Parental and environmental effects on the early life history of a tropical reef fish, *Amphiprion melanopus*

PhD thesis submitted by

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STATEMENT OF CONTRIBUTION OF OTHERS

This thesis includes some collaborative work with my supervisor Dr Mark McCormick, Dr Kenneth Anthony and Dr Rebecca Fisher. While undertaking these collaborations, I was responsible for the project concept and design, carrying out the experiments, their analysis and interpretation and synthesis of the results into a format suitable for publication. My co-authors assisted financially, with editorial advice and technical instruction for experimental equipment.

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General abstract

Tropical coral reef fish larvae are characterised by high mortality, which is predominantly driven by size- and growth- selective processes. While recent studies of environmental correlates have explained 7 - 36 % of the variation in larval growth rate in wild populations, the majority of the variation in growth rate and recruitment remains unexplained. This thesis used a series of laboratory experiments to assess the contribution of environmental and parental influences on embryonic, larval and juvenile growth and development in a tropical marine fish species, *Amphiprion melanopus* (Pomacentridae).

Maternally determined egg size coupled with clutch micro-environment was important in determining initial offspring size. By sampling embryos and larvae for morphometric measurement and metabolic rate, we found that size differences between offspring within a clutch were related to the clutch design. Eggs on the periphery of newly laid clutches were 2% smaller than eggs from the centre, and this size difference increased throughout embryonic development. Larvae hatched from the clutch periphery were 6 – 8 % smaller than larvae hatched from the clutch centre. Embryos on the clutch periphery had 63 % lower rates of oxygen consumption. Changes in oxygen consumption throughout development were related to developmental changes within the embryo.

Given that the study species, like many demersal spawning fishes, has parental care of the eggs, we explored whether parental tending modified the oxygen microenvironment of the embryos, and subsequently, whether tending was modified according to ambient dissolved oxygen (DO), increasing metabolic demands of developing embryos and water temperature. There was a time lag of 1 second between fanning and increases in the amount of oxygen within the nest, demonstrating that DO is directly affected by parental tending. Males invested more time tending nests (40 % initially) than did females (20 – 30 %), and male investment increased to 70 % as embryo development progressed. Additionally, male fish adjusted fanning effort on a diel cycle as ambient DO fluctuated. The female's investment in nest tending was minor in comparison to

the males and did not change with ontogeny, with the exception of a small increase in activity just prior to hatching. Nest tending appears to be an important mechanism whereby males can invest in the survival of their offspring.

To determine the relative importance of maternal, paternal and environmental (specifically temperature) influences on early life history traits, we experimentally examined their interactive influences on larval growth, swimming ability and developmental rate using a full factorial (diallel) breeding design. There were strong paternal and maternal influences in size at hatching and metamorphosis, and surprisingly, paternal affects were responsible for 52 % of the variation in growth rate, while 30 % was attributable to the combination of temperature*female*male. We speculate this was due to the significant male contribution through their key role in nest tending. Pre-hatch egg size, post-hatching larvae size and size at metamorphosis all showed significant influences from male and female, and the interaction of these, while temperature had minimal influences on size at particular development stages. Temperature did, however, reduce developmental rate, increasing the time taken to reach metamorphosis by 50%. Larvae reared in water 25 $^{\circ}$C (3 $^{\circ}$C below ambient) were smaller than larvae reared at ambient temperature (28 $^{\circ}$C) at the same age (7 days after hatching, dah), and had slower critical swimming performance but took longer to metamorphose (mean: 8.9 ± 0.06 days at 28 ºC and 11.6 ± 0.09 days at 25°C). When this slower developmental time was factored in to size and swimming, fish reared at 25 $^{\circ}$C were larger at similar developmental age (11dah, pre-metamorphosis). This stage-specific size increase did not result in better performance as there was no difference in swimming ability immediately prior to settlement (11dah), despite slower swimming for larvae raised at 25 $^{\circ}$C, 7dah.

This thesis shows that position of an embryo within a clutch, maternally-determined egg size and subsequent parental care were important in influencing the condition and performance of marine fish embryos and larvae. Size advantages began in the embryonic stage due to maternal investment through gametogenesis and the allocation of endogenous reserves to the egg, and were enhanced throughout development. This thesis suggests that parental contributions to the embryo were important to size, growth and performance of

larvae, and may be the source of previously unexplained variation in larval growth and survival.

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General Introduction

Marine fish are characterised by high fecundity and almost equally high mortality. Despite the importance of larval survival to successful recruitment and replenishment of fish stocks, the processes underlying mortality are poorly understood. Commencing with Hjort's influential paper in 1914, nearly 100 years of research has related population-level recruitment (as the end-product of larval survival) to broad-scale environmental variation within the larval period (Houde 1974). The primary focus of such research was to predict the strength of recruitment by correlating larval growth (Tupper & Boutilier 1995) and condition (Cushing 1972, Theilacker 1978) to environmental variability such as food availability (Lasker 1975, Hunter 1981) and temperature (Brett 1967, Houde 1989a). These critical studies determined that variation in daily growth and mortality rates can lead to orders of magnitude difference in survival and recruitment (Houde 1989b, Cushing & Horwood 1994).

The multitude of factors that act on the early life history stages of marine fishes, affecting growth, condition and survival until recruitment, fall into three key categories: 1) genetic inheritance; 2) environmental influences; and 3) parental effects. Selection based on these factors operates on individuals, and is consequently expressed in populations. This thesis examines how parental and environmental influences are borne into the early life history traits of fishes within clutches in tropical marine fishes, investigating differences within and between clutches at all stages of development leading up to recruitment. Genetic inheritance is beyond the scope of the present study and will not be considered further here.

Environmental influences comprise a broad spectrum of factors that impact on growth and condition of larval fish, including abiotic influences, such as: temperature (Green & Fisher, 2004), salinity (Swanson 1996) and turbidity (Fiksen & Folkvord 1999); and biotic factors, such as: food availability (Green & McCormick 1999a), predators (Margulies 1989) and competition (Bystrom & Garcia-Berthou 1999). As it is not possible to examine all components of environmental variation that a developing fish might encounter, we have

selected the two most influential traits on fish metabolism in early development: temperature and dissolved oxygen (Rombough 1988, Jobling 1995), for examination in this thesis.

The majority of research on factors affecting the early life history of marine fishes and the recruitment/mortality relationship has focused on temperate northern hemisphere species, generally of commercial importance. Although these studies have provided a solid framework of theory for investigations into recruitment in tropical fishes, several critical environmental and biological attributes of tropical marine fishes differ from their temperate counterparts. Key developmental stages are much shorter (Green & McCormick 2001) and the larval stage finishes with a distinct habitat shift from plankton into the benthic reefal habitats of adults (see discussion in Bergenius et al. 2002). In the tropics, temperatures are higher and fluctuate less than temperate waters (McGregor & Nieuwolt 1998). For example, sea surface temperatures on the Great Barrier Reef, Australia, fluctuate from 4-6°C seasonally, and 1°C diurnally (McGregor & Niewolt 1998). The relative importance of temperature change in the tropics has been alluded to (Rombough 1997, Hunt von Herbing 2002), but rarely tested. Early development such as the larval phase is especially susceptible to temperature change (Jobling 1995, Rombough 1997), as it influences metabolism, growth and development (Meekan et al. 2003). Within this thesis, the effects of temperature change on growth, development and performance will be examined for the first time in a tropical marine fish.

