning	0.05 ± 0.02	10.27 ± 6.76
Ventral leaning	0.02 ± 0.02	5.23 ± 5.23
Exploratory swimming	2.83 ± 0.79	388.20 ± 21
Nest cleaning (male)	0.16 ± 0.05	34.19 ± 11.18
Nest cleaning (female)	0.02 ± 0.02	7.92 ± 6.60
Parallel swimming	0.09 ± 0.03	25.20 ± 9.45

Discussion

The failure of *A. akallopisos* to spawn in this experiment suggests that anemones were not the final missing factor in inducing spawning. The lack of behavioural predictors of spawning (belly touching, mutual nest cleaning, biting of the anemone away from a nesting site) indicate that spawning was not imminent in any of the pairs. Although individual nest cleaning was exhibited by both sexes under both treatments, Analysis of Variance showed that the levels recorded were significantly lower than those recorded for spawning *A. akallopisos*. The mean frequency of nest cleaning by male fish maintained with, and without anemones was 0.23 ± 0.05 and 0.16 ± 0.05 respectively, per 10 minute observation period compared to 2.56 ± 0.25 for spawning pairs. Similarly, the mean duration of nest cleaning by male *A. akallopisos* was 45.36 ± 13.46 and 34.19 ± 11.18 seconds per 10 minute observation period compared to 148.89 ± 33.51 seconds for spawning fish. Frequency of nest cleaning by female fish was only 0.01 ± 0.01 and 0.02 ± 0.02 compared to 1.67 ± 0.22 per observation period, while the mean duration of this behaviour was 5.90 ± 5.90 and 7.92 ± 6.60 seconds compared to 95.26 seconds for female *A. akallopisos* in spawning condition. The low levels of these activities exhibited by *A. akallopisos* in this study are further signs that spawning was not imminent.

Anecdotal literature indicates that the absence of an anemone may retard the acclimation of wild-caught adult *Amphiprion* to captivity (Davis 1993, Hoff 1996). While this may have been a factor in acclimatising newly-caught *A.akallopisos,* the fish used in this study had been maintained in aquaria for 11 months prior to the start of the experiment. Therefore, it is unlikely that they were still adapting to life in captivity. Bok (1997) noted that the presence or absence of anemones had no noticeable effect on spawning of *A. akallopisos*. He also noticed no

differences in the time taken for the fish to begin spawning. These observations were not quantified and the information must be regarded as anecdotal. Hoff (1996) also speculates that *Amphiprion* maintained without anemones may lack the nutritional and psychological benefits afforded by the host, and that this may affect their ability to reproduce in the long term. This has not been tested and this study suggests that *A.akallopisos* do not need to be maintained with host anemones. The reasons for the differences in the frequency and duration of interactive behaviour between treatments are not clear. *A.akallopisos* maintained without an anemone may spend a greater amount of time interacting with each other because they do not have a host in which to nestle.

The collection of anemones from the wild at present is not done on a sustainable basis and should be discouraged (Fautin and Allen 1992). Collection of anemones for the aquarium trade has also caused extinction of certain populations of *Amphiprion* species and *Premnas biaculeatus* in the Phillipines (Fautin and Allen 1992). This practise, together with the disadvantages associated with maintaining anemones in captivity (lighting, water quality, danger to eggs) makes a strong case for keeping *Amphiprion* without anemones wherever possible. The results of this study show that *A. akallopisos* exhibits more interaction when maintained without *H.magnifica.* This suggests that anemones are not necessary for pair bonding and conditioning for spawning in *A. akallopisos*. Subsequent spawning of an *A. akallopisos* pair maintained without an anemone, together with Bok's (1997) observations, indicates that anemones are not essential for spawning purposes. The death of an anemone maintained with a spawning pair was recorded in five instances and in all cases there was no noticeable effect on spawning rhythm (pers. obs.).

A. akallopisos should be maintained without anemones wherever possible, but the possibility that the presence of an anemone in the early stages of conditioning wild *A. akallopisos* may be beneficial cannot be discounted and should be examined. The long-term effects of fish maintained with or without anemones and the performance of breeding pairs should also be examined.

CHAPTER 7

EFFECT OF LIGHT INTENSITY ON THE REPRODUCTIVE BEHAVIOUR OF *A.akallopisos*

Introduction

Literature concerning the effects of light intensity on the gonadal development and reproductive behaviour of teleosts is limited. The only available literature concerns a study on *Fundulus heteroclitus*. In that investigation, increased light intensity resulted in better final maturation in mature ovaries and better recrudescence in developing ovaries. There was a threshold intensity of 0.1 lux below which gonadal development did not occur (Taylor, Hyde, Platt and Day 1985).

Amphiprion akallopisos occurs on coral reefs in the tropics where the light intensity at the water surface at noon can reach 150 000 lux (Spotte 1992). This approximates to roughly 3.81×10^{23} quanta.sec^{-1}.cm^{-2} (1 lux $\approx 2.54 \times 10^{17}$ quanta.sec^{-1}.cm^{-2}). This value is only an estimate as lux cannot be directly converted to quanta.sec^{-1}.cm^{-2}. Commercial clownfish hatcheries such as Instant Ocean Hatchery and Sea World Hatchery in Florida, U.S.A, employed solar lighting supplemented with fluorescent tubes (Hoff 1996). Dynasty Marine Hatchery employs full spectrum metal halide lighting (Henningsen 1989). While intensities are not mentioned, the intensity of similar 250 watt metal halide lamps used at the Rhodes University Hatchery is 1.54×10^{16} quanta.sec^{-1}.cm^{-2} at the water surface.

The role of light intensity as a possible factor necessary to stimulate gonadal development

and spawning in *A.akallopisos* was considered after a study of two systems in which skunk clownfish had been successfully spawned. One of the systems was the Rhodes University Hatchery in Grahamstown and the other was at Sea World Aquarium, Durban. The Rhodes University Hatchery system uses direct natural daylight and the Durban system supplemented indirect natural daylight with fluorescent tubes. Water quality parameters in these systems were similar to those of the experimental system. Foods and feeding regimens were also similar in all three systems. Given that lunar periodicity had no effect on spawning, the higher light intensities caused by the presence of natural lighting were identified as possible factors necessary for conditioning and gonadal development in *A.akallopisos*.

The main objective of the study was to test if the existing lighting in the experimental system could have been insufficient to induce spawning in *A.akallopisos* in previous studies. For this reason, increasing light intensity and/or supplementing the illumination with natural daylight was tested for conditioning of the fish. The primary aim of the experiment was to document the effect of increased light intensity on the reproductive behaviour of *A.akallopisos*. The hypothesis for the experiment was as follows: Increasing light intensity would have no effect on the reproductive behaviour of *A.akallopisos*.

