Trans-generational marking of clownfish larvae via maternal transmission of stable isotopes

Thesis submitted by
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STATEMENT OF CONTRIBUTION OF OTHERS

This thesis included some collaborative work with my supervisor Professor Geoffrey P. Jones, Dr. Simon R. Thorrold from the Woods Hole Oceanographic Institute, and Ashley Frisch from James Cook University. Overall, I was primarily responsible for the project concept and design, carrying out the experiments, their analysis and interpretation. Professor Jones assisted in the revision of chapters into a format suitable for publication. Dr. Thorrold carried out the ICP-MS analyses for Chapter 2 at Woods Hole. Ashley Frish assisted with the hormone analyses in Chapter 3.

STATEMENT ON ETHICS

DECLARATION

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Nationoal Sttement on Ethics Conduct in Research involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and *the James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1134)

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Thank you

Abstract

Recent studies on coral reef fishes have successfully employed chemical tagging techniques to quantify local patterns of larval retention and dispersal. Experiments in which larvae were marked via tetracycline immersion of embryos have shown larval dispersal to be more limited than previously thought. However, this technique is limited to fishes that lay eggs on artificial substrata. More recently, a new chemical marking technique has been developed which can be applied to all reef fishes. Females are injected with enriched stable isotopes, such as ^{137}Ba, and the chemical signature is maternally transmitted to embryos and is deposited at the core of the otoliths of larvae. While this technique has been validated for a few species and applied in the field to estimate local dispersal patterns, further laboratory experiments are necessary to determine appropriate injection concentrations and assess any negative effects on larval and adult condition.

The goal of this study was to conduct a series of laboratory experiments to validate the use of trans-generational marking in clownfishes (genus *Amphiprion*). In the first experiment, the minimum dose of ^{137}BaCl for successful marking of *Amphiprion percula* larvae and the period over which females continue to produce marked larvae were evaluated. The effects of barium injections on clutch size, clutch area, size at hatching and subsequent larval growth were also assessed. The fish were subject to three dose levels of ^{137}Ba (0.5, 1.0 and 2.5 µg ^{137}Ba/g fish weight) and the effectiveness of the mark was quantified by measuring the ^{138}Ba/^{137}Ba ratio at the core of the otoliths of recently metamorphosed larvae. All dose levels were 100% successful in providing unequivocal chemical signatures on offspring otoliths. The two highest dose levels, 1.0 and 2.5 µg ^{137}Ba/g fish weight, continued to mark larvae over 6 consecutive clutches, extending over a period of 80 days after a single injection. In females injected with the lowest concentration, 0.5 µg ^{137}Ba/g fish weight, the ^{138}Ba/^{137}Ba ratio returned to the natural barium ratio of 6.385 after 2 clutches (≈40 days). Therefore, while all dose levels could be used to mark larvae, the low dose may require females to be re-injected if longer-term

marking is required. Barium injections had no consistent effects on the clutch size (number of eggs) or the clutch area. A significant interaction between treatment and time was detected for both the length and weight of larvae. The females with the highest ^{137}Ba dose, 2.5 µg ^{137}Ba/g fish weight, produced smaller larvae, but the effect disappeared after the fourth generation. As larval size may be a critical parameter affecting survival, this dosage is not recommended for field studies.

In the second experiment, the effects of ^{137}Ba injections on levels of barium in the tissues of adult females and the period over which barium levels remained elevated were assessed for *Amphiprion melanopus*. In addition, potential effects on adult condition and stress were also evaluated using plasma cortisol analysis. The barium ratios in four tissues (gonads, liver, muscles and bones) were analysed to determine the retention period of barium following two injection doses (2 µg and 4 µg ^{137}Ba/g fish weight). The retained barium level was higher in the bones than in the soft tissues (gonads, liver and muscles) for most sampling times (2, 21 and 56 days). Following the initial elevated barium levels, the ratio of ^{138}Ba/^{137}Ba gradually approached the natural ratio of 6.835, although there was some retention even at 56 days post-injection. The plasma cortisol analysis showed that neither the injection nor the chemical induced any stress to females.

In conclusion, these results suggest barium marking will be 100% effective for marking clownfishes larvae, and provided dose levels are kept to a minimum, there will be no adverse effects on adult females or their offspring.

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1. General introduction

The majority of marine organisms have broad geographic ranges that encompass numerous, seemingly isolated, populations. Given that the majority of marine species have a dispersive larval stage, it has been assumed that each population is demographically "open" and connected to others via larval dispersal (Gaines & Lafferty 1995, Caley et al. 1996, Hixon et al. 2002, Gaines et al. 2007). Knowledge of the magnitude and spatial extent of larval dispersal is considered critical information for understanding how marine metapopulations operate and how they should be managed (Fairweather 1991, Botsford et al. 2003, Hastings & Botsford 2003, Jones et al. 2007). Dispersal is a fundamental process to understanding population dynamics, the management of exploited species, and the conservation of marine biodiversity (Caley et al. 1996, Jones et al. 1999, Swearer et al. 1999, Armsworth et al. 2001, James et al. 2002, Swearer et al. 2003, Zacherl et al. 2003, Fowler et al. 2005, Gillanders 2005b, Jones et al. 2005, Ruttenberg et al. 2005). Information on dispersal is essential for the optimal design of marine protected area networks and to evaluate fishing quotas (from recruitment rates) (Palumbi 1999, Campana et al. 2000, Palumbi 2005, Sale et al. 2005). Dispersal is also an important process to understand and minimise the impact of habitat loss and fragmentation (Hughes et al. 2005, Jones et al. 2007). Despite its significance, there is little direct information on larval dispersal because of the difficulties associated with tracing minute larvae from one population to another.

There are many different approaches that provide clues as to the magnitude and extent of dispersal. Population genetics (Hellberg et al. 2002, Hedgecock et al. 2007), biophysical models (Cowen et al. 2000, Cowen et al. 2006, Werner et al. 2007), spatially explicit or metapopulation models (Armsworth 2002), otolith microchemistry (Thorrold et al. 2002, Thorrold et al. 2007) and studies on larval behaviour (Leis 2006) have all been employed to make inferences about the extent of larval dispersal over different spatial and temporal scales (Hellberg et al. 2002, Hedgecock et al. 2007, Thorrold et al. 2007, Werner et al. 2007). However, none of these approaches provide direct estimates of larval dispersal,

and all have limited utility for quantifying retention within or exchange among local populations. Ultimately, the only way to determine exactly where larvae go is to tag them (Jones et al. 1999, Swearer et al. 2002, Thorrold et al. 2002). While tagging will always be limited to a few species at a few places and times, this direct approach is the only way to ground-truth the estimates of dispersal that are being made from the wide range of indirect approaches.

An understanding of the extent of larval connectivity is particularly important for coral reef fishes, as adult populations are associated with discontinuous coral reef habitats that may be separated by 10's to 100's of kilometres. Most coral reef fishes have a pelagic phase that commonly lasts from 10 to 50 days (Swearer et al. 2002, Irisson et al. 2004, Patterson & Kingsford 2005). Although adults are relatively sedentary, it has traditionally been assumed that there is a high level of connectivity among subpopulations at the larval stage (Sale 1980, Mora & Sale 2002). Most of the information we have on coral reef fish larval dispersal comes from indirect methods such as population genetics (Thorrold et al. 2002, Taylor & Heelberg 2003, Sekino et al. 2005), natural chemical markers (Swearer et al. 2002, Thorrold et al. 2002, Patterson & Kingsford 2005, Arai & Hirata 2006), coupled biophysical models (Cowen et al. 2000, James et al. 2002, Cowen et al. 2006), spatially explicit models (Armsworth et al. 2001) and observations on larval behaviour (Irisson et al. 2004, Simpson et al. 2005a, Simpson et al. 2005b, Leis 2006). However, the problem with these approaches is that none can discriminate populations that are linked by small amounts of larval connectivity on a local scale (Swearer et al. 2002). Despite increasing attention to the subject in the last decade, the extent of dispersal in coral reef fishes remains poorly understood (Swearer et al. 1999, Jones et al. 2005, Palumbi 2005, Ruttenberg et al. 2005).

In the last 10 years, larval tagging studies have challenged the assumption that the pelagic larval stage in coral reef fishes is necessarily associated with a high level of dispersal. Jones et al. (1999, 2005) marked damselfish larvae by immersing developing embryos in tetracycline, and found a significant proportion of the larvae settled on a reef were spawned by parents from the same population. More recently, Almany et al. (2006)

employed a new transgenerational chemical marking technique and showed that as many as 60% of juvenile coral reef fishes on an isolated island were locally spawned. In each case, the origin of larvae arriving from other locations was unknown. Clearly, direct larval marking studies provide a new means of assessing the range of dispersal patterns in reef fishes, particularly those with more limited dispersal.

Larval tagging techniques rely on the approach of marking calcified structures such as otoliths that are formed early in larval development. Otoliths are marked with elements or compounds not present or rare in the organisms' environment, so it can be assumed that juveniles with the right chemical signature have been marked. These markers can potentially include dyes, fluorescent compounds, elemental markers, radioactive isotopes, and ideally, they should be harmless, long-lasting, non expensive, and easily applied (Jones et al. 1999, Bath et al. 2000, Thorrold et al. 2001, Thorrold et al. 2002, Eldson & Gillanders 2005, Gillanders 2005a). The most common method used to mark coral reef fish larvae has been to immerse eggs of demersal spawning fishes in a solution of the fluorescent compound tetracycline (Jones et al. 1999). However, immersing embryos in fluorescent compounds is restricted to species that lay their eggs on the substratum (Thorrold et al. 2002). Given that the majority of reef fishes are pelagic spawners, this limits the utility of this technique.

Recently, Thorrold et al. (2006) established that larvae of both pelagic and demersal spawners can be marked via maternal transmission of stable isotopes of elements that occur naturally in fish otoliths. They injected a rare stable isotope of barium (^{137}Ba) in a soluble chloride form into females of *Amphiprion melanopus* and *Centropristis striata*. They found that all concentrations of barium injected provided an unequivocal mark (a lowered ratio of ^{138}Ba/^{137}Ba) in the core of the otoliths of all offspring from injected females. This demonstrated that the material laid down at the core of the juvenile otolith is maternally derived. The marking technique is powerful because the barium isotope is retained in adult females long enough to mark multiple clutches from a single injection (Thorrold et al. 2006). The field study by Almany et al. (2007) showed that larvae marked by this method can survive and be recaptured in the natural environment.