While environmental influences have long been considered critical in determining variation in growth rate and survival (Hjort 1914), parental influences have received attention relatively recently (Blaxter 1969, Solemdal 1970). Parental effects are the “non-genetic influences derived from parental phenotypes or environments that have an impact on offspring phenotypes” (Heath & Blouw 1998, p178), although it is not always possible to separate the genetic influence from parental effects. Non-genetic *maternal* effects have been detected more frequently than *paternal* effects, and thus parental effects are generally referred to as ‘maternal effects’.

Maternal effects can occur through a variety of pathways including cytoplasmic inheritance, nutrition, transmission of pathogens and antibodies and

behavioural interactions with offspring (Bernado 1996). These effects may be manifested in many traits of offspring, including quality, quantity and behaviour. For example, the occurrence of diapause in flesh fly offspring is determined by the day length experienced by the mother (Denlinger 1998), and sprint speed in lizard offspring is determined by maternally aliquoted egg size (Sinervo 1990). Maternal influences have been identified as central in determining condition and survival of offspring in a large range of taxa including vertebrates (Bernado 1996), insects (Mousseau & Dingle 1991) and plants (Mazer & Wolfe 1998). The variation in propagule characteristics due to maternal effects occurs at all scales within a (meta)population, that is, between full siblings within clutches, between clutches from the same female, within species and between populations in fishes (Chambers & Leggett 1996) and frogs (Kaplan 1997).

While evidence of significant maternal influences on offspring phenotype and survival have been well documented both in plant and animal kingdoms, they are seldom considered as a source of variation in growth of marine fish larvae. Over three decades ago, maternal effects in fishes were noted as one of the most important characteristics in developing marine fishes, particularly in that the size of the female can influence the viability of offspring through conditions of incubation, fecundity and egg size (Blaxter 1969). However, it was not until recently fisheries biologists and ecologists have begun to consider that quantifying variation in the condition of individuals may be the key to understanding variable recruitment (rather than eliminating it as 'experimental noise' Falconer 1981). Subsequently, maternal effects have been examined relative to variation in offspring size, condition, viability and abundance, and strong relationships have been identified between attributes of the female and the life-history characteristics and body condition of her offspring, particularly at hatching. These mother/offspring relationships include: female size and egg size (Chambers & Leggett 1996, Benoit & Pepin 1999); female condition and egg yolk volume (Chambers et al. 1989, Kerrigan 1997); female condition and egg size (Chambers & Waiwood 1996); and female size, condition and age and egg and larval size (Marteinsdottir & Steinarsson 1998). Examination of maternal/parental effects is not commonplace, however, these studies have established parental effects as an aspect of individual history that is crucial for the comprehensive understanding of population dynamics of fish populations.

While these studies suggest that the maternal contributions to egg and early larval quality are important, the progression of these initials differences is rarely followed to determine maternal effects on the condition, growth and performance of late stage larvae and new recruits. Additionally, paternal effects are seldom considered and rarely identified, (with a few notable exceptions, e.g. Rakitin et al. 1999, Rakitin et al. 2001, Rideout et al. 2004), despite evidence that paternity can determine recruitment success (Knouft et al. 2003), hatching success, larval size, and yolk size (Rideout et al. 2004). While the egg is initially provisioned by the female through gametogenesis, the male parent contributes half of the genetic material, and the majority of the nest care in demersal brooding species (Clutton-Brock 1991).

Prior to the pelagic larval period (which has been the focus of most research in the last 100 years), is the embryo stage where offspring subsist on endogenous resources, provisioned by the mother. Female provisioning is a key source of variation in size of fishes at hatching (Kerrigan 1997). The embryo stage varies in length in tropical fishes from 24 - 48 hours for pelagic eggs from broadcast spawners, and up to 8 days for benthic eggs. For broadcast spawners, input into the early life history ceases after they have selected a suitable spawning site and released their clouds of gametes into the water column. Alternatively, benthic spawners attach their eggs to the substratum, often in discrete monolayered clutches and intensively guard these nest sites (Robertson 1991). While egg provisioning and spawning site selection are critical to benthic and pelagic eggs, benthic spawners can further influence their offspring survival through maintenance of the embryos' environment. Benthic eggs develop in the parentally chosen environment, which can differ markedly from the pelagic larval environment. It follows that different environmental processes may influence growth, condition and survival in benthic eggs than those which influence the pelagic larval stage. Due to the extended nature of embryogenesis in the 14 % of tropical species that develop from benthic eggs (Leis 1991), embryonic processes are important to subsequent larval growth and survival. Since growth trajectories are often established during early development, selective processes in the larval phase can act on growth variability derived from the benthic embryo phase and determine which individuals survive through to recruit as juveniles.

The species that is the focus of the present study, *Amphiprion melanopus* (Pomacentridae), is a benthic spawning reef fish with three distinct early life history stages: an 8-day egg stage, provisioned by the female and tended by both parents; an 8-day free swimming larval stage in the variable pelagic environment; and benthic juvenile where individuals compete within a size-selective hierarchy for limited space within their host anemones. This species provides a unique opportunity to separate factors affecting a tropical marine fish during its early life history stages, considering the interaction of paternal, maternal and environmental effects on growth development and performance at all life stages.

Recent studies of tropical species linking size at hatching and larval growth to post-settlement survival (Suthers 1998, Vigliola & Meekan 2002, McCormick & Hoey in press) found that both food and temperature contributed significantly to larval growth rates and survival (Booth & Hixon 1999, Meekan et al. 2003). While these studies have shown conclusive evidence that environmental variation influences growth in the larval stage, which in turn, is critical to survival and recruitment strength in tropical marine fishes, these relationships only explain part of the variation in growth. That is, only 7 - 36% of growth variation in larvae can be attributed to variation in water temperature, rainfall and wind in a Caribbean damselfish (Wilson & Meekan 2002). Due to an historic focus on larval biology as the key determinant of recruitment dynamics (see Houde 1987 and Robertson 1991 for discussion) and the logistic difficulties of sampling larvae and tracing parentage in wild populations, these correlations only address part of the lifecycle (the pelagic larval period), and therefore only answer part of the mortality/recruitment question. Broadly, this thesis investigates the causes and correlates of the remaining variation in growth of marine fish larvae by considering the relationship between parentage and environment for all stages of reef fish life cycle: eggs, larvae and juveniles.

This thesis examines some of the variability found within and between clutches of tropical reef fish and determine how parental influences interact with environmental conditions to influence the size, growth and performance of offspring from egg to juvenile. It further addresses the variability within and between clutches of fishes as they ultimately determine life history traits at the

population level, testing the key hypothesis *that variations in larval characteristics originate from parental influences and are subsequently modified by their environment.*

In order to test this hypothesis, I constructed 3 specific aims:

1. To determine the importance of the embryonic period to development and condition at hatching I investigated:
    - the structural and physiological changes an embryo undergoes through development in relation to changing oxygen requirements.
    - the relative variation in size and physiological measures such as oxygen consumption amongst embryos within a clutch;
2. To describe relative roles of male and female parents in nest tending in response to minor environmental variation, summarising the parental contribution to their offspring through nest tending behaviour;
3. To investigate how parental and environmental influences interact and affect embryo and larval and juvenile condition by differentiating between paternal, maternal and environmental contributions to larval condition and performance.