Materials and Methods

Nine pairs of *A.akallopisos* were housed in 114 L glass aquaria which measured 600x500x380 mm. One pair of fish was kept in each tank. The pairs were maintained in the recirculating system described in Chapter 2. The behavioural programme described in Chapter 2 was used for all observations in this experiment. Observations of the behaviour

outlined in Table 2.3 were made for 10 minutes/tank and the observations were made on the basis of a random design. All pairs were observed every three days for a period of three months. A one-way Analysis of Variance was used to test for differences between treatments. Tukey's multiple range test was used to identify where these differences occurred. Regression analysis was used to test for changes in a given frequency or duration of an activity as a function of time over the experimental period.

The aquaria were isolated from one another by means of black plastic cardboard to avoid the fish from being affected by the activity of fish in other tanks or by different light intensities. Decorations and the substratum in all tanks were standardised. Substratum consisted of a thin layer of shale, the surface of which could be vacuumed to remove debris. Decoration consisted of a 200x100 mm piece of 110 mm PVC pipe and a 200 mm section of a concave terra cotta tile to provide nesting sites. All tanks were covered with clear 1,5-mm acrilan plastic to prevent the fish from jumping out and to reduce evaporation. Flow rates were kept constant in all aquaria at 5 L/min which resulted in a turnover time of 23,8 minutes.

Water temperature was kept at 26 °C ± 1 °C for the duration of the experiment. Salinity was kept constant at 34 ppt ± 1 ppt with the addition of rain water. Ammonia-nitrogen (NH_4^+-N) levels ranged from 0 - 0.01 mg/L, nitrite-nitrogen (NO_2^--N) levels from 0.0 - 0.010 mg/L and nitrate-nitrogen (NO_3^--N) levels remained below 25 mg/L. pH ranged from 8,1 - 8,3 and dissolved oxygen from 6,7 - 7,1 mg/L.

Three aquaria were illuminated by a single Biolux fluorescent tube. This was the standard

lighting arrangement for the three previous experiments. Light intensity was doubled in six aquaria with the addition of a second Biolux tube. Three of these aquaria also received three hours of direct sunlight at an oblique angle. A thick black curtain was erected across the laboratory to prevent sunlight affecting any of the other aquaria. A thick black curtain was also lowered over the window at night to prevent fish being affected by the natural photoperiod change. The tubes had peaks in the 450 and 550 nm range of the spectrum. Light intensity was measured at the surface and at 25 cm below the water surface. Light intensities were measured using a QSL-100 Quantum Scalar Irradiance Meter and are shown in Table 7.1 Intensities were measured without sunlight entering the laboratory.

Table 7.1 Light intensities in quanta.sec^{-1}.cm^{-2} under which *A.akallopisos* was maintained. L1- Double tubes plus direct sunlight; L2 - Double tubes; L3 - Single tubes (original light intensity).

Intensity	Surface ± SD	Submerged at 25 cm depth ± SD
L 1	$8.85 \times 10^{15} \pm 0.19 \times 10^{15}$	$2.45 \times 10^{15} \pm 0.18 \times 10^{15}$
L 2	$8.83 \times 10^{15} \pm 0.28 \times 10^{15}$	$2.4 \times 10^{15} \pm 0.17 \times 10^{15}$
L 3	$4.16 \times 10^{15} \pm 0.28 \times 10^{15}$	$1.2 \times 10^{15} \pm 0.10 \times 10^{15}$

Results

One pair of *A.akallopisos* spawned during the course of the experiment under the treatment of increased light intensity but without direct sunlight (L2). No other spawnings were recorded. Behaviour patterns identified in Chapter 3 as predictors for spawning - belly touching by the female and nest cleaning by the female were only exhibited by fish maintained under that light intensity. Mutual nest cleaning and belly touching by the male fish were not recorded in

any of the other treatments during the course of the experiment. Regression analysis showed a significant change in behaviour over the 81 day experimental period for frequency of exploratory swimming exhibited by fish maintained under L3, and frequency of head shaking by the male fish under L1. The results of the regression analyses are summarised in Tables 7.2 to 7.4.

Different light intensities had a significant effect on the frequency and duration of nest cleaning by female and male fish, and parallel swimming. Light intensity also had a significant effect ($p < 0.05$) on the mean frequencies and durations of head shaking by the male fish, and frequency of exploratory swimming. Fish kept under L2 exhibited a significantly higher frequency and duration of nest cleaning and parallel swimming compared to L1 and L3. Mean values for frequency of head shaking and frequency of exploratory swimming in L1 were significantly different from those in L3 (Figures 7.1-7.4). The means and standard deviations for all the behaviour patterns exhibited by *A.akallopisos* in this experiment are shown in Tables 7.5 and 7.6

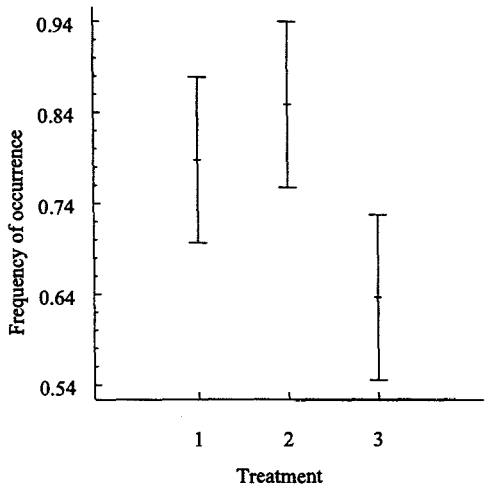

Figure 7.1. Mean frequency of head shaking exhibited by male *A.akallopisos* maintained under three light intensities. Error bars indicate 95 % confidence levels. Values shown are for a ten minute period. Treatment 1 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}. + limited natural light; Treament 2 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}; Treatment 3 - 4.16 x 10^{15} quanta.sec^{-1}cm^{-2}.