The aim of this thesis was to further develop the technique of maternal transmission of the stable isotope (^{137}Ba) for reliably and safely marking *Amphiprion* larvae. While Thorrold et al. (2006) found no negative effects on adult survival, adult reproduction or larval survival in *A. melanopus*, they stressed that this technique needs to be validated for all species, before being applied in the field. Here, two laboratory experiments were carried to answer the following important questions: (1) What is the minimum dose of ^{137}Ba that can be used to mark larvae, and how many clutches are marked using different dose levels? (2) At what dose levels are there negative effects on adult reproduction, hatch size or larval growth? (3) What is the temporal profile of ^{137}Ba levels on the different tissues of injected adult females? And (4) Do barium injections increase physiological stress on adult females, and if so, does this affect offspring quality?

This study focuses on two clownfish species, *Amphiprion percula* and *Amphiprion melanopus* as the larvae of these two species can be successfully reared through to settlement in laboratory, making a full assessment of the marking technique possible (Godwin 1994a, 1994b, Wilkerson 1998, Thorrold et al. 2006). Furthermore, the females of these species are easily identified in the field, facilitating the injection (Godwin 1994b). Chapter 2 focuses on *A. percula*, with the aim of determining the minimum dose of ^{137}Ba needed for effective marking and to evaluate the possible negative effects of barium on larval growth/survival and adult condition. The aim of Chapter 3 was to examine the retention of barium isotopes in the different tissues of *A. melanopus* and to evaluate the cortisol level as an indicator of barium-induced physiological stress.

2. Evaluation of the effects of different injected doses of stable barium isotopes on larvae of the clownfish *Amphiprion percula*.

2.1 Synopsis

A new technique of chemically marking larval fish otoliths via maternal transmission of an enriched stable isotope provides a means of quantifying connectivity among coral reef fish populations. However, appropriate dosages and the potential for negative effects on reproduction or larval quality need to be assessed prior to field studies. Here I evaluate the efficiency of different injected dosages of ^{137}BaCl on the successful marking of *A. percula* larvae, and evaluate any negative effects on adult reproduction (clutch size and area) or larval size at hatching and metamorphosis. Three different treatments of ^{137}BaCl (0.5, 1.0 and 2.5 µg ^{137}Ba/g fish weight) were injected in female *A. percula* and the ratios of ^{138}Ba/^{137}Ba were measured by laser ablation ICP-MS at the core of the otoliths of larvae reared through to metamorphosis. All larvae from injected females were unequivocally marked with the barium isotope. The offspring from females given the lowest dose were marked, but only for the first 2 clutches (~40 days post injection). At the highest dose, as many as 6 clutches were marked post-injection, showing effective tagging for over 80 days. No main effects of the different dosages were found on clutch size or clutch area. However, there was a significant interaction between treatment and time on larval weight, attributable to an effect of the highest dose rate on the first few clutches. The results indicate that an extremely low dose of ^{137}Ba can be used to mark larval *A. percula* without any adverse effects.

2.2 Introduction

Most coral reef fishes exhibit a bipartite life history, beginning with a dispersive larval stage, followed by a relatively sedentary adult phase (Sale 1980). Until recently, it has been assumed that the larval stage is associated with long distance dispersal, providing a high level of connectivity among discrete and distant populations (Caley et al. 1996, Mora & Sale 2002). There is considerable indirect evidence for long distance dispersal, including high levels of genetic connectivity over 100's of kilometres (Shulman & Bermingham 1995, Planes 2002, Shanks et al. 2003) and strong physical transport processes that can link populations at these scales (James et al. 2002, Bode et al. 2006). However, the average extent of larval dispersal in each generation is poorly understood and a complete dispersal kernel has never been quantified for a coral reef fish.

Over the last decade, evidence that pelagic larvae do not always disperse far from their natal reefs has been accumulating (Jones et al. 1999, Swearer et al. 1999, Cowen et al. 2000, Swearer et al. 2002, Thorrold et al. 2002, Irisson et al. 2004, Jones et al. 2005, Cowen et al. 2006). The most compelling evidence for limited dispersal has come from new approaches for the mark-recapture of larvae. For example, Jones et al. (1999, 2005) marked damselfish larvae by immersing developing embryos in tetracycline, and found that a significant proportion of the larvae (~30%) returned to the natal populations. In both studies, the origin of larvae arriving from other locations was unknown. Clearly, direct larval marking studies provide a new means of assessing the range of dispersal patterns in reef fishes, particularly those with more limited dispersal. However, mark-recapture studies are based on a number of assumptions which must be validated before estimates of self-recruitment can be considered reliable.

The tetracycline marking technique has limited applicability, since it can only be applied to demersal spawners that lay eggs on artificial substrata (Jones et al. 1999, Jones et al. 2005, Almany et al. 2007). Recently, Thorrold et al. (2006) developed a larval marking technique based on the maternal transmission of stable isotopes that are incorporated into larval otoliths. This technique can be applied to all fish species, provided adult females

can be captured alive and injected with trace amounts of rare, stable isotopes. Laboratory experiments have shown that soluble forms of ^{137}Ba, when injected into gravid females, are subsequently transmitted through the egg and incorporated into the core of the otoliths of offspring larvae. These marks can be detected (using Inductively Coupled Plasma Mass Spectrometry ICP-MS) as a low ratio of $^{138}Ba/^{137}Ba$ that is unique to the otoliths of juveniles derived from injected females. Field trials for the clownfish, *Amphiprion percula* and the butterflyfish *Chaetodon vagabundus* show that large numbers of barium tagged juveniles can be recaptured at natal locations (Almany et al. 2007). Thorrold et al. (2006) presented some qualitative observations that there were no negative effects on the adult survival or reproduction, nor were there any effects on the larval growth and survival. However, further laboratory studies are required to confirm that barium unequivocally marks larvae of a range of species and that barium toxicity does not affect adult reproduction or larval growth or survival.

The overall goal of this chapter was to fully validate the procedure for stable isotope marking larvae of the clownfish *A. percula*. It assesses the effectiveness of different barium dosages in marking larvae from multiple clutches and evaluates the potential effects on reproduction and larval hatch size and growth. The following specific questions were addressed: (1) What is the minimum dose required to mark 100% of larvae from a females' first clutch post-injection? (2) How many subsequent clutches result in marked larvae and how does this vary with different dose levels? (3) How do different doses affect the clutch size (number of eggs) and clutch area laid by each female and do these effects persist for subsequent clutches? (4) How do different doses affect the length and the weight of the larvae at different stages of development?

2.3 Methods

2.3.1 Maintenance of breeding pairs

Adult *A. percula* were kept in plastic aquaria (100 L) in an open water system with constant water flow, constant aeration, controlled temperature (29-30° C), controlled pH (8.0) and controlled salinity (30-32 ppt) at the Marine and Aquaculture Research Facilities Unit (M.A.R.F.U.) of James Cook University. The experiment was conducted between September 2005 and December 2006. The tanks containing breeding pairs were supplied with a terracotta pot plant holder, two bricks and an anemone (*Heteractis crispa* or *Heteractis magnifica*) to create a suitable habitat for breeding and to provide a removable surface for egg laying. The removable structure as substrate for the eggs allowed for the subsequent transfer of eggs to a controlled hatching environment. Also, the incubation period of the eggs from each breeding couple was recorded for several months prior to the experiment to be able to predict the hatching date. Adults were fed a daily ration of high quality frozen food.

2.3.2 Experimental design and injection procedure

Three replicate females from stable pairs of *A. percula* were injected with different dosages of enriched ^{137}BaCl (0.5, 1.0 and 2.5 µg ^{137}Ba/g fish weight). All the injected fish were observed regularly for a period of 24 hours after the injection to record any reactions to the chemical or to the manipulations, however, no adverse effects were observed. Females were injected with barium from a sterile syringe in the abdominal cavity, below the pectoral fin (Figure. 2.1). All females were injected with a standard ^{137}BaCl solution (1375 µg/ml) and different dosages were delivered by varying the volume of the injected solution. The appropriate volume of each injection was calculated individually in relation to the weight of each female (measured to nearest gram).

2.3.3 Breeding and assessment of embryo production

The size and the number of eggs for up to 6 clutches post-injection were estimated to evaluate any effects of different barium dosages. To record clutch size and area, the clutches were photographed as soon as laid and then, analysed. Embryos were monitored very closely to predict accurately when the eggs were going to hatch. When the majority of the embryos had silver eyes (over 60%), around 7 days post-laying, the embryos were ready to hatch and were transferred to rearing tanks.

2.3.4 Larval hatching, rearing and sampling:

At sunset on the predicted hatching day, each clutch was transferred to hatchery tubs in a temperature controlled room to optimise conditions for larval survival. Embryos usually hatched sometime during the following night. The hatching tubs were kept at temperatures between 29°C and 30°C, with constant aeration and water flow. The egg substrate was placed in position so as to expose the embryos to a moderate level of aeration, so as to maximise oxygen exchange and minimise dislodgement.

After hatching, the photoperiod was artificially maintained at a twelve hour cycle. As larvae were sensitive to strong light, green algae were added to the tubs before lights were turned on to reduce stress (Wilkerson 1998). The tanks water salinity was kept between 30 ppt and 36 ppt with a pH of around 8.0. The larvae were fed every twelve hours following a strict diet composed of rotifers (*Brachionus* sp.) and *Artemia* (Nauplii) (Day 1 to Day 4 = 5 rotifers/ml; Day 5 = 5 rotifers/ml + drops of artemias; Day 6 = 4 rotifers/ml + 1 artemias/ml; Day 7 = 3 rotifers/ml + 2 artemias / ml; Day 8 = 3 rotifers/ml + 2 artemias/ml; Day 9-21 = 3 artemias/ml).

To examine the effects of the different barium dosages on size at hatching and growth, samples of five larvae from each clutch were taken on day 1 (day after hatching) and day 14 (after metamorphosis). All remaining embryos were then reared through to the third week of development (21 days), when 10 individuals were

sampled for growth and otolith examination. The specimens were preserved in 90% ethanol for otolith extraction.