Each of these aims is comprehensively addressed in a chapter as detailed below.

This thesis commences by examining embryological details and progresses through the early life history stages of fish development with each ensuing chapter. **Chapter 1** provides some essential background material on embryo development and the concurrent general trend in embryological oxygen consumption. **Chapter 2** follows on by investigating how size differences of embryos within a clutch are related to oxygen consumption and the initial maternal aliquot; and are consequently the source of size variation in larvae at hatching. Further parental contributions throughout embryological development are considered in **Chapter 3**, through an examination of nest-directed tending behaviour in response to variable temperature and oxygen content of the water, and then related back to the changing embryonic oxygen requirements examined in Chapter 1. Chapter 3 explores the paternal contribution to embryonic development by examining the contribution of males to nest tending. In **Chapter 4** we further examined the effect of environmental factors on

performance of larvae through critical swimming trials, and whether decreased temperature decreased rates of growth and development. To elucidate how parental effects interact with temperature variation to influence egg and larval traits, we used a diallel (or full factorial) cross breeding experiment. This design enabled me to partition the variance in larval condition and swimming performance due to temperature change and to parentage (female and male) (**Chapter 5**). Details of the study species, *Amphiprion melanopus* (Pomacentridae) are included in each chapter, highlighting the features that make it a good model for each specific question addressed.

This dissertation is written as a series of stand-alone, though conceptually interconnected, publications (see Appendix for full list of publication details), tracing through the life cycle of the fishes from conception to metamorphosis. Each chapter considers how parental and environmental influences interact and affect each phase of development, and in turn, how this is expressed as growth, condition or performance of the offspring. The idea that variability in juvenile size and quality at recruitment may stem from embryonic or larval stages of development underpins this thesis. To this end, both larval and embryonic life stages are explored as sources of variability, and both stages are investigated in relation to environmental and parental influences. This thesis uses a series of laboratory experiments to manipulate parentage and environmental variables while holding all else constant to determine whether events and influences ***prior*** to the oft-sampled pre-settlement larval stage might be important in determining condition of larvae and recruits. No studies have yet addressed nest care, maternal aliquot and environmental variation to separate the sources of variability in larval growth and size and performance at metamorphosis, and as such, this thesis represents the first comprehensive attempt to consider all parts of the life cycle as sources of variation in larval growth.

Chapter 1: Embryogenesis and oxygen consumption in benthic egg clutches.

Publication: Green B.S. Changes in respiration during embryogenesis of a tropical reef fish. *Accepted Comp.Phys. Biochem A.*

Synopsis

Variation in size at hatching is common in demersally spawning organisms, suggesting that processes during embryonic development may be critical in determining growth and development. To examine critical periods during embryonic development in the demersal spawning reef fish *Amphiprion melanopus*, the rate of oxygen consumption within an egg clutch was related to morphometric changes in the embryos. Changes in oxygen consumption by egg clutches corresponded to the timing in the development of key organs and expansion of the circulatory system. Oxygen consumption was least on day 1 of development where organ differentiation had not begun (mean 1.73 ± 0.34 x 10^{-5} µmol O_2 egg^{-1} sec^{-1}). Tail movement throughout the perivitelline fluid began on day 3 and is likely to assist in moving oxygen around the embryo, complementing diffusive transport. The appearance of haemoglobin in the blood corresponded to a peak in oxygen consumption on day 4, where the highest mean rate of oxygen consumption was recorded (6.73 ± 0.82 x 10^{-5} µmol O_2 egg^{-1} sec^{-1}). This could be a critical period in development whereby risk of mortality is increased through increased embryo requirements at developmental thresholds.

Introduction

Patterns in oxygen consumption corresponding to morphological changes are well studied in marine fish larvae (e.g. Nelson & Wilkins 1994, Finn et al. 1995b). However, physiological processes and their impacts on the life history stage that precedes the larval stage are poorly understood (Rombough 1988, Pelster 1999). The morphological changes that occur during

embryogenesis have been described (Balon 1985, Masuma et al. 1993), but seldom related to oxygen consumption. Conversely, metabolic changes (measured as oxygen consumption) are recorded throughout development in many kinds of embryos such as crabs (Fernández et al. 2003), cuttlefish (Cronin & Seymour 2000), echinoids, bivalves and asteroids (Hoegh-Guldberg & Manahan 1995), yet few studies describe the accompanying morphological changes. Physiological, morphological and metabolic developments during embryogenesis are inter-dependent, and studies examining these systems together will greatly advance our understanding of the mechanisms underlying ontogenetic development and variation in size at hatching.

The development of benthic egg masses account for proportionately little of overall longevity of fishes, but encompasses a large amount of morphological and physiological change (Blaxter 1988). Embryos from externally fertilised oviparous species such as demersal spawning fishes represent partially closed systems, exchanging only heat, respiratory gases and limited amounts of solutes and water with the external environment (Kjorsvik 1990). The embryo is encapsulated in a semi-permeable membrane and unlike pelagic or brooded eggs, demersal eggs remain in the parentally chosen environment throughout the embryonic period. Whilst development of fish embryos from benthic clutches is rarely examined, asynchronous embryo development occurs commonly within clutches or broods of invertebrate embryos in response to parental care, oxygen availability, nest construction and site (Chaffee & Strathmann 1984, Booth 1995a, Fernández et al. 2003). Embryo development of organisms within benthic clutches represents a period of risk of developmental abnormalities and mortality (Steer 2002, Gowland et al. 2002), not yet considered in fishes with benthic eggs.

Critical periods are intervals of fish development with elevated mortality rates and relate to periods where morphological and functional development must stay abreast of changes in larval requirements or environment (Thorisson 1994). Tropical larvae from demersal eggs undergo a large amount of development within the embryo capsule, hatching in a shorter time and as better developed larvae than fishes from temperate waters (Rombough 1988, Green & McCormick 2001). Changes in embryo requirements during this period of rapid development must be met by morphological and functional development to

avoid elevated mortality rates. Understanding these changes is crucial for recognising the ramifications of early life-history changes to recruitment (Rombough 1988).

This study considered gross morphological changes in relation to the changes in oxygen consumption throughout development. Specifically, this paper examined embryogenesis in a tropical teleost to determine: a) how oxygen consumption changed throughout ontogeny; and b) whether there were distinct critical periods of high oxygen consumption associated with major stages of organogenesis and development.

Materials and methods

Study species and preparation of eggs clutches

In its natural environment, the tropical reef fish *Amphiprion melanopus* (Bleeker) attaches clutches of 300-700 eggs to the benthos in semi-cryptic habitats under the tentacles of their host anemone. *A. melanopus* are capable of producing an egg clutch approximately every two weeks. The parentally tended embryos develop for 7.5 days. *A. melanopus* was chosen because of its relatively long embryonic period, large initial egg sizes and level of skeletal, organ and sensory development during embryogenesis (Green & McCormick 2001). Captive broodstock maintained in flow-through tanks held at 28 ºC ± 0.5ºC in the James Cook University Research Aquarium were conditioned to lay their clutches onto roughened acetate sheets attached to the inside of hollow cement blocks (internal 16 cm x 16 cm x 19 cm) simulating a protected nest. Egg clutches were removed from parents by detaching the acetate sheet from the cement block, and were transported into the condition-controlled laboratory in a bucket of water taken from their natal tank. A total of seven egg clutches obtained from five broodstock pairs were used to measure oxygen consumption.