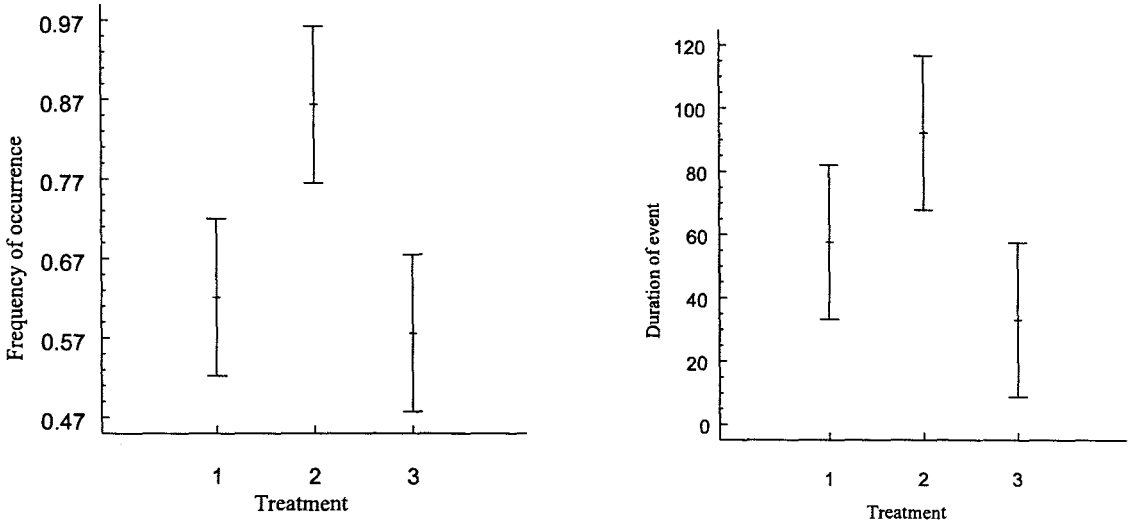

Figure 7.2. Mean frequency and duration in (in seconds) of nest cleaning exhibited by male *A.akallopisos* maintained under three light intensities. Error bars indicate 95 % confidence levels. Values shown are for a ten minute period. Treatment 1 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}. + limited natural light; Treament 2 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}; Treatment 3 - 4.16 x 10^{15} quanta.sec^{-1}cm^{-2}.

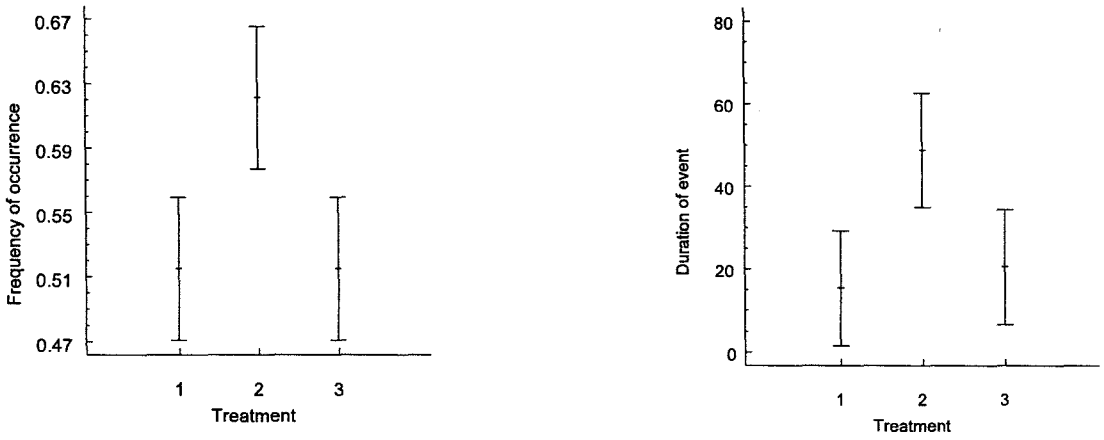

Figure 7.3. Mean frequency and duration (in seconds) of parallel swimming exhibited by *A.akallopisos* maintained under three light intensities. Error bars indicate 95 % confidence levels. Values shown are for a ten minute period. Treatment 1 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}. + limited natural light; Treament 2 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}; Treatment 3 - 4.16 x 10^{15} quanta.sec^{-1}cm^{-2}.

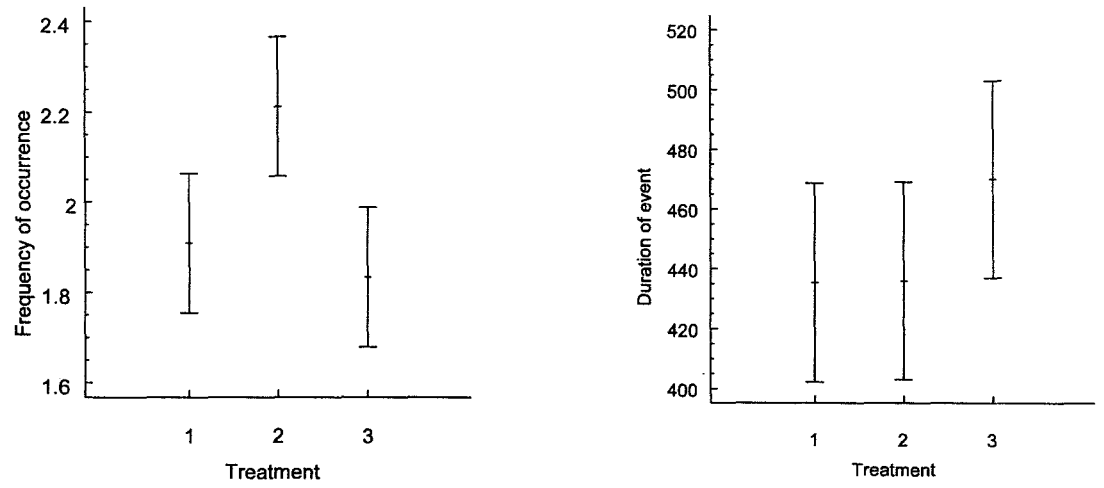

Figure 7.4. Mean frequency and duration (in seconds) of exploratory swimming exhibited by *A.akallopisos* maintained under three light intensities. Error bars indicate 95 % confidence levels. Values shown are for a ten minute period. Treatment 1 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}. + limited natural light; Treament 2 - 8.84 x 10^{15} quanta.sec^{-1}cm^{-2}; Treatment 3 - 4.16 x 10^{15} quanta.sec^{-1}cm^{-2}.