2.3.5 Otolith extraction, preparation and analysis

The sagittal otoliths were extracted by dissecting the dorsal section of the skull, then affixed to adhesive tape and stored in Eppendorf tubes. The tubes were sent to the Woods Hole Oceanographic institution for analysis. The analysis consisted of measuring the barium isotope ratios in the otolith cores using a Thermo Finnigan Element2 ICP-MS using a New Wave Research UP213 laser ablation system. The otoliths were prepared for ICP-MS analysis following the procedure outlined in Thorrold et al. (2006). The ICP-MS extracts the different isotopes from the core of the otolith by laser-ablation. Tagged larvae were indicated by a sharp reduction in the ratio of $^{138}Ba/^{137}Ba$ at the core of the otolith. The ratio returns to the natural level further away from the core, corresponding to the time larvae begin feeding and assimilating barium material from the external environment. The altered barium resulting from maternal transmission is permanently locked in the core of the otolith.

2.3.6 Data analysis

A mixed model, hierarchical analysis of variance was carried out on untransformed data considering Treatment, Pair and Clutch as factors. Treatments were specified as fixed, and Pairs and Clutches were specified as random factors. The variable Pair was nested in Treatment for all tests and the variable Clutch was nested in Pair for the tests on the measurements of larval weights and length; because of the sub sample of the clutch.

2.4 Results

2.4.1 Influence of ^{137}BaCl injection dosage on ^{138}Ba/^{137}Ba ratios in the core of juvenile otoliths

An identifiable ^{137}Ba signature was detected in all (21-day old) juveniles raised from the first clutch laid by females injected with ^{137}BaCl, regardless of the dosage (Figure 2.2). All dosages had the same mean lowered ^{138}Ba/^{137}Ba ratios in the first post-injection clutch. However, the different treatments tracked back towards control ratios at different rates, with the lowest dosage 0.5 µg ^{137}Ba/g fish weight indistinguishable from control levels by the third clutch (Figure 2.2). The two higher dosages, 1.0 and 2.5 µg ^{137}Ba/g fish weight, produced progressively higher ratios with each clutch, but even by the sixth clutch, juveniles remained distinguishable from controls.

2.4.2 Breeding and assessment of embryo production

There was no statistically significant effect of the barium dosage level on clutch size (number of eggs) (Figure 2.3) or clutch area (Figure 2.4). However there were significant differences among reproductive pairs within the different treatment levels, both for clutch size (Table 2.1) and clutch area (Table 2.2). The mean clutch sizes and clutch area for the 0.5 and 2.5 µg ^{137}Ba/g fish weight treatments were consistent for all clutches before and after injection (Figure 2.3). However, pairs injected with 1.0 µg ^{137}Ba/g fish weight experienced a larger variation in clutch size and clutch area over time, although no significant effect of treatment was detected.

2.4.3 Effect of ^{137}BaCl treatments on the length and weight of larvae

There were no significant effects of the ^{137}BaCl injection dose on the mean length of the larvae derived from the different clutches post-injection, either at the time of hatching (Figure 2.5a, Table 2.3a) or at 14 days post-hatching (Figure 2.5b, Table 2.3b). However, at day 21 there was a clear trend for a reduction in mean length for

juveniles raised from the first clutch post-injection (Figure 2.5c, Table 2.3c). This effect progressively declined and was similar to the other treatments by the 4th clutch post-injection.

There was no significant effect of the different ^{137}Ba treatments on the mean weights of larvae raised from any of the post-injection clutches (Figure 2.6, Table 2.4). However, there was a significant difference in the weights of larvae produced by individual females, and a significant interaction between females and the post-hatching clutch number (Table 2.4). Although there is an apparent trend for a lower weight of juveniles for the highest dosage, this was not statistically significant.

2.5 Discussion

This study has shown that enriched ^{137}Ba isotopes are clearly an effective means of marking larval *A. percula*, even at extremely low dosages of just 0.5 ug ^{137}Ba/g fish weight. This supports the use of the barium marking technique in field experiments to evaluate larval dispersal patterns (Almany et al. 2007). ^{138}Ba/^{137}Ba signatures were detected in the otoliths of all the first progeny from injected females, confirming that the elemental composition of the core of juvenile otoliths is maternally derived.

For the first clutch produced by treated females, the lowered ^{138}Ba/^{137}Ba ratio in the otolith core was the same for three injection dosages that were trialled. For subsequent clutches, the higher the dosage, the longer the female continued to produce marked larvae following a single injection. That is, at the lowest dosage, female *A. percula* will retain sufficient ^{137}Ba to mark 2 clutches (~40 days), whereas at higher dosages, females will continue to produce marked larvae after 6 clutches (~80 days). This pattern is consistent with the findings of Thorrold et al. (2006) for *A. melanopus*, where all injection doses produced marked larvae, but higher dosages produced marked larvae over a longer period (up to 84 days). Thus, the choice of dosage in any field trial may depend on the period over which successful marking is required. The reason that ^{138}Ba/^{137}Ba ratios in juveniles from first laid clutches are the same, regardless of the injected dose, is unclear. Thorrold et al. (2006) speculated that there may be an upper limit to which the barium ratio can be manipulated.

No statistically significant adverse effects of barium injections on adult reproduction or larval development were detected in this experiment. The hatching time of the embryo pre-injection and the hatching time of embryos post-injection were similar, i.e. between 7 and 8 days. The clutch size (number of eggs) and the clutch area are not influenced by the dosage applied to females. In addition, the sizes of larvae at hatching and at 14 days were not influenced by barium dosages, for any of the clutches produced post-injection. However, although not statistically significant, there was a strong indication that the highest barium dosage resulted in females producing smaller 21-day old juveniles, but

only for the first few clutches post-injection. Further work, with larger sample sizes, will be needed to verify this result. Given that settlement size can have a major effect on subsequent juvenile growth and survival (Jones 1991, Jones & McCormick 2002), barium dosages must be kept well below any level that induces any effect on larval growth.

Considerable variation among replicate pairs of fish was found for a number of the traits examined. Much of the variation among successive clutches, in clutch size, clutch area and larval size, was explained by intrinsic differences among the pairs. Variation among females in egg and larval quality may result from differences in maternal investment, which may affect hatching size and developmental rate. Environmental factors, conditions and physiological processes can influence the development and the growth of larvae. Some of the variation among pairs may have been explained by differences in the assimilation of barium among individuals or error in the applied dose. Hence, greater replication will be required in future experiments to fully account for sources of variation among adult females.

Further work is needed to assess the affects of barium injections on larval survival rates and to examine the threshold dosages after which such affects will be observed. While survival was not quantified in this study, no obvious effects of the different treatments were observed, either for hatching success or numbers surviving to 21 days. Although Choudhury and Cary (2001) found a higher mortality rate of adults exposed to barium, no deleterious effects were recorded in two other animal studies (Yamada & J. 1987, Levin et al. 1993). Despite the fact that barium may have a toxic effect on embryos above a certain threshold (Spangenberg & Cherr 1996), the results for *A. percula* suggest the barium dosages being used are well below this level.

In conclusion, this chapter confirms that enriched barium isotopes are a suitable and an effective means of marking coral reef fish larvae. The strength of this technique is that it is simple to apply and a single injection can mark larvae from multiple clutches. In terms of dosage levels, there appears to be a large window between the lowest dose for effective marking and the lowest dose that causes any demonstrable effects on

reproduction or larval growth. However, given that there were signs that the highest dose used here may reduce the size of juveniles at settlement, this dosage is not recommended unless its safe use can be verified. Also, further validation of this technique is required, with the focus on possible health issues for injected females (see Chapter 3).

2.6 Figures and tables

Figure 2.1 Injection technique; picture taken of *Amphiprion percula*.

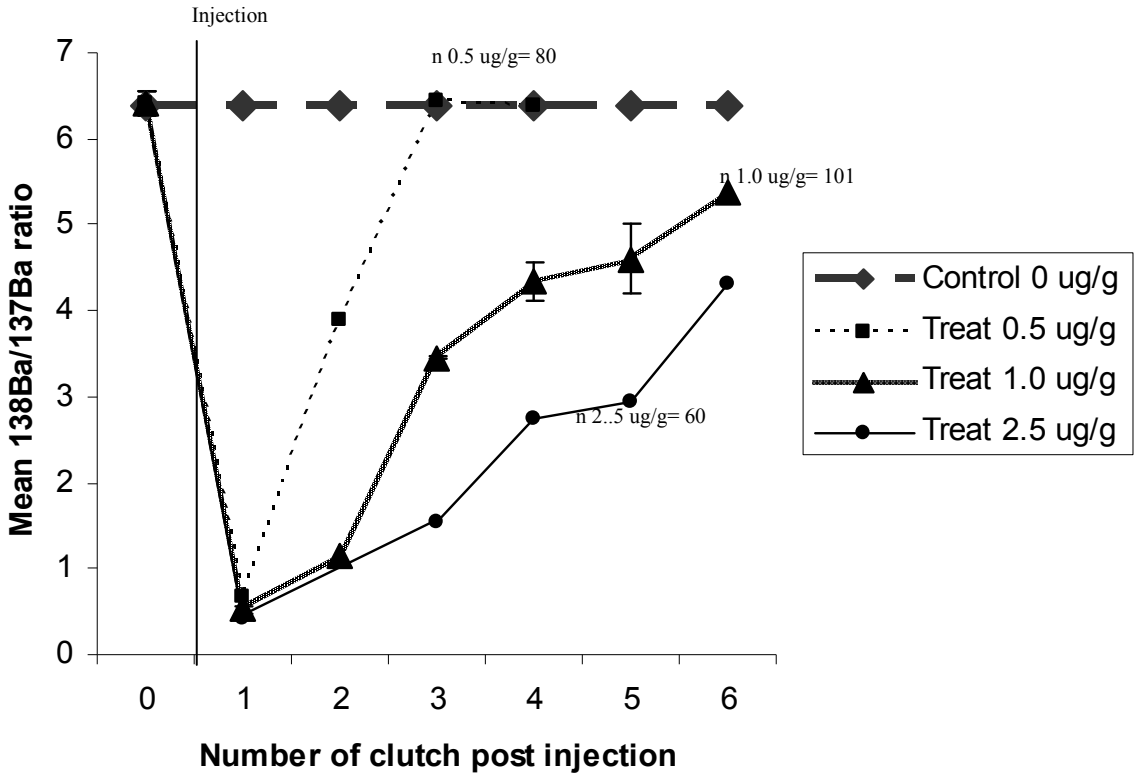


Figure 2.2 Mean $^{138}Ba/^{137}Ba$ ratios +/- SE in the core of the otoliths of juvenile *Amphiprion percula* raised from different clutches laid by females subject to three different doses of $^{137}BaCl_2$. The mean ratio for all control fish was 6.385.