Oxygen Consumption

Oxygen consumption was quantified using a micro-optode and the diffusive boundary layer method (Klimant et al. 1995, Kuhl et al. 1995). This method quantifies the flux of oxygen through the semi-stagnant boundary layer above a respiring surface, relative to the physical laws of diffusion, allowing the

consumption of oxygen below the boundary layer to be quantified. The micro-optode (140 µm tip, Presens, Neuburg, Germany), measures oxygen concentration by dynamic fluorescence quenching, whereby the presence of oxygen decreases the fluorescence of an immobilised fluorophore (Klimant et al. 1995).

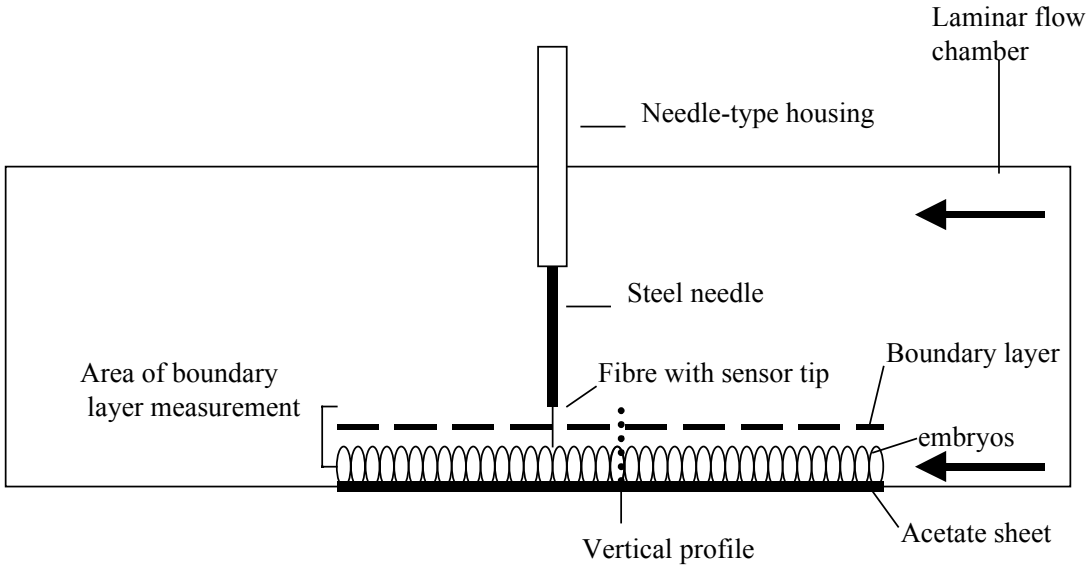

Fig. 1. Schematic representation of the diffusive boundary layer technique used to measure oxygen consumption within demersal egg clutches of *Amphiprion melanopus*, including a representation of the micro-optode fibre within the egg clutch inside a laminar flow chamber. Bold arrows indicate the direction of the flow within the chamber, dashed line indicates the upper limit of the semi-stagnant boundary layer, and dotted line represents a vertical profile where 2 minutes of oxygen measurements were taken at each point through the boundary layer above the eggs.

The fluorophore is excited by emitting light from a blue LED (light emitting diode) through a fibre optic cable to the luminophore matrix. This method is highly accurate, as the optode does not consume oxygen and is independent of changes in flow velocity (Klimant et al. 1995). The fibre is housed within a plastic syringe and stainless steel needle and protruded from the needle by pushing the plunger on the syringe.

Whole egg clutches were placed into an open, laminar-flow flume channel (L 370mm, W 151mm, H 68mm) similar to that described by Patterson et al. (1991). Oxygen consumption recordings were performed on embryos maintained under identical temperature, salinity and light conditions:

temperature 28 ºC ± 0.5 ºC; salinity 30 ppt; 14:10h light-dark cycle. All oxygen consumption measurements were performed in light conditions. Water flow was generated at a velocity of 3.5 cm sec^{-1} by a submerged bilge pump (1000W, Johnson®) attached to a variable, regulated power supply. Flow velocity was calibrated by visibly tracking neutrally buoyant particles over time. Individual acetate sheets with an egg clutch attached were clamped onto the channel floor approximately 150 mm from the flow straighteners (Fig. 1). Once the boundary layer had formed over an egg clutch (approximately 30 minutes), 3 vertical profiles (increments of 0.2 mm) of oxygen concentration were measured above the centre of the egg clutch eggs using a micro-optode supported by a micromanipulator (MM-33, Sutter, California, USA) with an accuracy of 5 µm. The 3 vertical profiles were taken at the approximate centre of the clutch and 0.5 cm either side, perpendicular to the water flow.

The flux (J) of oxygen through the boundary layer was calculated based on Fick's first law of diffusion

$$J = k\, d[O_2]/dx, \qquad (1)$$

where k is the kinematic viscosity of seawater, approximately 2.5×10^{-5} , at 28 °C and 30 ppt salinity (Broecker & Peng 1974), reflecting the experimental conditions, and x is the vertical position within the boundary layer. The upper limit of the boundary layer was estimated as the point at which oxygen concentrations approximate mainstream values (Patterson 1992). The oxygen gradient (cm^{-2}) at the level of the eggs was determined by the slope of the O_2 concentration vs x profile near the egg surface (Epping et al. 1999). The flow chamber restricted the use of simultaneous clutches so a sub-sample of 'day of development' within 7 clutches were sub-sampled according to Table 1. Seven clutches were measured to reduce the effect of within clutch variation. All egg clutches were photographed with a digital camera and number of embryos and total clutch area was measured using UTHSCSA Image Tool graphics package (University of Texas, San Antonio), egg density (cm^{-2}) of each clutch was calculated from these measurements. Flux rates for each clutch were standardised for embryo density, therefore oxygen consumption rates are presented per egg.

Table 1. List of samples from each experimental clutch where oxygen consumption was measured per day of development

	Day						
Clutch sampled	1	2	3	4	5	6	7
1							*
2						*	*
3		*	*	*	*	*	*
4	*	*	*				
5					*	*	
6			*	*	*	*	
7	*	*	*	*			

Calibration

The micro-optode was calibrated daily at 100 % oxygen saturation (air saturated with water) and 0 % oxygen saturation (water supersaturated with sodium sulphite zero DO_2 solution). Calibration was regularly checked throughout the measurement process.

Embryonic development and growth

To describe the overall embryonic development in relation to the rate of oxygen consumption, every siz hours from fertilisation until hatching ten live embryos were sampled and observed. To avoid disturbing monitored clutches, these samples were taken from 5 clutches not used to describe the oxygen environment. Embryo samples were placed on concave slides in sea-water and viewed under a high powered microscope at 40 and 100 x magnification. To determine if egg size changed throughout development, embryos were sampled

on day 1 (n=20) and on day 8 (n=20) and were photographed on a dissecting microscope using UTHSCSA Image Tool graphics package (University of Texas, San Antonio) to capture the images. Egg length was measured on all images of embryos using Image Tool graphics package. Wet weights of 10 embryos were measured on a Mettler balance (± 0.0001, Model AE, USA). Observations were made on development of structures and circulation patterns within each embryo (see Allen 1972 for detailed drawings of embryos). These results were summarised for each 24h period. Heart rate was measured at 06:00h every day, by counting the number of beats per 10 sec and averaging this per min, repeating this measure 5 times for each age of embryo.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to test for the effect of egg age and position within a clutch on the rate of oxygen consumption. Variances were heterogenous so data were square root transformed to achieve homogenous variances. Post-hoc tests were performed by Fisher's LSD multiple means comparisons. All statistical analyses were performed using Statistica (V6 Statsoft Inc, Tulsa OK USA).