Table 7.2. Results of regression analysis of change in behaviour exhibited by *A.akallopisos* over the 81 day experimental period under a light intensity of 8.85×10^{15} quanta.sec^{-1}.cm^{-2}. supplemented with limited natural light (n = 81).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.03	7.30
	duration	0.22	2.38
Chasing by female	frequency	0.45	0.90
	duration	0.10	4.06
Ventral leaning	frequency	0.11	3.99
	duration	0.11	3.99
Dorsal leaning	frequency	0.07	4.95
	duration	0.07	4.86
Vertical swimming	frequency	0.33	1.45
	duration	0.33	1.45
Exploratory swimming	frequency	0.89	0.03
	duration	0.11	4.02
Nest cleaning (male)	frequency	0.43	0.98
	duration	0.33	1.46
Parallel swimming	frequency	0.18	2.69
	duration	0.18	2.69

Table 7.3. Results of regression analysis of change in behaviour exhibited by *A.akallopisos* over the 81 day experimental period under a light intensity of 8.83×10^{15} quanta.sec^{-1}.cm^{-2} (n = 81).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.46	0.85
	duration	0.89	0.03
Head shaking (female)	frequency	0.31	1.58
	duration	0.12	3.74
Chasing by female	frequency	0.93	0.01
	duration	0.76	0.14
Ventral leaning	frequency	0.97	0
	duration	0.45	0.86
Belly touching (female)	frequency	0.49	0.72
	duration	0.49	0.72
Exploratory swimming	frequency	0.09	4.56
	duration	0.71	0.22
Nest cleaning (male)	frequency	0.29	1.72
	duration	0.83	0.07
Nest cleaning (female)	frequency	0.20	2.54
	duration	0.27	1.87
Parallel swimming	frequency	0.53	0.62
	duration	0.91	0.02

Table 7.3. Results of regression analysis of for change in behaviour exhibited by *A.akallopisos* over the 81 day experimental period under a light intensity of 4.16×10^{15} quanta.sec^{-1}.cm^{-2}(n = 81).

Behaviour pattern		**p value**	**r^2 (%)**
Head shaking (male)	frequency	0.38	1.16
	duration	0.32	1.56
Head shaking (female)	frequency	0.11	3.99
	duration	0.11	3.99
Chasing by female	frequency	0.09	4.48
	duration	0.60	0.43
Ventral leaning	frequency	0.97	0
	duration	0.69	0.24
Dorsal leaning	frequency	0.51	0.67
	duration	0.50	0.73
Exploratory swimming	frequency	0.04	6.29
	duration	0.61	0.41
Nest cleaning (male)	frequency	0.18	2.73
	duration	0.53	0.60
Parallel swimming	frequency	0.55	0.57
	duration	0.55	0.57

Table 7.5 Mean frequencies of behaviour exhibited by *A.akallopisos* for a ten minute observation period (n = 81). Intensity 1 indicates a light intensity of 8.85 x 10^{15} quanta. sec^{-1} .cm^{-2} supplemented by natural light; Intensity 2 - 8.85 x 10^{15} quanta. sec^{-1} .cm^{-2}; Intensity 3 - 4.16 x 10^{15} quanta. sec^{-1} .cm^{-2}

Behaviour pattern	L1± SD	L2 ± SD	L3 ± SD
Head shaking (male)	0.78 ± 0.06	0.84 ± 0.06	0.63 ± 0.04
Head shaking (female)	0	0.07 ± 0.03	0.03 ± 0.02
Chasing (female)	1.07 ± 0.07	1.01 ± 0.10	1.04 ± 0.07
Ventral leaning	0.02 ± 0.02	0.05 ± 0.03	0.05 ± 0.03
Dorsal leaning	0.03 ± 0.02	0	0.03 ± 0.02
Belly Touching (female)	0	0.02 ± 0.02	0
Vertical swimming	0.02 ± 0.02	0	0
Exploratory swimming	1.91 ± 0.08	2.21 ± 0.12	1.83 ± 0.06
Nest cleaning (male)	0.62 ± 0.05	0.86 ± 0.08	0.12 ± 0.03
Nest cleaning (female)	0	0.09 ± 0.04	0
Parallel swimming	0.51 ± 0.02	0.62 ± 0.04	0.51 ± 0.02

Table 7.6 Mean durations (in seconds) of behaviour exhibited by *A.akallopisos* for a ten minute observation period (n = 81). Intensity 1 indicates a light intensity of 8.85 x 10^{15} quanta. sec^{-1} .cm^{-2} supplemented by natural light; Intensity 2 - 8.85 x 10^{15} quanta. sec^{-1} .cm^{-2}; Intensity 3 - 4.16 x 10^{15} quanta. sec^{-1} .cm^{-2}

Behaviour pattern	L1± SD	L2 ± SD	L3 ± SD
Head shaking (male)	65.94 ± 17.35	78.27 ± 17.83	38.50 ± 12.73
Head shaking (female)	0	24.85 ± 11.65	9.68 ± 6.95
Chasing (female)	109.78 ± 19.97	99.40 ± 20.53	119.60 ± 22.41
Ventral leaning	1.11 ± 1.11	3.43 ± 2.33	4.57 ± 3.40
Dorsal leaning	6.89 ± 4.78	0	6.98 ± 4.90
Belly Touching (female)	0	1.24 ± 1.24	0
Vertical swimming	0.02 ± 0.02	0	0
Exploratory swimming	434.86 ± 21.17	435.54 ± 19.21	469.55 ± 19.05
Nest cleaning (male)	57.61 ± 15.56	92.18 ± 18.18	33.08 ± 8.07
Nest cleaning (female)	0	16.34 ± 7.53	0
Parallel swimming	20.41 ± 0.41	53.80 ± 13.22	25.48 ± 5.48

Discussion

Although one pair of fish spawned under L2, this event did not prove that light intensity was the critical factor in inducing spawning. Factors that could influence development such as changing natural photoperiod or lunar influence were eliminated by hanging a black curtain over the window at night. The 14L:10D photoperiod used was longer than the natural photoperiod, preventing the possibility that *A.akallopisos* could be influenced by change in natural photoperiod, which was approaching the winter solstice. If light intensity was indeed the critical factor in inducing spawning, the absence of spawning under the treatment where light intensity was doubled and supplemented with natural light is puzzling. However, in the two months following this experiment, the pairs were maintained under the same three light intensities and two more pairs began spawning. Both of these pairs were maintained under the increased light intensity with natural light. While not within the timespan of the light intensity study, these spawnings provide further evidence for the importance of light intensity in conditioning *A.akallopisos* for spawning. Although it has not been conclusively proven that light intensity was the critical factor in inducing spawning in this study, the spawning event that occurred under increased light intensity and the subsequent spawnings under the identical light intensity conditions suggest that light intensity may have been a necessary cue for conditioning *A.akallopisos*.

Significant changes in behaviour over the experimental period were obtained for only two behaviours - frequency of head shaking under L1 and frequency of dorsal leaning under L3. This lack of change in behaviour over time suggests that light intensity does not have a major influence on general behaviour patterns of *A.akallopisos*.