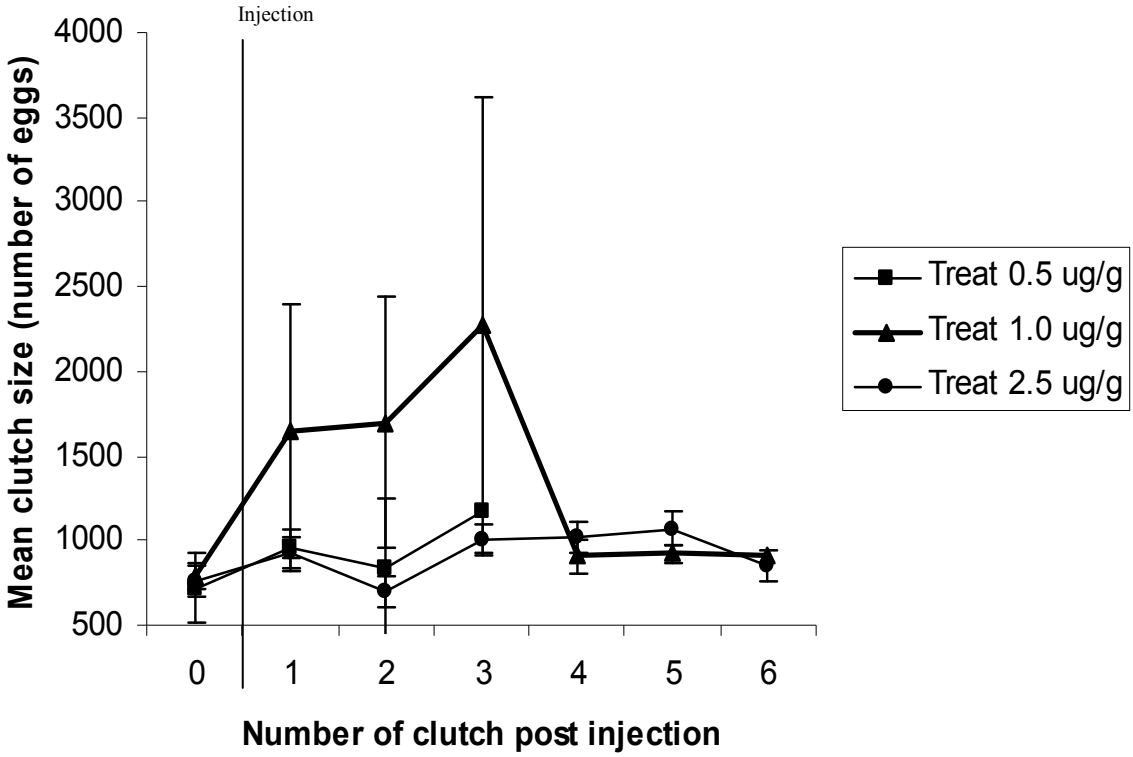


Figure 2.3 Clutch size (number of eggs) +/- SE produced by *Amphiprion percula* pairs, treated with different concentration of ^{137}Ba in function of the number of clutch post injection.

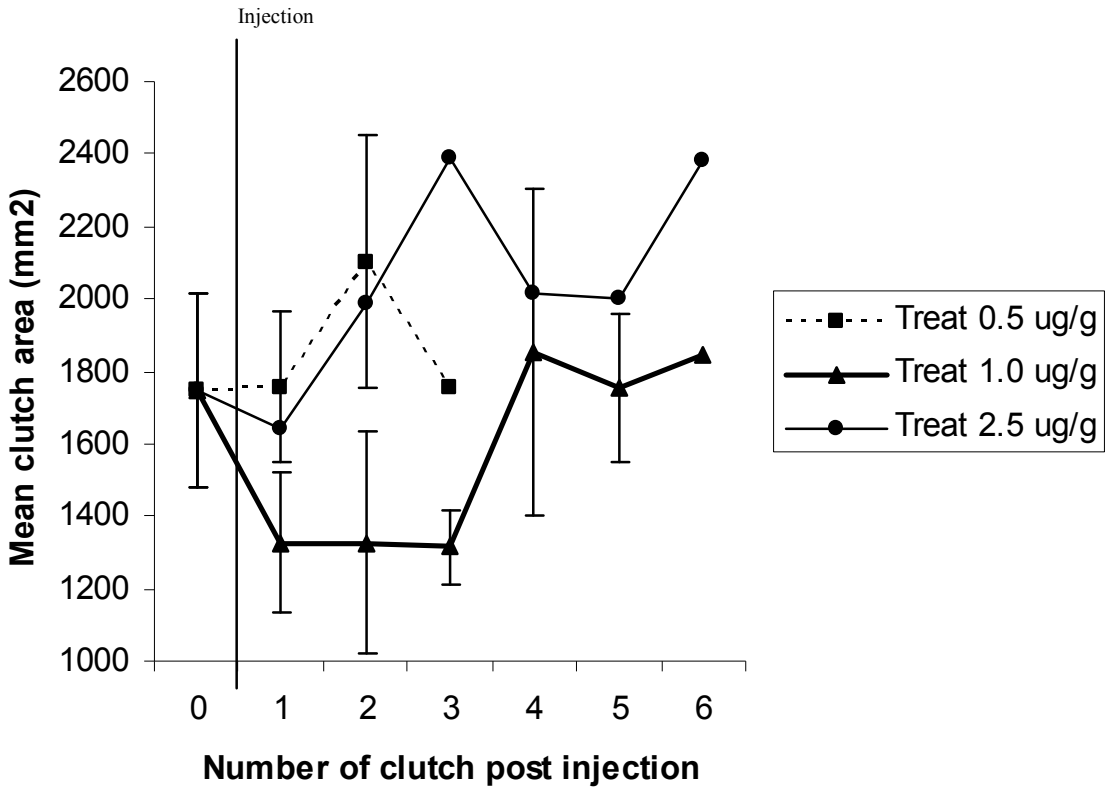


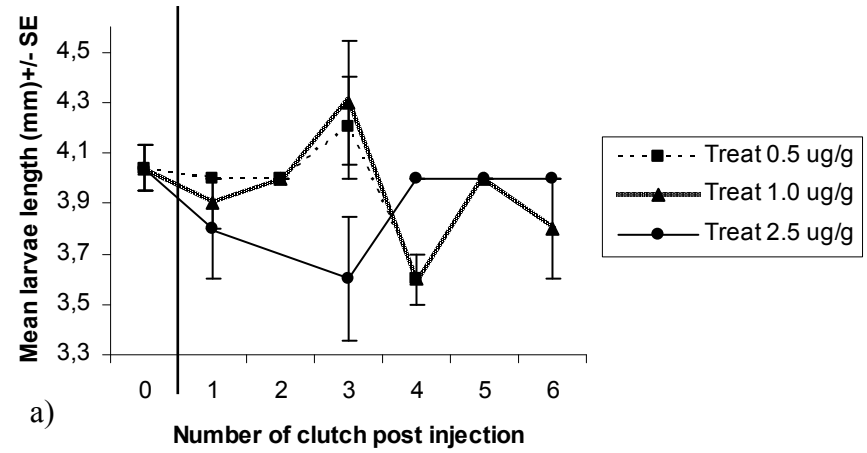
Figure 2.4 Clutch area (mm^2) +/- SE produced by *Amphiprion percula* pairs, treated with different concentration of ^{137}Ba in function of the number of clutch post injection.

Table 2.1 Mixed model, hierarchical analysis of variance for the effect of barium dosage and reproductive pair on the clutch size (number of eggs) of *Amphiprion percula*, for successive clutches at time zero (prior to injection) and for 6 successive clutches post-injection.

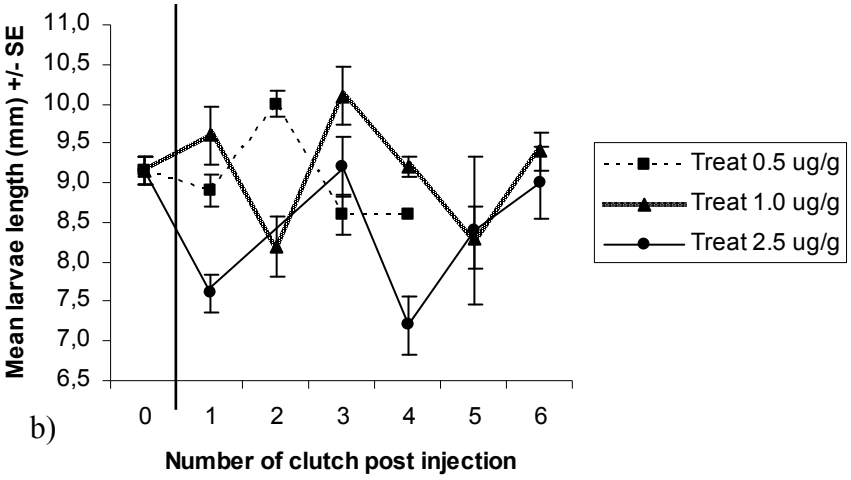
Source	DF	Type III SS	Mean square	F value	P
Treatment	2.1.99	12850	6424.939	0.02	0.977
Pair(Treatment)	2.21	514011	257006	11.72	0.000
Error Ms(Error)	21	460554	21931		

Table 2.2 Mixed model, hierarchical analysis of variance for the effect of barium dosage and reproductive pair on the clutch area of *Amphiprion percula*, for successive clutches at time zero (prior to injection) and for 6 successive clutches post-injection.