Results

A. melanopus embryos are spherical and initially contain multiple oil globules within a large yolky mass. Eggs were spawned at around 08:30h and hatched 7.5 days later at dusk. Egg length did not change substantially throughout embryological development, ranging from 1.76 - 2.51 mm (mean 2.3 mm) at spawning increasing to 2.24 – 2.67 mm (mean 2.5 mm) just prior to hatching. Mean weight (SE) of sampled embryos was 0.975 mg (± 0.05)

Embryogenesis and Oxygen Consumption

A. melanopus embryogenesis involves a transition from a yolk-dominated embryo without obvious organ structure on the day of spawning to a larva with significant morphological development 8 days later (Table 2). These morphological changes coincided with an increase in O_2 consumption in the first half of development (Fig. 2). Embryonic age was the source of significant changes in the rate of O_2 consumption (Fig. 2, Table 3). The lowest rate of O_2 consumption was measured on day 1 (mean ± stdev: 3.74 ± 1.2 $x10^{-5}$ µmol O_2

egg^{-1} sec^{-1}, Fig. 2, at 28 ºC, or standardised to mean embryo weight 3.8 x10^{-5} µmol O_2.mg^{-1} sec^{-1}.), corresponding to initial cell divisions and gastrulation (and lack of definitive morphogenesis or form structure) within the egg (Table 2). On day 3 the embryonic tail begins to move throughout the perivitelline fluid regularly.

Table 2. Major stages of development in embryos of *Amphiprion melanopus* from fertilisation to hatching**.** Age is days after fertilisation, day 1 is the day eggs were fertilised.

Age	Major stage of development
1	Blastula stage: Visible separation of the cytoplasm into blastomeres and non-cleaving yolky mass. Gastrulation is occurring as blastomeres from the animal pole engulf the yolk.
2	Gastrulation is complete and morphogenesis has begun as an embryo with rudimentary head and trunk with somites have formed at the animal pole. Vertebral cord and segmented brain (an enlargement of the anterior end of the spinal cord) develop late in the day.
3	A rudimentary single chambered heart begins to beat as wave-like contractions, pushing colourless blood which lacks erythrocytes/haemoglobin into the proximal end of the dorsal aorta. Circulation increases throughout the day as circulation fluid moves from the heart and around edge of yolk-sac and through the dorsal aorta to the carotid arteries to supply the spinal cord, brains and eyes. The otic capsule and two pairs of otoliths (sagitta and lapillus) form. Trunk segmentation is complete and the rapidly growing tail has extended beyond the yolk-sac and is moving regularly through the perivitelline fluid. The eye cup has formed and eye is pigmented. The heart rate (HR) is approximately 110-120 beats per minute (bpm).
4	Erythrocytes develop in the blood plasma, colouring the heart and blood orange, and the intensity of this color deepens throughout the day. The heart has two visible chambers and HR = 110-120bpm. The ends of eye cup overlap.
5	Embryonic respiratory organs have developed, irregularly heaving rudimentary opercula have formed and blood flows to this area. The ends of the eye-cup have joined, small blood vessels run off the spinal artery and feed the trunk muscles, and pectoral fins appear. The third set of otoliths – the asteriscus - are visible. A rudimentary gut is developing. HR = 160-170bpm.
6	Nascent jaw and orbital bones and gill rakers appear and arteries to the branchial basket are transporting blood. The pectoral fins are elongated, however no fin rays are present, the alimentary tract is looped and by the end of the day irregular gill ventilation has begun. HR = 168bpm
7	Four rows of gill rakers are visible, the gills are ventilating regularly, moving the jaw with it indicating that the interhyal articulation is functional. HR = 160-170
8	Development of the eye is completed and eyeshine from the tapetum is visible, the lens protrudes substantially from surface of eye. The hindgut forms. The larval finfold is still present and the spinal cord in not flexed just prior to hatching. HR = 160-170

The rate of embryo O_2 consumption peaked on day 4 (mean ± stdev: 8.49 ± 1.1 x10^{-5} µmol O_2 egg^{-1} sec^{-1}, Fig. 2, or standardised to mean embryo weight 8.7 x10^{-5} µmol O_2 mg^{-1} sec^{-1}), coinciding with the initiation of blood circulation throughout the cardiovascular and neural systems and the presence of oxygen-carrying erythrocytes throughout the circulatory system (Table 2). O_2 consumption decreased after day 4 and remained constant until hatching. The embryonic gills formed on day 5 (Table 2), and organ differentiation continued throughout embryogenesis and structures increased in size and complexity (Table 2). The heart rate increased throughout development, ranging from 110 - 120 beats per minute (bpm) on day 3 to 160 – 170 bpm on day 8.

There was no difference in O_2 consumption detected between the three positions at the clutch centre (Table 3).

Table 3. Summary of two-way ANOVA testing the effect of egg age and position within a clutch on the rate of oxygen consumption. Bold type denotes significant differences

Source of variation	df	MS	F	p
Egg age	6	0.000027	5.77	**<0.001**
Position within a clutch	2	0.000004	0.80	0.45
Egg age x position	2	0.000002	0.43	0.95
Error	101	0.000005		