The behaviours identified as predictors for spawning - nest cleaning by the female fish and belly touching, were exhibited only by the pairs under the L2 conditions, i.e. the treatment under which spawning occurred. The frequency and duration of nest cleaning activities by both male and female fish in this treatment were significantly higher than those exhibited by fish maintained under both the original light intensity and the light intensity supplemented with natural light. The frequency and duration of nest cleaning exhibited by fish under L2 did not however, fall within the range predicted for spawning pairs. These results indicate that increased light intensity of 8.85×10^{15} quanta.sec^{-1}.cm^{-2}. without the benefit of natural lighting, is instrumental in inducing significant levels of nest cleaning activity.

The other behaviours for which significant differences were recorded were frequency of head shaking by the male and parallel swimming. The results showed the same pattern, with a significantly higher frequency and duration of event being exhibited by fish kept under L2. As a consequence of these findings, it should be tested further if an identical light intensity, supplemented with natural light would produce similar results. Although the behaviours were not significantly different, *A.akallopisos* maintained under the higher light intensity with natural light exhibited more nest cleaning activity, as well as more interaction in total, than fish maintained under the lower light intensity. It was also evident from the duration of exploratory swimming that fish under the two increased light intensities exhibited similar levels of interaction in total and that a greater proportion of time was spent in exploratory swimming by fish under the low light intensity, i.e. less interaction in total than under the other two light intensities.

The results of this study suggest that the light intensity of 4.16×10^{15} quanta.sec^{-1}.cm^{-2} employed in previous experiments was possibly insufficient to condition *A.akallopisos,* and that this species should be maintained at light intensities equal to or greater than 8.83×10^{15} quanta.sec^{-1}.cm^{-2}. Even this light intensity is low when compared to that of metal halides lamps, 2.54×10^{16} quanta.sec^{-1}.cm^{-2} or natural daylight that occurs on tropical reefs, approximately 3.81×10^{17} quanta.sec^{-1}.cm^{-2} (Spotte 1992). The light intensity provided by bio-lux fluorescent tubes should therefore be regarded as a minimum intensity under which to maintain *A.akallopisos* for breeding purposes in the absence of natural lighting.

CHAPTER 8

GENERAL DISCUSSION

The primary objectives of this study were to describe the spawning behaviour of *A. akallopisos* and to document and compare behaviour of *A.akallopisos* under various environmental conditions. The aim of the second objective was to identify behaviour patterns that could be used as predictors for spawning and to obtain a reference point for comparison with behaviour of *A. akallopisos* under various environmental conditions. The experiments were run in a logical sequence in order to find the factors necessary to induce pre-spawning behaviour.

The first experiment investigated the behaviour of *A. akallopisos* under three photoperiods. Photoperiod was investigated as it is one of the basic cues necessary to stimulate gonadal development (Lam 1983). Spawning was not achieved in this experiment, but behaviour was recorded and quantified, and a photoperiod of (14L:10D) was recommended on the basis of the results obtained. *A. akallopisos* began spawning at a later date on a regular basis under the photoperiod recommended in this experiment.

The second factor as recommended by Lam (1983) which should be manipulated under culture conditions to induce spawning is temperature. For practical purposes this was not possible in this study. The temperature selected (26°C) for the duration of the study was based on the natural sea temperature of the area where the fish were collected at the time at which they were likely to be spawning. This temperature also falls within the range of 26-28 °C at which most commercial clownfish hatcheries operate (Hoff 1996).

After photoperiod and temperature manipulation, hormone treatment is most often used to induce spawning. The second experiment investigated the behaviour of *A. akallopisos* under four dosages of Gonadotropin Hormone Releasing Hormone analogue. Although hormone treatment affected behaviour, it did not induce spawning. It is possible that the method of applying the hormone was not suitable for this species.

Rather than pursue further hormone application methods it was decided to investigate the behaviour of *A. akallopisos* maintained with and without their natural anemone hosts. The results showed that fish maintained without their hosts exhibited significantly more interaction that those maintained with anemones. It was therefore suggested that, although anemones may be useful for acclimatising wild-caught *A.akallopisos*, they are not necessary for breeding purposes. The high cost of anemones, and the fact that anemones are not collected from the wild on a sustainable basis, are further reasons for maintaining *A.akallopisos* without their hosts. Successful spawning of *A.akallopisos* maintained in the experimental system without anemones in the months following this experiment proved that anemones are not necessary for spawning in this species.

The final experiment tested the behaviour of *A. akallopisos* under three light intensities. Unlike the previous experiments, light intensity was not chosen because it was an established cue such as photoperiod or temperature for the induction of spawning. Light intensity was chosen as an environmental cue after two systems were studied where *A. akallopisos* were spawning. Environmental conditions in these systems were compared to those in the experimental laboratory. All three systems are compared in Table 8.1. It was found that light intensity was

the major difference between the systems studied. For this reason light intensity was

Table 8.1. Comparison of three systems in which *A.akallopisos* was spawned..

Variable	Experimental system	Sea World	R.U. Hatchery
Number of tanks	12	12	8
Size of tanks (mm)	600x500x380	900x500x480	970x580x580
Tank volume (L)	114	215	340
Tank material	Glass	Glass	Polyethylene
System type	Closed	Closed	Closed
Water changes	15 % /fortnight	20% /4 days	5% /week
Exchange rate	2/hour	2/hour	1,25/hour
Broodstock	Wild-caught	Wild-caught	Wild-caught
Anemones present	Never	Sometimes	Always
Substrate type	Crushed shell	Coral sand	Crushed shell
Lighting	Fluorescent	Fluorescent/Natural	Natural
Photoperiod	14L:10D	12L:12D/Natural	Natural
Temperature (°C)	26 ±1	26 ±1	25 ±1
Salinity (ppt)	34	35	34
pH	8.1-8.3	8.0-8.3	8.2-8.3
NH_4^+-N (mg/L)	≤ 0.01	< 0.01	< 0.1
NO_2^--N (mg/L)	≤ 0.01	< 0.01	< 0.1
NO_3^--N (mg/L)	< 25	< 10	25-50

investigated as a factor in conditioning of *A.akallopisos*. The fish spawned under an increased light intensity and significant differences in behaviour of pairs under the various intensities were recorded. In the months following the end of experiments, *A. akallopisos* began spawning on a regular basis in the experimental system under the increased light intensity. Larvae were reared to the juvenile stage in the same system. The fact that a number of pairs spawned and larvae were reared was not of great importance to the study but it highlighted light intensity as a potential environmental cue for spawning in *A.akallopisos*. It also proved that the eggs and larvae produced by the experimental animals were viable and that the system that had been used for the study was suitable for the breeding and rearing of this species.

The reproductive behaviour and the spawning process of *A.akallopisos* was described and quantified. Three behaviour patterns were identified as predictors for spawning. These were belly touching (by both sexes), nest cleaning by the female fish, and mutual nest cleaning. These behaviour patterns were used as a reference point with which to compare the behaviour of *A. akallopisos* maintained under various environmental conditions.