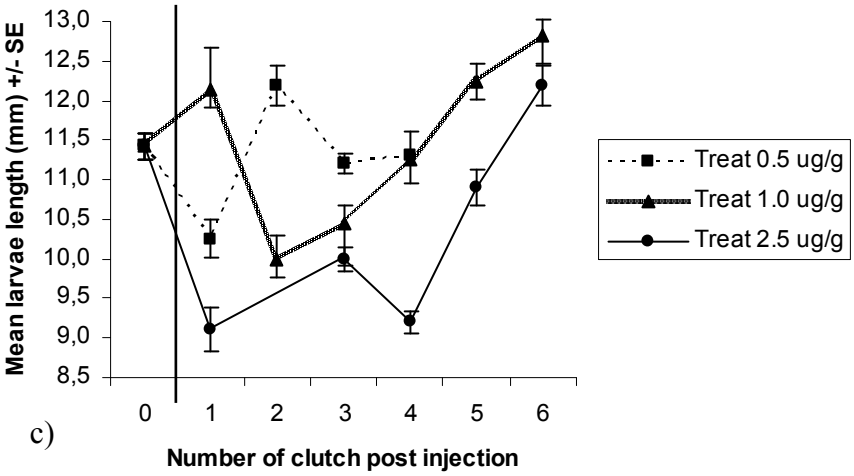
Source	DF	Type III SS	Mean square	F value	P
Treatment	2.1.98	286680	143340	0.09	0.917
Pair(Treatment)	2.21	3034354	1517177	10.53	0.001
Error MS (Error)	21	3025433	144068		



a)



b)



c)

Figure 2.5 Mean larvae length (mm) +/- SE of *Amphiprion percula* pairs, treated with different concentration of ^{137}Ba, sampled at 3 successive post hatching intervals. a) day 1 post hatching b) day 14 post hatching c) day 21 post hatching.

Table 2.3 Summary of ANOVA testing the effect of treatment, pair and clutch on the mean length of *Amphiprion percula* larvae at 3 successive post hatching time a) day 1, b) day 14 and c) day 21.

a)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.83	0.278	0.139	0.416	0.667
Pair(Treatment)	2.83	1.784	0.892	2.681	0.099
Pair*Clutch_post(Treatment)	16.83	5.319	0.332	3.285	0.000
Error	83	8.400	0.101		

b)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.84	12.417	6.209	1.345	0.283
Pair(Treatment)	2.84	31.429	15.714	4.032	0.033
Pair*Clutch_post(Treatment)	19.84	103.885	5.468	8.633	0.000
Error	84	53.200	0.633		

c)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.208	46.53	23.26	1.786	0.196
Pair(Treatment)	2.84	14.87	4.96	0.404	0.752
Pair*Clutch_post(Treatment)	19.84	242.43	13.47	16.275	0.000
Error	84	172.14	0.83		

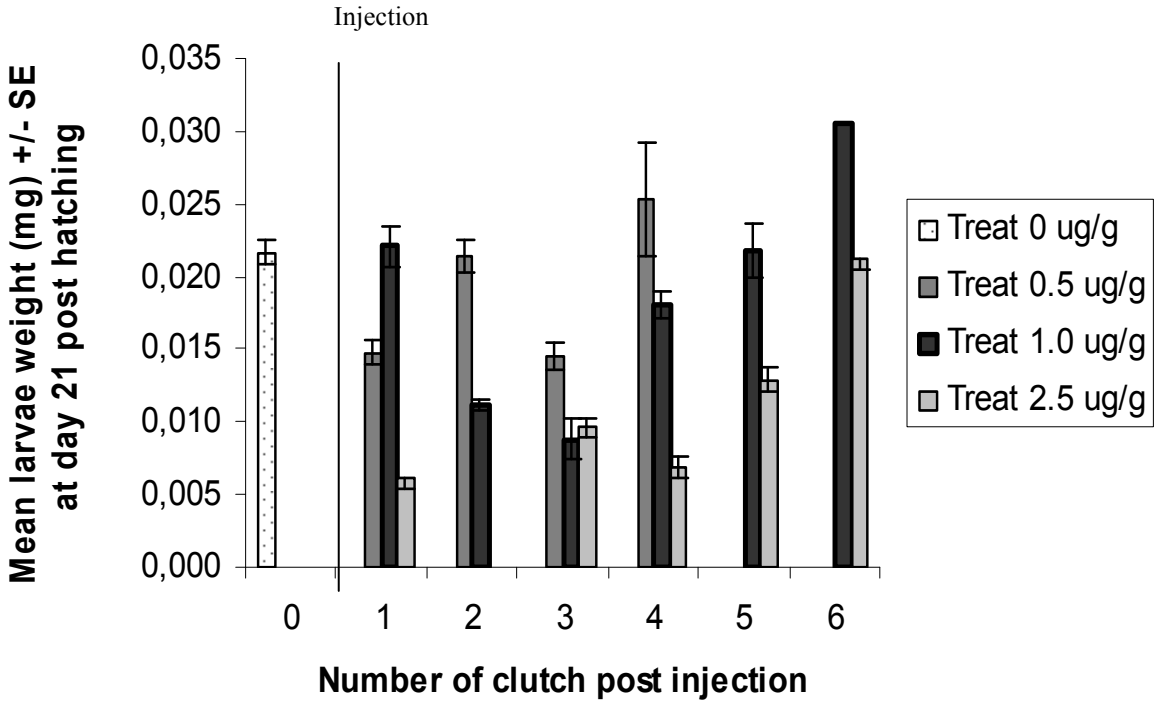


Figure 2.6 Mean weight (mg) ± SE of *Amphiprion percula* larvae at 21 days post-hatching pairs, treated with different concentration of ^{137}Ba.

Table 2.4 Summary of ANOVA testing the effect of treatment, pair and the clutch on the mean weight of *Amphiprion percula* larvae at 21 days post-hatching.

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.1.84	0.001478	0.000739	0.46	0.689
Pair (Traitment)	2.19.25	0.002635	0.001318	3.71	0.044
Pair*Clutch_post(Treatment)	19.207	0.008025	0.000422	30.17	0.000
Error	207	0.002898	0.000014		

3. Incorporation of enriched barium isotopes in tissues of female *Amphiprion melanopus* and assessment of physiological stress.

3.1 Synopsis

Injection of females with enriched isotopic solutions of barium has provided a new means of batch-marking larval marine fishes. The isotopes are maternally transferred to embryos and are incorporated into the core of otoliths of juvenile fishes. However, while the technique appears to have minimal effect on larvae, potential stress on females and long-term health require further investigation. In this study, the effect of injecting two different doses of ^{137}BaCl on plasma cortisol levels in female *Amphiprion melanopus* was evaluated. In addition, the temporal profile of elevated ^{137}Ba was measured for 4 different body tissues – bones, muscles, gonad and liver using ICP-MS analysis. It was hypothesized that altered isotopic ratios of barium would be fixed in bone tissue, but would be rapidly assimilated on soft body tissues. Neither the injection procedure nor the barium treatment appeared to induce stress, as indicated by plasma cortisol levels. Unexpectedly, there was a strong signal of ^{137}Ba in all body tissues that remained even 56 days after injection. There was no significant difference between the two dosages, for any of the tissues sampled. While ^{138}Ba/^{137}Ba ratios were increasing toward control levels, it is clear that ^{137}Ba retention may last many months. The long retention of ^{137}Ba in gonad tissue may explain why barium is so effective in marking multiple clutches from a single injection.

3.2 Introduction

An understanding of the degree of larval retention and connectivity among coral reef fish populations ultimately requires a means of marking larvae (Jones et al. 1999, Thorrold et al. 2002, Jones et al. 2005). While this approach may be limited to a few species or a few locations, mark-recapture methods are necessary to ground-truth other approaches to estimating larval dispersal, including genetic approaches (Shulman & Bermingham 1995, Hellberg et al. 2002, Planes 2002, Shanks et al. 2003, Hedgecock et al. 2007), physical or coupled biophysical models (Cowen et al. 2000, James et al. 2002, Bode et al. 2006, Cowen et al. 2006) and natural variation in otolith microchemistry (Swearer et al. 1999). These indirect methods of assessing dispersal are based on assumptions that can potentially be validated if larvae can be marked and recaptured. Recently, larval tagging approaches have exposed much greater local retention of larvae than would have been predicted or detected by indirect methods (Jones et al. 1999, Jones et al. 2005, Almany et al. 2007). However, mark-recapture techniques are based on their own assumptions, which must be validated in order that estimates of self-recruitment or connectivity can be considered reliable.

One successful approach to tagging coral reef fish larvae has been to immerse embryos of demersal-spawning fishes in tetracycline (Jones et al. 1999, Jones et al. 2005). Tagged juveniles, which have a fluorescent core to their otoliths have been recaptured close to their natal sites. However, this technique is limited to species in which females lay eggs on artificial substrates that can be immersed in tetracycline. Also, when cross-validated with parentage analysis, the method was not 100% reliable (Jones et al. 2005). Recently, Thorrold et al. (2006) developed a method to mark larvae of both pelagic and demersal spawners. It involves the maternal transmission of stable isotopes of elements that occur naturally in fish otoliths into the eggs, and subsequently the larvae of offspring. Rare stable isotopes of barium (e.g., ^{137}Ba), when injected in trace quantities into females of the clownfish *A. melanopus*, were found to be deposited in the core of the juvenile otoliths, and could readily be detected using laser ablation Inductively Coupled Plasma Mass Spectrometry (ICP-MS). This marking technique was successfully applied in the

field to estimate larvae retention at an isolated island for two coral reef fishes (Almany et al. 2007).

Estimates of larval dispersal and retention from larval mark-recapture assume that sufficient isotope is retained in the adult tissues to continue marking offspring for the course of an experiment. While barium is primarily deposited in the calcified bones of teleost fishes, it must be retained in soft tissues or the bloodstream long enough to be absorbed into developing eggs. In addition, the technique assumes that injecting females does not stress females to the point that it has a detrimental affect on reproductive behaviour, egg production or offspring quality. In fishes, initial signs of stress are indicated by a neuroendocrine response, with affected fish producing elevated levels of cortisol within 30 to 60 minutes of exposure to the stress-inducing agent. The affect of barium injections on levels of barium in adult tissues, and potential stress responses, have not been evaluated.

The aim of this chapter was to experimentally examine the effects of single injections of different dosages of ^{137}BaCl on the levels of ^{137}Ba in different tissues of adult females in *A. melanopus*. This species was chosen as it has been the laboratory model for the development of the barium marking technique and it is known that barium isotopes are transferred to larval offspring (Thorrold et al. 2002). The specific questions were: (1) What is the temporal profile of elevated levels of ^{137}Ba in the different tissues of injected adult females, including bones, muscles, gonads and liver? And, (2) Do barium injections increase physiological stress on adult females, as indicated by plasma cortisol levels?