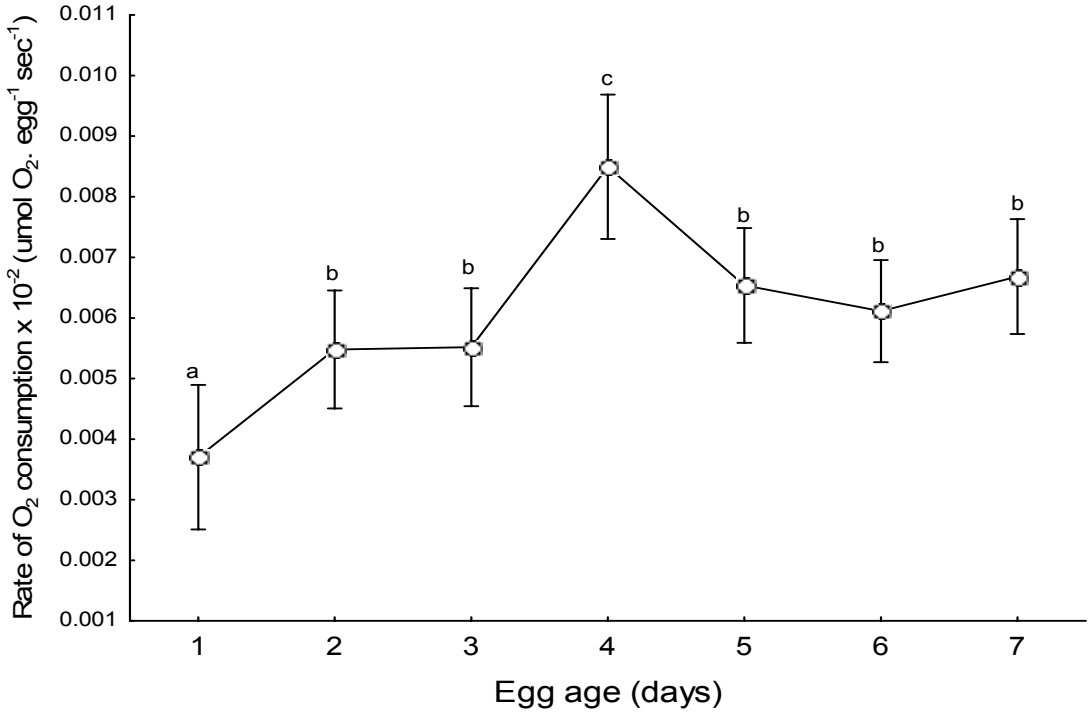

Fig. 2. The average rate of oxygen consumption within seven clutches of *A. melanopus* eggs measured in a laminar flow chamber on each day of development from fertilisation (day 1) until the day prior to hatching. Superscripts denote significant differences calculated by Fishers LSD at 0.05.

Discussion

Embryogenesis in *A. melanopus* proceeds with the transformation of embryos from yolk-dominated eggs to larvae with significant morphological and functional development over an 8-day period. This is a relatively long embryonic period compared to many tropical species (Job & Bellwood 2000) and subsequently *A. melanopus* hatches with the ability to feed, swim and catch prey, unlike most other fishes described (Green & McCormick 2001). This advanced state of development at hatching means the rate of processes throughout embryogenesis are faster than reported rates for many fishes. The link between embryogenesis and oxygen consumption represents a gross relationship, as the physiological delivery system is not the rate-limiting step, (rather metabolic rate is regulated by key steps in carbon and O_2 flux, and ATP turnover, Darveau 2002). Extrinsic, or environmental factors interact with metabolic pathways driving respiration (e.g. Finn et al. 1996, Darveau 2002, Finn et al. 2002), of which growth and development are an end product.

Oxygen consumption, ontogeny and critical developmental stages

Metabolism generally scales with the size of the organism, as oxygen requirement increases with increasing size and increasing mass of metabolically active material (e.g. Wieser 1984). In particular, rate of oxygen consumption (as a proxy for metabolism) generally increases with embryogenesis in fishes (e.g. Atlantic cod *Gadus morhua,* (Finn et al. 1995a); milkfish *Chanos chanos*, (Swanson 1996); Senegal sole *Solea senegalensis*, (Parra 1999)). The oxygen consumption of *A. melanopus* embryos held at constant temperature, did not increase linearly with development. The two days on which respiration was significantly different from the rest of development were on day 1 (day of fertilisation) where no organ development has occurred and day 4 where the major circulation and blood transport system began operating.

On day 1 of embryogenesis, *A. melanopus* embryos were undergoing cell division, gastrulation and cleavage; however there was no visible circulation system, consequently diffusion would meet their oxygen demands (Hughes 1984, Rombough 1988). Early development can continue for a short time in anoxic conditions (Pelster 1999) and it appears that the initial stages of

embryonic development are not tightly coupled to environmental changes and therefore may not represent critical thresholds in *A. melanopus* development.

Ontogeny of movement within an embryo is a critical part of development to bridge the oxygen requirements between major stages of organogenesis (Peterson & Martin-Robichaud 1983). Tail movement through the perivitelline fluid (tail flicking) as observed in *A. melanopus* on day 3, increases the oxygen circulation within the embryonic capsule (salmon *Salmo salar* (Peterson & Martin-Robichaud 1983); zebrafish *Danio rerio* (Pelster & Burggren 1996); and damselfish *Pomacentrus amboinensis* (McCormick & Nechaev 2002). Without this tail-flicking, the perivitelline fluid would create a barrier to oxygen diffusion to the embryo (Cronin & Seymour 2000) as benthic eggs act as an oxygen 'sink' (Blaxter 1969, Rombough 1988). If a developing embryo removes oxygen from a stationary water mass, then the layer of water immediately adjacent to it, the boundary layer, will quickly become oxygen depleted (Schmidt-Nielsen 1997). Oxygen replenishment is therefore assisted by convection created by the movement of the embryo within the egg capsule (see Cronin & Seymour 2000).

A. melanopus developed a circulatory system on day 3 of development, followed by the appearance of haemoglobin on day 4, coinciding with a spike in oxygen consumption. Haemoglobin is the site of oxygen binding and transport within erythrocytes in blood (Pelster & Burggren 1996). This absence of haemoglobin in the initial circulatory system suggests that early development of the circulatory system has functions other than gas transport, such as moving metabolites or as an aid to angiogenesis (Pelster & Burggren 1996). The increase in haemoglobin throughout the day may account for the relatively large amount of oxygen consumed on this day; however, this contrasts with zebrafish embryos that develop haemoglobin, but show no concurrent increase in oxygen consumption (Pelster & Burggren 1996). The heart rate of *A. melanopus* increased throughout embryonic development, similarly to confamial, *Pomacentrus amboinensis* (McCormick & Nechaev 2002). However, uncoupling of structure and function demonstrate that larval circulatory function is not essential for oxygen transport, at least in zebrafish (Pelster & Burggren 1996).

A. melanopus undergo a large proportion of their early development within the embryonic stage. As embryos develop, new structures become

specialised for oxygen transport throughout the embryo, streamlining the efficiency of oxygen consumption, but also creating critical periods whereby embryonic oxygen demands must be met by increased embryonic transport capacity (Rombough 1988), or parental tending (Green & McCormick in review-a, Chapter 3), to avoid retarded rates of embryonic development such as those found in invertebrates (e.g. Chaffee & Strathmann 1984, Booth 1995a, Fernández et al. 2003).

Chapter 2: Variation in size at hatching has maternal origins.

Publication: Green, B.S., Anthony K.R.N., McCormick, M.I. (in review) Variation in size at hatching in marine fishes has maternal and environmental origins. *Oecologia.*

Synopsis

Size variation among propagules is ubiquitous in oviparous animals and small initial differences can be critical to survival. Despite this, the source of size variation is rarely investigated in marine fish embryos. This study examines the relative importance of maternal investment and clutch microenvironment to size of larvae of the benthic spawning fish *Amphiprion melanopus*. Newly hatched larvae from the periphery (0.5cm from edge) of 2-dimensional clutches had smaller standard length, head depth, eye diameter and body area (7, 8, 4 and 11 % respectively) than larvae from the clutch centres. Size of eggs within two hours of fertilisation was measured to determine if this was a source of size variation in larvae. Eggs from the clutch periphery were >2 % smaller in length and 4-6 % smaller in volume than embryos at the clutch centre. There was a consistent relationship between initial egg size, position within clutches and larval size, implying that variation in egg and subsequent larval size has maternal origins. Rates of embryo oxygen consumption throughout development, measured using a micro-optode, were 63 % lower at the egg clutch peripheries than at centres, reflecting smaller egg sizes and lower growth rates at the peripheries.

The small degree of differential investment in embryos by the female was thus amplified throughout development. Size variation within clutches of fish may originate from maternal endowment interacting with the clutch micro-environment during embryogenesis.