This study has both practical and academic relevance. On a practical level, hatchery managers and aquarists will be able to use the results obtained to detect predictors for spawning. They will then be able to prepare for the spawning event and rearing process, i.e. culture of live food or setting up of rearing systems. When predictors for spawning are noted, managers will be able to use the observation technique to record frequencies and durations of these events which will enable them to estimate a date on which spawning may occur. As general reproductive behaviour in *Amphiprion* species does not vary very much (Allen 1972), the methods outlined in this study may be useful in assessing the gonadal development of other *Amphiprion* species. In order to do this, observation would first have to be made on other species, and the results compared with those obtained in this study. The observation method used in this study can also be used by fish farmers or aquarists as a means of assessing the development of *A.akallopisos* under various conditions, without sacrificing valuable broodstock. The study has contributed toward the isolation of a suite of conditions necessary for spawning of this species on a regular basis. Although reliable breeding of this species was not achieved, the environmental conditions outlined in Table 8.1 can serve as a guideline for keeping and conditioning of *A.akallopisos.*

This study also has academic merit as little work has been done in this field. Apart from

Okuno's (1963) study, behaviour of *Amphiprion* in captivity has been mainly anecdotal and descriptive. Work done has often been focussed on the relationship between the fish and its host anemone (Mariscal 1970). The behaviour of *A.akallopisos* under different environmental conditions has been documented and quantified. This work can serve to compare the behaviour of other *Amphiprion* species in captivity, or to study the behaviour of *Amphiprion* species in the wild.

Although the ideal approach to this study would be to test all the environmental variables simultaneously, this was not possible. In order for all the factors in this study to be investigated concurrently (3 photoperiods x 5 hormone dosages x 2 anemone treatments x 3 light intensities x 3 replicates), a total of 270 tanks were needed and finances and space for such a large number of aquaria was not available. For such a study, 270 pairs of fish were needed and collecting such a large number of adult pairs from the wild would have had a negative impact on the local population. For these reasons only one factor could be tested at a time. The significance of this is that small changes may occur in the system or to the fish over time. For example, the pairs may have needed a long period of time to acclimatise fully to captivity and to begin spawning, even if conditions were favourable.

Although the observational method used in this study was adequate for the purposes of the investigation, observations may need to be combined with other methods of documenting gonadal development in order to obtain more conclusive results. Methods involving sacrifice of fish were not possible in this study due to the small number of pairs available and the large costs and the legal difficulties involved in collecting numbers of fish in a Marine National Park

(1500 km away from our laboratory). In future studies, sufficient fish should be maintained so that behaviour can be properly correlated with gonadal development. For instance, in a hormone study such as the one described in this study, correlation of blood hormone levels with behaviour could show conclusively whether behaviour influences endocrine status or whether it is the other way round. Similarly, the sacrifice and gonadal staging of fish at various points in a study, such as the light intensity experiment, would substantiate the results obtained from the behavioural observations. In conclusion, while behavioural observations of *A.akallopisos* were able to satisfy the aims of the study, further experiments should include gonadal staging methods to obtain a complete picture of the gonadal development of this species under captive conditions.

REFERENCES

Alava, V.R. and L.A. Gomes. 1989. Breeding marine aquarium animals: The anemonefish. *NAGA, Iclarm* 12(3): 12-13.

Allen, G.R. 1972. *The anemonefishes - their classification and biology.* T.F.H. publications. 286 pp.

Allen, G.R. 1980. *Anemonefishes of the world.* Aquarium systems, Mentor, Ohio. 101pp.

Andrews, C. 1990. The ornamental fish trade and conservation. *J. Fish Biol.* 37: 53-59.

Arnold, C.R. 1991. Precocious spawning of red drum. *Prog. Fish-Cult.* 53: 50-51.

Baggerman, B. 1980. Photoperiodic and endogenous control of the annual reproductive cycle in teleost fishes. In: *Environmental physiology of fishes* (Ed.) Ali, M.A. Plenum Press, New York. pp 533-568.

Bell, L.J. 1976. Notes on the nesting success and fecundity of the Anemonefish *Amphiprion clarkii* at Miyake-Jima, Japan. *Jpn. J. Icthyol.* 22(4): 207-211.

Bok, A. 1997. Marine ornamental fish breeding. Oceanogr. Res. Inst. Spec. Publ. 140:44-45.

Bower, C. E. and T. Holm-Hansen 1980. A Salicylate-hypochlorite method for determining ammonia in seawater. *Can. J. Fish. Aquat. Sci.* 37(5): 794-798.

Boyd, C.E and H.V.Daniels 1988. Comparison of the Hach company fish farmer's water quality kit. *J. World. Aquacult. Soc.* 19(2): 21-26.

Burton, S.M. 1998. The effect of environmental factors and hormone treatments on ovulation rate and spawning success in cardinal tetras, *Paracheirodon axelrodi* (Pisces: Characidae). Msc. thesis. Department of Ichthyology and Fisheries Science, Rhodes University. 142 pp.

Chang, C., Hu, H., and H.Tang 1992. Effects of photoperiod and temperature on testicular development in male ayu, *Plecoglossus altivelis*. *Environ. Biol. Fishes* 34: 309-314.

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

Davis, J. 1993. Report on a visit to Kronborg hatchery, Norway. Unpublished Internal Report. Department of Ichthyology and Fisheries Science, Rhodes University. 4 pp.

Duston, J. and N.Bromage 1986. Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout. *Fish Physiol. Biochem.* 2(1-4): 35-51.

Fautin, D.G. 1991. The Anemonefish Symbiosis: What is known and what is not. *Symbiosis* 10:23-46.

Fautin, D.G. and G.R.Allen 1992. Field guide to anemonefishes and theirhost sea anemones. Western Australia museum, Perth. 160 pp.

Fraile, B., Saez, F.J., Vicentini, C.A., Gonzalez, A., de Miguel, M.P. and R.Panigua 1994. Effects of temperature and photoperiod on the *Gambusia affinis holbrooki* testis during the spermatogenisis period. *Copeia* 1994(1): 216-221.

Frakes, T. and Hoff, F.H. 1983. Mass propogation techniques currently being used in the spawning and rearing of marine fish species. *Zoo Biol.* 2:225-234.

Fricke, H.W. 1973. Individual partner recognition in fish: field studies on *Amphiprion bicinctus. Die Naturwissenschaften* 4: 204-205.

Fricke, H.W. 1974. Öko-Ethologie des monogamen Anemonenfisches *Amphiprion bicinctus* (Freiwasseruntersuchung aus dem Roten Meer). *Z. Tierpsychol.* 36:429-512.