3.3 Methods

3.3.1 Experimental design

A total of 61 specimens of *A. melanopus* were maintained at the James Cook University Marine and Aquaculture Research Facility Units (MARFU) between June and December 2006. In August 2006, 46 replicate fish were injected with one of two different dosages of ^{137}BaCl, based on the range that had previously been used to mark larvae (Thorrold et al. 2002, Thorrold et al. 2006): (1) 2 ug ^{137}Ba/g fish weight, and (2) 4 ug ^{137}Ba/g fish weight. In addition, 15 fish were maintained as controls. Five replicate fish were sampled at four different times: 2, 7, 21, and 56 days post injection to examine levels of ^{137}Ba in the different tissues and to quantify levels of blood cortisol. Three control fish and six fish from each of the two dosage treatments were sampled at each sampling time to assure a minimum of 3 females pre treatment and 1 per control.

All fish were maintained in plastic aquaria (100 L) in an open water system with constant water flow, constant aeration, controlled temperature (29-30° C), controlled pH (8.0) and controlled salinity (30-32 ppt). Injections were made beneath the pectoral fin following the protocol outlined in Chapter 2. The different dosages were delivered by varying the volume of a standard ^{137}BaCl solution injected into fish of known weights.

3.3.2 Gender identification

The 61 fish used in the experiment were classified as female according to the externally visible criteria in Figure 3.1. The fish were anaesthetized with clove oil solution (10 ml clove oil, 50 ml ethanol 90%, 1000 ml salt water) and the genital papilla morphology was examined under a binocular microscope. Male *A. melanopus* usually have a small pinnacle. In contrast, fully developed females have an orifice and when pressure is applied, the ovipositor can be observed. However, this

technique is not 100% accurate, and so some males were inadvertently included in the sample of 61 experimental fish. Hence, to balance the sampling design, only 3 replicate females were analysed for each treatment at each sampling time.

3.3.3 Blood and hormone analysis

In this experiment, fish were caught with small nets and blood samples were taken immediately after the capture. It was predicted that cortisol levels may be high shortly after injections, but decline over time. If barium injections result in additional stress, differences in cortisol levels should be apparent among the different treatments. To test this, blood samples were collected from the caudal artery of each individual sampled. To extract blood, fish were secured in a foam cradle using a 1 ml syringe containing fluoride heparin (Sigma, St. Louis, U.S.A.) with a hypodermic needle (27 G x 1/2). The blood was immediately transferred to a 2 ml vial and kept on ice until centrifugation for 5 min at 3000 rpm. The plasma was removed and stored at -20°C until further analysis. Unfortunately, because of the size of the specimens, only a small amount of blood was available for collection. The average volume of blood collected was 0.5 ml. However, the collected volume of blood was just enough to analyze one hormone (cortisol).

The cortisol concentration was measured by radioimmunoassay (RIA) following extraction from plasma with ethyl acetate using the protocol described by Pankhurst and Carragher (1992). Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid from triplicates of a plasma pool, and assay values for each steroid were adjusted accordingly. Assay specificity was verified by confirming parallelism in the binding curves of serially diluted plasma extracts and steroid standards. The minimum detectable concentration for each assay was 0.075 ng/ml.

3.3.4 Tissue sampling and analysis

Four different tissues (gonads, liver, muscles and bones) were sampled from the females to evaluate the barium level and retention throughout time. Following the extraction, the tissues were freeze-dried to avoid deterioration until further analysis. The analysis consisted of digesting the samples using microwave ovens and analysing the extracts using an ICP-MS spectrometric analysis. An approximately 0.1 g sample of each tissue was placed in a digestion vessel. A solution of 2.5 ml Superpure 70% HNO_3, 1 ml HF and 1ml HClO4 was then added and left to react for 2 hours. Thereafter, the digestion vessels were sealed and put into a microwave oven on high power for 2 minutes. The resulting solution was transferred into a beaker and subsequently heated until dry. Another 1 ml of $HClO_4$ was added and heated again until dry. The dry contents were then dissolved in 10 ml of HNO_3 (20%) and re-diluted to 50 ml. Finally, a 1:1 dilution was carried out before the ICP-MS analysis was done. The samples were analysed by a Varian New ICP-MS and the instrument was externally calibrated using a multi-element standard. Three internal standards, Ga, Y and In, were used to correct instrumental drifts and potential matrix effects, and an independent multi-element standard was used as a quality control sample.

3.3.5 Data analysis

A two-way Analysis of variance was carried out on both the mean cortisol levels and mean $^{138}Ba/^{137}Ba$ ratios in the different tissues considering Treatment and Time as fixed factors. The data was log10 transformed to remove heterogeneity in the variances. All figures and values cited in the text are based on untransformed data (means ± SE).

3.4 Results

3.4.1 Effect of ^{137}Ba on plasma cortisol

There were no statistically significant effects of either treatment level (0, 2 and 4 μg ^{137}Ba/g fish weight) or sampling times (2, 7, 21 and 56 days after injection) (Figure 3.2, Table 3.1). The predicted short-term increase in cortisol due to handling did not arise for any of the treatments. Little of the variation in cortisol levels could be explained by a difference among the two barium treatments and the control. While there is a suggestion of raised levels of cortisol in the barium treatments at 7 and 21 days, this was not statistically significant. The highest mean concentrations, 6.59±2.79 ng/ml and 6.61±3.47 ng/ml were observed for the enriched barium treatments at 21 days. However, most of this variation in cortisol levels appeared to be attributable to the level of individual fish.

3.4.2 Levels of ^{137}Ba in different tissues

The mean ratio of ^{138}Ba/^{137}Ba in the gonads of barium injected females was significantly lower than for the controls, indicating that ^{137}Ba was being retained in gonad tissues (Figure 3.3a). There was no significant effect of sampling time, or an interaction between treatment and sampling time, indicating that ^{137}Ba was being retained in the tissues for 56 days, regardless of the dosage of barium given to the females (Table 3.2a). There was no significant difference in barium ratios for the two different dosages, indicating that a double dose of ^{137}Ba had no effect on the ^{138}Ba/^{137}Ba ratio in the gonad (Table 3.2a). Both the enriched barium treatments resulted in a dramatic reduction in the ^{138}Ba/^{137}Ba ratio in muscle tissues (Figure 3.3b and Table 3.2b). While levels of ^{137}Ba were being depleted over time, the ^{138}Ba/^{137}Ba ratio was substantially lower than controls after 56 days and there was no significant difference between the two barium treatments. The patterns in the ^{138}Ba/^{137}Ba ratio in the liver were similar to the gonad (Figure 3.3c, Table 3.2c). There was a substantial reduction in the ratios for barium injected fish that was maintained across all

sampling times. However, the significant effect of time suggests that the levels of ^{137}Ba were declining by 56 days. Also, there was no significant difference between the two enriched barium treatments. As expected, barium injections also resulted in a large reduction in the ratio of $^{138}Ba/^{137}Ba$ in the bones (Figure 3.3d, Table 3.2d), regardless of the barium concentration. Unlike the other tissues, there was no tendency for levels of ^{137}Ba to decline over time, and the $^{138}Ba/^{137}Ba$ ratios remained steady over time. There was no consistent difference among the two barium levels, indicating that a doubling of the barium dose is ineffective in increasing ^{137}Ba levels in bony tissues. Measurements of barium were more variable among replicates, compared with the soft tissues.

3.5 Discussion

There were no indications from this study that experimental administration of ^{137}Ba has any negative effects on adult females that are likely to compromise the use of this technique for quantifying larval processes. While this technique has been validated by laboratory rearing studies (Thorrold et al. 2006) and applied in situ to measure larval retention in two coral reef fish populations (Almany et al. 2007), potential effects on females were previously unknown. Here I show that levels of stress caused by barium injections are not atypical of laboratory handling procedures. The long period of retention of ^{137}Ba in all body tissues suggests that females can continue to sequester a barium signature in the eggs over at least 2 months, providing a large window of opportunity for recapturing marked larvae in field applications.

Levels of plasma cortisol are recognised as a good indicator of stress in fishes (Pankhurst & Carragher 1992, Pankhurst & Sharples 1992, Godwin & Thomas 1993, Frisch & Anderson 2000, Barton 2002, Frisch & Anderson 2005). High cortisol levels can suppress reproductive hormones and reproductive behaviour, causing a disturbance in breeding and can also influence the size of larvae (Barton 2002). However, the different barium treatments had no significant effect on cortisol levels, nor were there any temporal patterns that could be attributed to handling or injection. Hence it is unlikely that reproduction will be adversely affected by low doses of ^{137}Ba.

The absolute levels of cortisol observed across treatments were not indicative of severe stress. All the cortisol levels obtained in this experiment, between 0.27 ± 0.09 and 6.61 ± 3.47 ng/ml, are under the expected cortisol values for normal active fish of 1.7 to 8.0 ng/ml (Pankhurst & Sharples 1992). While it has been shown that capturing and handling fish can induce stress (Frisch & Anderson 2000, Barton 2002, Frisch & Anderson 2005), there was no elevated levels of cortisol at the time of first sampling in this experiment. The mean plasma cortisol level for other species of fish sampled immediately after being captured was lower than 5 ng/ml (Pankhurst & Sharples 1992, Frisch & Anderson 2000). The results obtained in this experiment are consistent with this value.

The highest observed concentrations of cortisol, 6.59 ± 2.79 ng/ml and 6.61 ± 3.47 ng/ml, were at 21 days post injection for both injection doses (2 µg ^{137}Ba/g fish weight and 4 µg ^{137}Ba/g fish weight). While it is possible that the barium injections resulted in elevated cortisol at this time, these maximum values obtained in the experiment are lower than the ones found in other studies that have estimated cortisol levels (Robertson et al. 1988, Frisch & Anderson 2000, Frisch & Anderson 2005). Cortisol levels have also been found to vary in *A. melanopus* as a function of the lunar cycle and the stage of maturation (Godwin & Thomas 1993, Godwin 1994a, 1994b). The sample taken on September 7th , 7 days post injection, was taken on the day of the full moon and could explain the second highest level following 21 days, 2 µg ^{137}Ba/g fish weight had a value of 3.95 ± 3.00 ng/ml and 4 µg ^{137}Ba/g fish weight of 5.40 ± 1.68 ng/ml.