Introduction

Propagule size differences amongst siblings are ubiquitous in plant and animal systems (Bernado 1996). Large variation in number and size of offspring exists in marine and terrestrial animals as a result of differential maternal investment (Roff 1992). Within oviparous species such as lizards, amphibians, birds and fishes, differential maternal investment often results in variation in egg size within a clutch, which is directly related to hatchling size (e.g. Sinervo 1990, Price 1998). Initial propagule size is a critical determinant of survival in most organisms (Forester 1979, Beacham & Murray 1985, Kaplan 1997), and in marine fishes large size confers an advantage in the post-embryonic stages (Miller et al. 1988, Chambers & Leggett 1996, Vigliola & Meekan 2002). Larval fish size is a key parameter influencing the quality and magnitude of recruitment because bigger larvae have better chances of survival through their greater ability to capture food and escape predation (Miller et al. 1988). Early size advantages in marine fish are cumulative and may be amplified throughout development (Vigliola & Meekan 2002). Little is known about the influence of environmental conditions and physiological processes on the life-history stage that precedes the larval period (Pelster 1999), and the role these may play in determining variation in larval size at hatching (Kerrigan 1997, McCormick 1999) and subsequent survival.

Demersal embryos remain in the parentally chosen environment for the duration of the embryonic period. They are susceptible to a range of conditions in their surrounding environment yet cannot make defensive responses to it (Blaxter 1969). There are three likely sources of variation arising from demersal embryonic development including: maternal allocation (e.g. genetic, egg aliquot and nest design); environment (e.g. heat and gas exchange and nest design); or parental maintenance, which can modify the embryonic environment (e.g. through fanning and tending the clutch).

Studies of maternal effects have highlighted the importance of female allocation to offspring quality in many taxa, such as frogs (Kaplan 1997), fish (Beacham & Murray 1985) and salamanders (Forester 1979). Maternally aliquoted yolk (McCormick 1999) and maternally chosen nest sites (Jones & Reynolds 1999b) affect growth and developmental variation in fish embryos.

However, despite these strong influences on offspring size, the embryonic environment is rarely considered as a source of maternally derived size variation critical for post-embryonic survival (Bernado 1996, but see Burggren 1999 and Bize et al. 2002). Burggren (1999) found that in a range of vertebrate embryos, maternal effects interact with the oxygen environment to affect the cardiac rhythms, development and growth of embryos

Rates and synchrony of embryo development are affected by oxygen concentration interacting with maternally derived factors such as nest site, clutch size, and clutch density (e.g. gastropods, Lardies 2002, Cancino 2003; cephalopods, Cronin & Seymour 2000, Steer 2002, fishes, Jones & Reynolds 1999b, and amphibians Pinder & Friet 1994). Limited oxygen availability, for example, due to nest design, restricts the rate of development of individuals within an egg clutch, leading to asynchronous embryonic development within clutches of siblings. Propagule size is thus likely to be the result of the maternal allocation, subsequently modified by the propagules' interactions with its' environment (Bernado 1996). However, the relative roles of maternal influences and clutch microenvironment, particularly oxygen consumption and nest design, on embryonic development have not been examined in detail in marine fishes.

This paper investigates the influence of maternal allocation on size distributions of eggs within clutches (marginal versus central positions). We then examine the effect of position within clutches on rates of oxygen consumption as a proxy for embryonic metabolism and whether size differences across the clutches are maintained, lost or enhanced throughout development. This study provides the first detailed examination of the relative importance of clutch micro-environment and maternal factors in the growth of a demersal fish and explores the causes of size variation at hatching.

Materials and methods

Study species and preparation of egg clutches

In their natural environment, monogamous pairs of the tropical reef fish *Amphiprion melanopus* attach 300-700 capsule-shaped eggs in single-layer circular masses (diameter: 40-100mm) to the benthos in semi-cryptic habitats within the periphery of their host anemone. Embryos remain in the parentally

chosen environment and rely entirely on maternally aliquoted nutrition, which makes *A. melanopus* an ideal model for investigating the combined effects of maternal contribution and clutch micro-environment.

Captive broodstock were maintained in the James Cook University Research Aquarium and laid their clutches onto artificial nests as per Chapter 1.

Oxygen Consumption measurements

Oxygen consumption was measured using the equipment and boundary layer profiling technique as outlined in Chapter 1. By using Fick's first law of diffusion (Equation1) the rate of consumption of oxygen below the boundary layer can be quantified. Profiles of oxygen concentrations at seven positions within each clutch (Fig 1.) were determined using a micro-optode (140 µm tip, Presens, Germany, see Klimant et al. 1995), supported by a micromanipulator (MM-33, Sutter) with an accuracy of 5µm. Oxygen measurements using a micro-optode are highly accurate, as the optode does not consume oxygen and is independent of changes in flow velocity (Klimant et al. 1995).

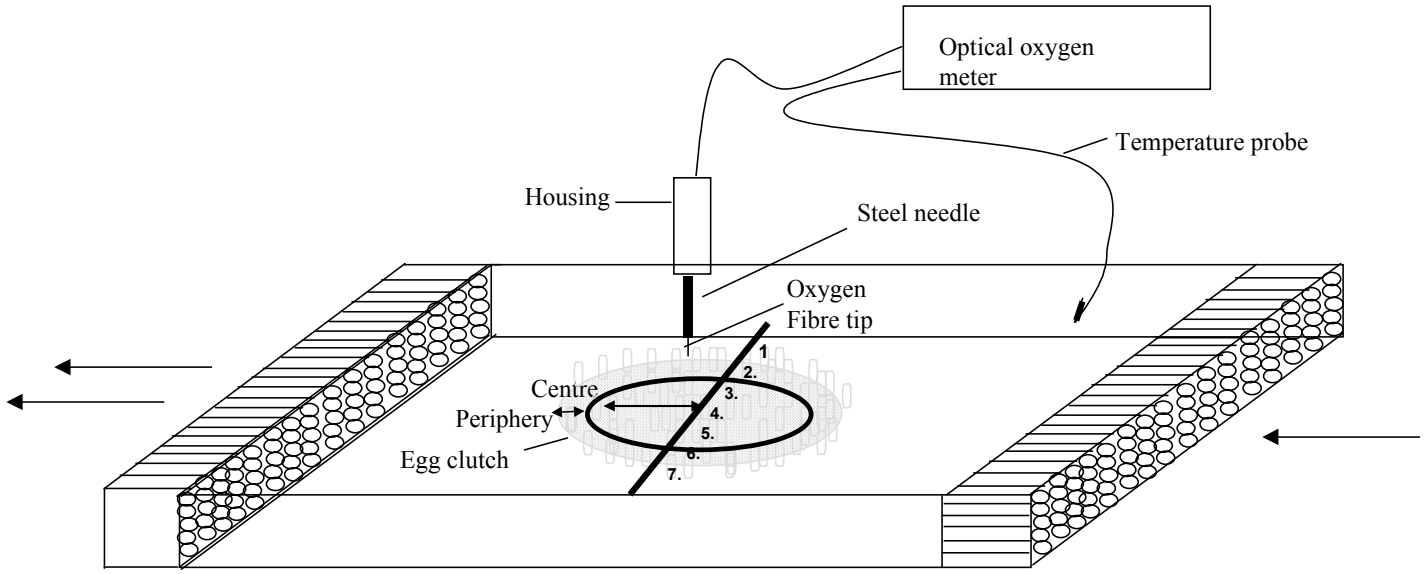

Fig. 1. Schematic representation of a benthic egg clutch attached to the floor of the laminar flow chamber. The micro-optode used to sample vertical oxygen profiles from the boundary layer above developing fish embryos is represented. Dark arrows indicate the direction of the water flow within the chamber. Positions of oxygen profile are numbered:1= far left; 2= 0.5 from far left; 3= 0.5 left of centre, 4= centre; 5= 0.5 right of centre; 6= 0.5 from far right; 7= far right, measurements are in centimetres. Sampling areas for embryo morphometrics are marked by bold oval inside of clutch periphery.