Fricke, H.W. 1979. Mating System, resource defence and sex change in the Anemonefish *Amphiprion akallopisos. Z. Tierpsychol.* 50: 313-326.

Fricke, H.W. 1983. Social control of sex: field experiments with the Anemonefish *Amphiprion bicinctus*. *Z.Tierpsychol.* 61: 71-77

Furukawa, K., Aida, K., Yoshioka, M., Satoh, H. and I.Hanyu 1991. Effects of water temperature and photoperiod on the spawning rhythm of *Shirogushu Silago japonica*. *Nippon Suisan Gakkaishi* 57(12): 2193-2201.

Gerking, S.D. 1980. Fish reproduction and stress. In: *Environmental physiology of fishes* (Ed.) Ali, M.A. Plenum Press, New York. pp 569-587.

Glubokov, A.I., Kouril, J., Mikodina, E.V. and T.Barth 1994. Effect of synthetic GnRH analogues and dopamine antagonists on the maturation of Pacific mullet, *Mugil so-iuy* Bas. *Aquacult.Fish. Manage.* 25: 419-425.

Goldstein, R. 1989. Breeding marine clownfish. *Aquarium Fish magazine.* June issue. pp 18-23.

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

Hattori, A. 1994. Inter-group movement and mate acquisition tactics of the protandrous anemonefish, *Amphiprion clarkii,* on a coral reef, Okinawa. *Jpn. J. Ichthyol.* 41(2): 159-165.

Haydock,I. 1971. Gonad maturation and hormone-induced spawning of the Gulf croaker, *Bairdiella icistia*. *Fish. Bull.* 69(1): 157-180.

Henningsen, A.D. 1989. An introduction to breeding clownfishes. *Tropical Fish Hobbyist* 37(5): 97-99.

Hodson, R.G. and C.V.Sullivan 1993. Induced maturationa and spawning of domestic and wild striped bass, *Morone saxatilis* (Walbaum), broodstock with implanted GnRH analogue and injected hCG. *Aquacult.Fish. Manage.* 24: 389-398.

Hoff, F.H., Rowell, C. and T.Pulver 1972. Artificially induced spawning of the Florida pompano under controlled conditions. *Proc. World Maricult. Soc.* 3: 53-64.

Hoff, F.H., Mountain, J., and T.Frakes 1973. Induced spawning of pompano and seabass. *FAO Aquacult. Bull.* 6(1): 4.

Hoff, F.H., Pulver, T. and J.Mountain 1978a. Conditioning Florida pompano (T*rachinotus carolinus*) for continuous spawning. *Proc. World Maricult. Soc.* 9: 299-309.

Hoff, F.H., Mountain, J., Frakes, T. and K.Halscott 1978b. Spawning, oocyte development, and larval rearing of the Florida pompano (*Trachinotus carolinus*). *Proc. World Maricult. Soc.* 9: 279-297.

Hoff, F.H. 1996. *Conditioning, spawning, and rearing of fish with emphasis on marine clownfish.* Aquaculture Consultants inc. Dade City, Florida. 212 pp.

Johnston, C.E., Hambrook, M.J., Gray, R.W., and K.G.Davidson 1992. Manipulation of reproductive function in Atlantic salmon (*Salmo salar*) kelts with controlled photoperiod and temperature. *Can. J. Fish Aquat. Sci.* 49: 2055-2061.

Juhl, T. 1992. Commercial breeding of clownfishes. *Sea Scope* 11: 2-4.

Kuo, C., Nash, C.E. and H.Shehadeh 1974. The effects of temperature and photoperiod on ovarian development in captive grey mullet (*Mugil cephalus* L.). *Aquaculture* 3: 25-43.

Lam, T.J. 1983. Environmental influences on gonadal activity in fish. p81-154. *In:* Hoar, W.S., Randall, D.J. and E.M.Donaldson (eds). *Fish Physiology*, vol 9B. Academic Press.

Liley, N.R. 1980. Patterns of hormonal control in the reproductive behaviour of fish, and their relevance to fish management and culture programs, p210-246. In: J.E.Bardach, J.J.Magnuson, R.C.May and J.M. Reinhart (Eds.) Fish behaviour and its use in the capture and culture of fishes. *ICLARM Conference Proceedings* 5: 512 pp. ICLARM, Manilla, Phillipines.

Liley, N.R. and E.S.P. Tan 1985. The induction of spawning behaviour in *Puntius gonionotus* (Bleeker) by treatment with prostaglandin $PGF_{2}\propto$. *J. Fish Biol.* 26:491-502.

Liley, N.R., Breton, B., Fostier, A. and E.S.P.Tan 1986. Endocrine changes associated with spawning behaviour and social stimuli in a wild population of Rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrin.* 62: 145-156.

Linhart, O. and R.Billard 1994. Spermiation and sperm quality of European catfish (*Siluris glanis* L.) after implantation of GnRh analogues and injection of carp pituitary extract. *J. Appl. Ichthyol.* 10: 182-188.

Mariscal, R.N. 1970. The nature of the symbiosis between Indo-Pacific anemone fishes and sea anemones. *Mar. Biol.* 6(1): 58-65

Marte, C.L., Sherwood, N.M., Crim, L.M. and B.Harvey 1987. Induced spawning of maturing milkfish (*Chanos chanos* Forsskal) with gonadotropin-releasing hormone (GnRH) analogues administered in various ways. *Aquaculture* 60: 303-310.

Marte, C.L., Crim, L.W. and N.M.Sherwood 1988. Induced gonadal maturation and rematuration in milkfish: Limited success with chronic administration of testosterone and gonadotropin releasing hormone analogues (GnRH-A). *Aquaculture* 74: 131-145.

Miyagama, K. 1989. Experimental analysis of the symbiosis between anemonefish and sea anemones. *Ethology* 80(1): 19-46.

Moyer, J.T. and C.E.Sawyers 1973. Territorial behaviour of the anemonefish *Amphiprion xanthurus* with notes on the life history. *Jpn. J. Ichthyol.* 20(2): 85-93.

Moyer, J.T. and L.J.Bell 1976. Reproductive behaviour of the anemonefish *Amphiprion clarkii* at Miyake-Jima, Japan. *Jpn. J. Ichthyol.* 23(1): 23-32.

Mylonas, C.C., Hinshaw, J.M. and C.V.Sullivan 1992. GnRHa-induced ovulation of brown trout (*Salmo trutta*) and its effects on egg quality. *Aquaculture* 106: 379-392.