The physiological retention of enriched barium is an important factor in evaluating the utility of the barium injection marking technique. While barium is primarily incorporated into calcified structures such as bones or otoliths (Dietz et al. 1992, Foster et al. 1998, Choudhury & Cary 2001, Elsdon & Gillanders 2002), it must remain free in the bloodstream and soft tissues to continue to be transmitted to eggs and larvae. The experiment showed that elevated levels of ^{137}Ba remained in all soft tissues of *A. melanopus* for the duration of the study (56 days). This explains why females given a single injection of ^{137}Ba can continue to produce marked larvae for several months (Thorrold et al. 2006).

The period over which ^{138}Ba/^{137}Ba ratios remained altered in soft tissues was much longer than that observed in the only other study on a coral reef fishes. Williamson et al., (submitted 2007) showed that for the pelagic spawning coral trout (*Plectropomus*), barium levels has returned to control levels over the same time period, except in bony structures. This may be because smaller quantities of ^{137}Ba are transferred to individual eggs in pelagic spawners, compared with the demersal spawning clownfishes.

In *A. melanopus*, different dosages of barium had no effect on the measured signal of ^{137}Ba (^{138}Ba/^{137}Ba ratio) in any of the tissues examined. This is significant, because it

means that there is no advantage to increasing the ^{137}Ba dose, either for the quality or duration of the mark. In Willianson's experiment the gonads retained ^{137}Ba for only 8 weeks which would suggest that after this period of time no more larvae would be marked. In this case an increase of the dosage would be favourable to mark more larvae of pelagic spawners.

The period of physiological retention in clownfishes was also much higher than recorded for other organisms. For example, barium levels in the skeletal muscles of rodents exhibited a significant decrease after 24-48 hours and returned to almost zero after few days. Harrison et al. studied humans exposed to ^{133}BaCl from injection and level zero was reached after 15 days (Harrison et al. 1966). The variation among different tissues and organs in the time taken for barium to reach control levels can be explained by the surface area hypothesis and by the study from Roza and Berman that tested the effect of intravenous injection of barium on the muscles of dogs (Choudhury & Cary 2001). It was suggested that larger organs will assimilate a higher concentration of barium because of their higher surface area. The specimens for this experiment were of different size and would have influence the amount of barium retained. Previous work has shown that barium is rapidly incorporated into developing bones (International Programme on Chemical Safety 1990, Foster et al. 1998, Choudhury & Cary 2001). Choudhury and Cary (2001) found in one experiment with rats that 78% of administered barium was found in the bones after only one day and 95% after 11 days. Barium is also known to be incorporated rapidly into kidneys, but can be found in all organs (Dietz et al. 1992, Koch et al. 2003).

As expected, elevated levels of ^{137}Ba were fixed in the bones of *A. melanopus* and were not assimilated over time. The ^{138}Ba/^{137}Ba ratios were not as low as detected in the study on the pelagic spawning coral trout (Williamson et al., submitted 2007), which suggests that prolonged retention in soft tissues is associated with reduced deposition in bony structure. Indeed, once barium is incorporated into bones it is not reworked or made available in other tissues (Borzelleca et al. 1988, Forbes 1989, HealthCanada 1990, International Programme on Chemical Safety 1990, Wones et al. 1990, Dietz et al. 1992,

NTP 1994, Foster et al. 1998, Shackleton et al. 2000, Choudhury & Cary 2001, Elsdon & Gillanders 2002, Koch et al. 2003, Montazeri et al. 2005).

Few previous studies have examined barium retention in different body tissues. Some of these studies have indicated, as the present study, that there are no adverse effects of barium ingestion on the reproduction and development of the parents or of the larvae (Dietz et al. 1992, Foster et al. 1998, Choudhury & Cary 2001). Dietz et al. (1992) observed sperm parameters and vaginal cytology of rats and mice and found no effects on reproduction or on the offspring after a long term ingestion of barium. He also found no significant trace of barium in the gonads or in other reproductive tissues (Dietz et al. 1992). Another study by Ridgeway & Kanofsky recorded no undesirable effects after injecting barium directly into chick egg yolks at early egg stage (Choudhury & Cary 2001); implying no effect of barium on larvae development. Some other studies have, on the other hand, observed a below normal pregnancy rate in many exposed species (Foster et al. 1998, Choudhury & Cary 2001). For example, Borzelleca et al. noticed a reduction in ovary weights of rats, but no effect on overall reproduction was measured (Borzelleca et al. 1988). Furthermore, Tarasenko et al. recorded some underdevelopment on rat embryos development after being exposed to a high dosage for a very long period time (Choudhury & Cary 2001). However, the mean barium ratios found in the gonads in this experiment suggest that concentrations were high enough to mark larvae but low enough to avoid causing any disturbance in the adults.

In conclusion, the plasma cortisol levels showed neither the injection nor the chemical caused any notable stress on adult female *A. melanopus*. The injection of enriched barium showed a prolonged period of physiological retention in all tissues, which explains why this technique is so effective in the production of marked larvae for so long after a single injection (Thorrold et al. 2006). The period of retention appears to be longer than for pelagic spawners (Williamson et al., submitted 2007), which may be because demersal spawners like clownfishes transfer greater amounts of maternally derived nutrients to individual progeny. Therefore, field studies involving pelagic spawners may require repeated injections of females over the same time periods. The

results of this study are consistent with the finding that there may be an upper limit to the degree to which the $^{138}Ba/^{137}Ba$ can be manipulated. That is, a doubling of the ^{137}Ba dosage does not lead to any significant decrease in the $^{138}Ba/^{137}Ba$ ratio in any of the adult tissues (this study) or in the otoliths of progeny (Thorrold et al. 2006). This is significant given the potential expense of barium isotopes and possible adverse effects of too much barium on larvae. This study strongly argues for evaluating the minimum barium dose required for significant retention in adult tissues over the period of a proposed experiment.

3.6 Figures and tables

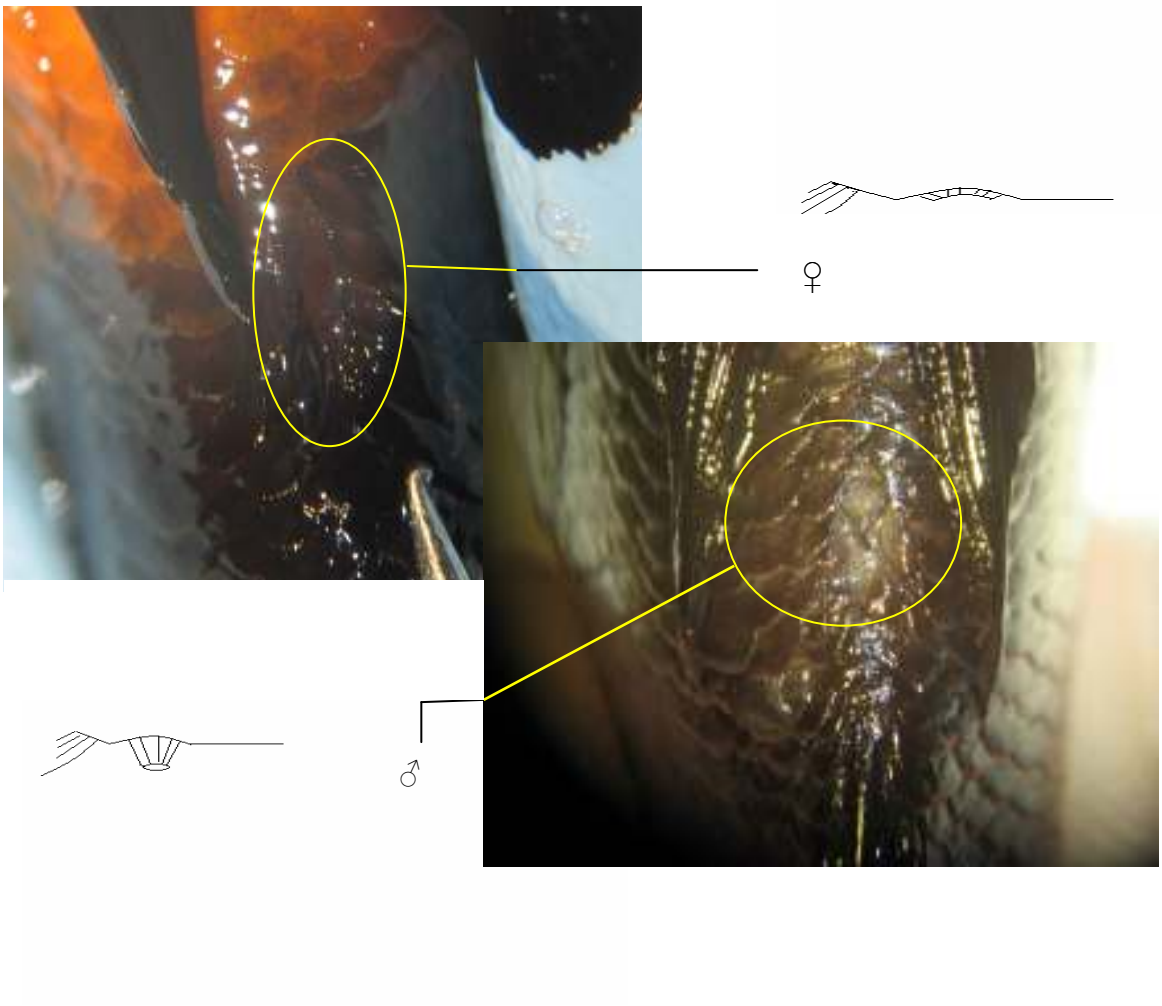


Figure 3.1 Sex determination of *Amphiprion melanopus* adult's.