Mylonas, C.C., Tabata, Y., Langer, R. and Y.Zohar 1995a. Preparation and evaluation of polyanhydride microspheres containing gonadotropin releasing hormone (GnRH) for inducing ovulation and spermiation in fish. *J. Controlled Release* 35: 23-34.

Mylonas, C.C., Zohar, Y., Richardson, B.M. and S.P.Minkkinen 1995b. Induced spawning of wild American shad *Alosa sapidissima* using sustained administration of Gonadotropin-releasing hormone analog (GnRHa). *J. World Aquacult. Soc.* 26(3): 240-251.

Ochi, H. 1985. Temporal patterns of breeding and larval settlement in a temperate population of the tropical anemonefish, *Amphiprion clarkii. Jap. J. Ichthyol.* 32: 248-257.

Ochi, H. 1989. Mating behaviour and sex change of the aneomonefish, *Amphiprion clarkii,* in the temperate waters of southern Japan. *Environ. Biol. Fishes* 26: 257-275.

Okuno, R. 1963. Observations and discussions on the social behaviours of marine fishes. *Publ. Seto Mar. Biol. Lab.* 11(2): 281-355.

Pankhurst, N.W. 1990. Changes in plasma levels of gonadal steroids during spawning behaviour in territorial male demoisellles *Chromis dispilus* (Pisces: Pomacentridae) sampled underwater. *Gen. Comp. Endocrinol.* 79: 215-225.

Pankhurst, N.W. 1995. Hormones and reproductive behaviour in male damselfish. *Bull. Mar. Sci.* 57(3):569-581.

Pankhurst, N.W. and J.F.Carragher 1995. Effect of exogenous hormones on reproductive behaviour in territorial males of a natural population of demoiselles, *Chromis dispilus* (Pisces:Pomacentridae). *Mar. Freshw. Res.* 46:1201-1209.

Pankhurst, N.W. and G.Van Der Kraak. 1997. Effects of stress on reproduction and growth of fish. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P. and C.B.Schreck (Eds). *Fish stress and health in Aquaculture.* Cambridge University Press. 278pp.

Peter, R.E., Lin, H.R. and G.van der Kraak 1988. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. *Aquaculture* 74: 1-10.

Polder, J.J. 1971. On gonads and reproductive behaviour in the cichlid fish *Aequidens portalegrensis*. *Neth. J. Zoo.* 21 (3): 265-365.

Reese, E.S. 1964. Ethology and marine biology. *Oceanogr. Mar. Biol. Annu. Rev.* 2: 455-488.

Roberts, D.E., Harpster, B.V. and G.E. Henderson 1978. Conditioning and spawning of the red drum (*Sciaenops ocellata*) under varied conditions of photoperiod and temperature. *Proc. World Mariculture Soc.* 9: 311-335.

Ross, R.M. 1978. Reproductive behaviour of the Anemonefish *Amphiprion melanopus* on Guam. *Copeia* 4: 103-107.

Rouger, Y. and N.R.Liley 1992. Effect of social environment on plasma hormones and availability of milt in spawning rainbow trout (*Oncorhynchus mykiss* Walbaum). *Can. J. Zool.* 71: 280-285.

Schreck, C.B., Olla, B.L. and M.W.Davis 1997. Behavioural responses and stress. In: G.K. Iwama, A.D. Pickering, J.P.Sumpter, and C.B.Schreck (Eds). *Fish stress and health in aquaculture.* Cambridge University Press. 278pp.

Sikkel, P.C. 1993. Changes in plasma androgen levels associated with changes in male reproductive behaviour in a brood cycling marine fish. *Gen. Comp. Endocrin.* 89: 229-237.

Spotte, S. 1992. Captive seawater fishes - science and technology. John Wiley & Sons, New York. 941 pp.

Smith, R.J.F. 1969. Control of prespawning behaviour of sunfish (*Lepomis gibbosus* and *Lepomis megalotis*). I. Gonadal Androgen. *Anim. Behav.* 17: 279-285

Smith, R.J.F. 1970. Control of prespawning behaviour of sunfish (*Lepomis gibbosus* and *Lepomis megalotis*). II. Environmental factors. *Anim. Behav.* 18: 575-587.

Smith, M.M. and P.Heemstra (eds). 1986. *Smiths' Sea Fishes.* Macmillan. Johannesburg. 1200pp.

Sukumasavin, N., Leelapatra, W., McLean, E. and E. M.Donaldson. 1992. Orally induced spawning of Thai carp (*Puntius gonionotus*, Bleeker) following co-administration of des gly 10 (D-Arg6) sGnRH ethylamide and domperidone. J. Fish Biol. 40(3): 477-479.

Taylor, M.H., Hyde, L.L., Platt, E.J. and J.R.Day 1985. Ovarian development in *Fundulus heteroclitus*: Photoperiod and light intensity effect. *Am. Zool.* 25(4):69A

Thomas, P and N.W.Boyd 1989. Dietary administration of an LHRH analogue induces spawning of spotted seatrout (*Cynoscion nebulosus*). Aquaculture 80 (4): 363-370.

Tongson, E.E and D.E.Mcallister 1997. Net vs. Cyanide capture of marine ornamental fishes. *AQUARAMA* 5 (4): 1-16. AQUARAMA conference proceedings, Singapore.

Tucker, J.W. 1984. Hormone induced ovulation of Black Sea Bass and rearing of larvae. *Prog. Fish-Cult.* 46 (3) 201-203.

Tucker, J.W. and D.E.Jory 1991. Marine fish culture in the Carribean region. World Aquaculture 22(1): 10-27.

West, G. 1990. Methods of assessing ovarian development in fishes: a review. Aust. J. *Mar. Freshwater.Res.* 41(2): 199-222.

Wood, E. 1985. Exploitation of coral reef fishes for the aquarium trade. *Marine Conservation Society*, Herefordshire, United Kingdom. 121pp.

Wood, E. 1992. Trade in tropical marine fish and invertebrates for aquaria. Proposed guidelines and labelling scheme. *Marine Conservation Society*, Herefordshire, United Kingdom. 35pp.

Zanuy, S., Carrillo, M., and F.Ruiz 1986. Delayed gametogenesis and spawning of sea bass (*Dicentrachus labrax* L.) kept under different photoperiod and temperature regimes. *Fish Physiol. Biochem.* 2(1-4) pp 53-63.

Zar, J.H. 1984. *Biostatistical analysis (2nd edition)*. Prentice Hall inc. Englewood Cliffs, N.J. 718pp

Zohar, Y. 1989. Fish reproduction: its physiology and artificial manipulation. In: M.Shilo and S.Sarig (eds), *Fish culture in warmwater systems: problems and trends.* CRC Press. pp 61-119.