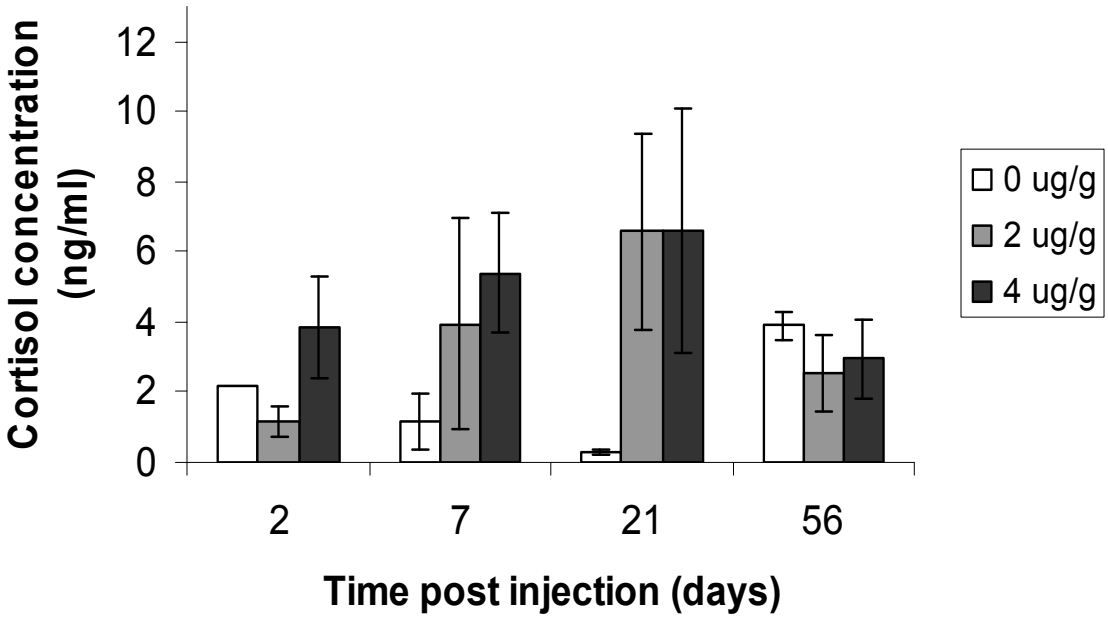


Figure 3.2 Cortisol concentration (ng/ml) ± SE in function of time and ^{137}Ba concentration (µg ^{137}Ba/g fish weight) injected in female *Amphiprion melanopus*.

Table 3.1 Summary of ANOVA presenting the test of the effect of treatment (2 µg ^{137}Ba/g fish weight and 4 µg ^{137}Ba/g fish weight) and sampling time (2, 7 21 and 56 days after injection) on the level of plasma cortisol in *Amphiprion melanopus*.

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.21	0.682	0.341	1.124	0.344
Time	3.21	0.322	0.107	0.354	0.786
Treatment*Time	6.21	2.056	0.343	1.130	0.379
Error	21	6.368	0.303		

Table 3.2 Summary of ANOVA presenting the test of the effect of treatment (2 µg ^{137}Ba/g fish weight and 4 µg ^{137}Ba/g fish weight) and time (2, 7, 21, 56 days post injection) on the Ba ratio in a) gonads b) muscles c) liver d) bones of *Amphiprion melanopus* females.

a)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.20	1.229	0.615	3.787	0.040
Time	3.20	1.127	0.376	2.314	0.107
Treatment*Time	6.20	0.456	0.076	0.468	0.824
Error	20	3.246	0.162		

b)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.20	0.972	0.486	6.870	0.005
Time	3.20	1.548	0.516	7.292	0.002
Treatment*Time	6.20	0.308	0.051	0.725	0.634
Error	20	1.415	0.071		

c)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.20	0.751	0.375	4.121	0.032
Time	3.20	1.719	0.573	6.290	0.004
Treatment*Time	6.20	0.464	0.077	0.848	0.548
Error	20	1.822	0.091		

d)

Source	DF	Type III SS	Mean square	F value	P
Treatment	2.20	1.048	0.524	4.546	0.024
Time	3.20	1.055	0.352	3.050	0.052
Treatment*Time	6.20	0.485	0.081	0.701	0.652
Error	20	2.305	0.115		

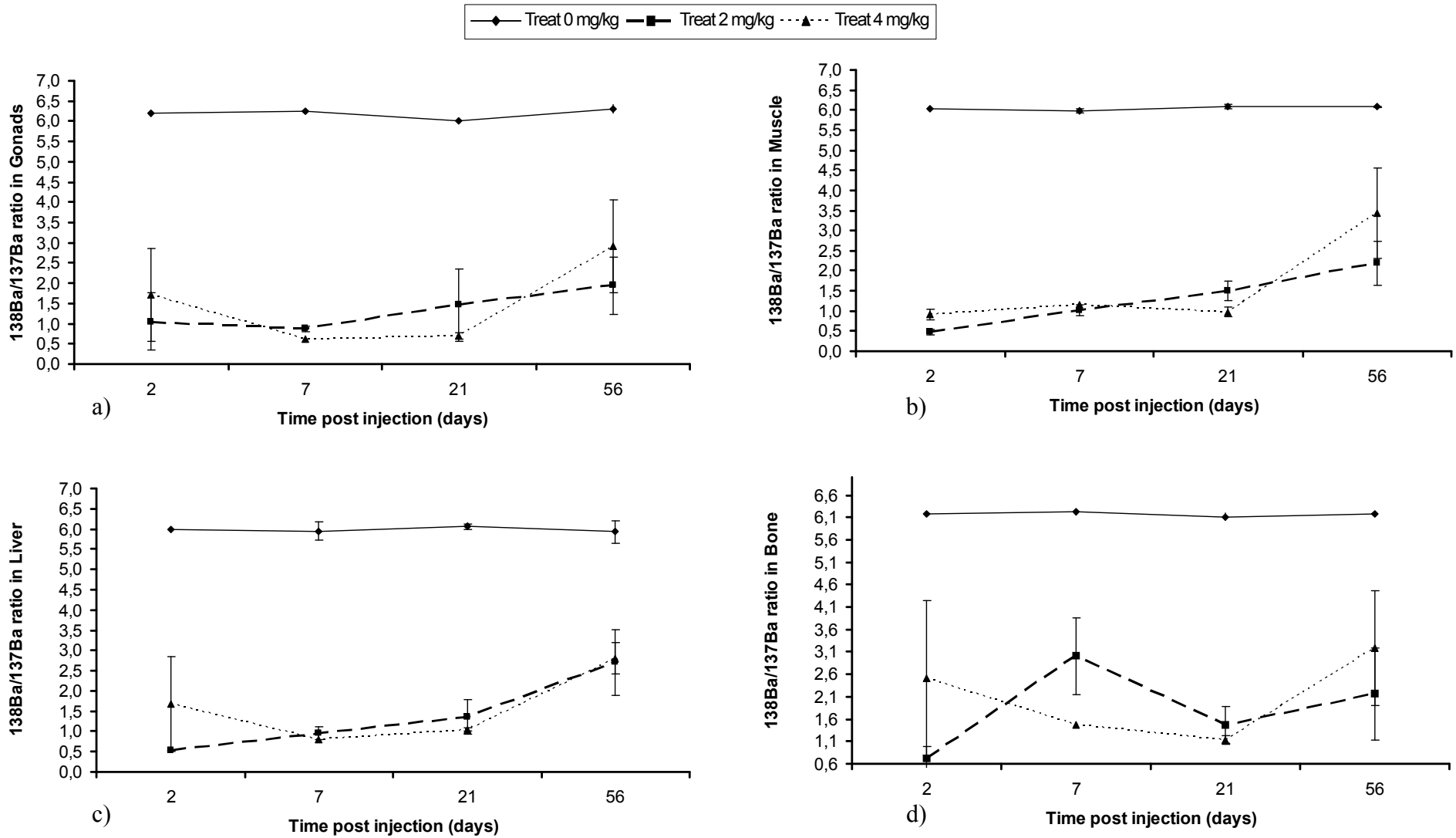


Figure 3.3 Mean $^{138}Ba/^{137}Ba$ ratio ± SE in a) gonads b) muscles c) liver d) bones of *Amphiprion melanopus* control females or injected females with 2 ug ^{137}Ba /g fish weight or 4 ug ^{137}Ba /g fish weight sampled at four different times post injection.

4. General conclusions

Overall, this thesis has confirmed that injections of enriched stable isotopes of barium are safe, reliable and effective means of mass-marking larval coral reef fishes (Thorrold et al. 2006, Almany et al. 2007). When provided barium is administered at the right dosage, no adverse effects on either larvae or adults of clownfishes (*Amphiprion*) should be encountered. The long period of retention in adult tissues of clownfishes corresponds to an equally long period over which females will pass on an enriched isotope signature to their larval progeny.

The first chapter confirmed previous work showing that injections are 100% effective in marking offspring over a broad range of dosages, without any ill-effects on larval progeny over most of this range. While there is some evidence that high doses of barium will eventually impair larval growth, increasing the dose of barium to such levels does not appear to increase the amount of barium transmitted to eggs and larvae. That is, the quality of the mark remains the same as for intermediate dosages. At the other extreme, while very low dosages are also effective in marking the first progeny, females given such doses may only mark larvae over a short period post-injection. Hence, it is very likely that there will be some intermediate dosage that will be the biological optimum. However, the technique is clearly robust to considerable variation around this value. Given the monetary cost of barium isotopes and their analysis, keeping the barium dosage to a minimum will be an important factor in any field application of this technique.

The second chapter showed clearly why barium injections are so successful in marking the larvae of successive clutches over periods of months after injections (Thorrold et al. 2007). Elevated levels of ^{137}Ba were observed in all tissues for up to 56 days after a single injection, making it available for transmission to eggs and larvae. This long period of retention was not associated with any significant increase in plasma cortisol levels. The period of retention was longer than that observed for a pelagic spawner (Williamson

et al., submitted 2007). However, further work on the influence of spawning mode on barium retention time is required.

While trans-generational marking of clownfish larvae via maternal transmission of ^{137}Ba is safe and effective, further work is needed to validate the use of this technique for other fishes with different reproductive modes. The technique clearly works for pelagic spawning groupers (Thorrold et al. 2006) and butterflyfishes (Almany et al. 2007), but possible effects on larval quality and adult condition require further investigation. Currently this is limited by our inability to successfully raised larvae through to metamorphosis and settlement. In addition, stable isotopes of over elements such as Strontium and Calcium should be evaluated, to increase the number of population markers available. Larval marking techniques may never be applied to all populations of all species, simply because they are expensive and time-consuming to carry out. However, they remain the best prospect we have of validating general models of larval dispersal in coral reef fishes.

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