Oxygen consumption measurements were taken within an open flume channel (Fig 1., L 370mm, W 151mm, H 68mm) similar to that described by Patterson et al. (1991). Water flow of 3.5cm s^{-1} was generated by a submerged bilge pump (Johnson, 1000W) attached to a variable, regulated power supply and flow straighteners at either end of the channel minimised turbulence. Individual acetate sheets with an egg clutch attached were clamped onto the channel floor approximately 150mm downstream of the flow straighteners. Flow velocity was measured using visual tracking of neutrally buoyant particles over a known distance, and laminar flow was visually checked by injecting milk into the water current. Once the boundary layer had formed over an egg clutch (approximately 30 minutes), vertical profiles of oxygen concentration were measured above the eggs (increments of 0.2 mm), (Klimant et al. 1995). The micro-optode was calibrated daily at 100% oxygen saturation (air saturated with water) and 0% oxygen saturation (water supersaturated with sodium sulphite zero DO_2 solution). Calibration was periodically checked throughout measurement. All oxygen measurements were carried out with automatic temperature compensation and ambient water temperature was 28^{o}C.

The flux (J) of oxygen through the boundary layer, and therefore oxygen consumption of embryos within the clutch, was calculated based on Fick's first law of diffusion

$$J = k\, d[O_2]/dx, \qquad (1)$$

where k is the kinematic viscosity of seawater (approximately 2.5×10^{-5} cm^2 sec^{-1}, at 28°C, 30ppt salinity Broecker & Peng 1974) and x is the vertical position within the boundary layer (measured from the tip of the eggs). The upper limit of the boundary layer was estimated as the point at which oxygen concentrations approximate mainstream values (Patterson 1992). The oxygen gradient at the level of the eggs was determined by the slope of the O_2 concentration versus x profile near the egg surface (e.g. Epping et al. 1999). Oxygen consumption rates for each clutch were standardised for embryo density, to compensate for different clutch size and density.

Sampling design

Vertical profiles of oxygen concentrations were taken at 7 points across the maximum diameter of the clutch, perpendicular to the flow direction

throughout development (Fig. 1), for 5 separate egg clutches. Not all clutches were measured on every day as the flow chamber restricted the use of simultaneous assays of clutches, however a broad sample of all days of development (day 1-7), except the day of hatching, was obtained from a range of clutches (see Table 1, Chapter 1). This design examined the combined effects of clutch age and egg position within a clutch on variation in oxygen consumption rates. Once a clutch was removed from the parents, it remained in the experimental chamber, as parent fish often eat their eggs if they were re-introduced.

Embryonic growth

To determine whether position within a clutch influenced embryo size and growth, embryos were sampled from two positions: periphery (5mm from the edge) and centre (Fig. 1) at two points during development (day 1 of embryogenesis and day of hatching). To avoid disturbing assayed clutches, these samples were taken from 8 clutches not used to describe the oxygen environment. On *day 1* (n=4 clutches) and the day embryos hatched, "*hatching*", (n=4 clutches), the entire clutch was sampled using a scalpel to remove embryos. The 4 clutches sampled on day one were different to those sampled at hatching to avoid interfering with clutch structure or influencing growth rates within the clutch by manipulating clutch design through embryo removal. Embryos from the peripheral 5mm of the clutch were sampled separately to embryos from the centre of the clutch (Fig. 1), and *day 1* samples were immediately preserved in Marine Bouins fixative. To sample size in *hatchin*g larvae, centre and peripheral samples of late stage embryos were each placed in a 500ml beaker with sea-water and aerated in darkness for one hour, simulating dusk which is their natural hatching time. Once hatched, all larvae were then preserved as for *day 1* embryos. Clutches were sub-sampled and 20 embryos from each position were photographed on a dissecting microscope with a camera attachment and Image Tool was used to capture and measure the images. To examine the relationship between clutch position and egg size at the time they were laid, egg length, maximum egg width and yolk area were measured on all captured images. These measurements were chosen because they encompassed the key visible morphometrics for fertilised eggs with no visible embryonic development, and I was confident in the accuracy of these

metrics. These measures also provided a range of size dimensions as a summary of maternal allocation and most recent growth processes. Egg volume was approximated using the formula for a cylinder

$$Egg_{vol} = \pi r^2 * L, \quad (2)$$

where r is egg width/2 and *L* is egg length. A sub-sample of eggs from the centre (n=10) and periphery (n=10) was weighed on a Meitler balance (model AE 240) to provide an initial standardisation of respiration per unit mass. To quantify size variation at hatching larval standard length, eye diameter, head depth and body area (excluding fins) were measured as for *day* 1 larvae. These measures differ from those at hatching because the embryos have developed from a relatively formless mass of embryonic material with a large yolk sac within an egg capsule, into a well-formed fish liberated from the egg capsule, and were a different shape and form. We compared the same dimensions, ie. length, width and volume, while accommodating for the organogenesis of the embryo. Therefore morphometrics of *hatching* fish were chosen to reflect the most accurately measurable dimensions of the newly hatched larvae. Eye diameter was included as a proxy for eye size. Eye size is not expected to change in response to environmental conditions (Fuiman et al. 1998), therefore eye diameter provides a standard for comparison with changes in the other morphometrics.

Lastly, I compared rate of oxygen consumption (from the clutches assayed for oxygen consumption) standardised to average egg size (from the 4 clutches measured) on *day 1* to determine if the difference in measured rates of respiration could be a factor of size alone. Average respiration rates for the peripheral embryos (‘far left’ and ‘far right’, Fig. 1) and central embryos (all other positions where respiration was measured) were grouped from all egg clutches and standardised for the average size of embryos from the periphery and the centre of the clutches for *day 1*.

Data analysis

The diffusion coefficients (D,) for each trial were determined empirically by estimating the initial slope of the oxygen concentration versus vertical position over the eggs. D and associated standard errors were estimated using linear, least-squares regression within the initial and approximately linear portion (X-Y mm) of the profile.

Two-way ANOVA was used to test for the effect of egg age and position within a clutch on the rate of oxygen consumption. Variances were heterogenous so data was transformed (square root) to achieve homogenous variances. Post-hoc test were performed by Tukey's HSD multiple comparisons. Further two-way ANOVA's were used to test for the effect of individual clutch and position within a clutch on morphometrics at day 1 and at hatching.

Results

Embryonic growth

Initial embryo size

On the day the eggs were laid and fertilised, (*day 1*), embryos on the clutch peripheries were significantly smaller (mean =2 % in length and 3 % volume) than embryos in the clutch centres (Table 1). This difference in egg volume was small, 3.75 cf. 3.71mm, (means for centre and periphery respectively) but consistent between clutches (Fig. 2a). Yolk area, a measure of yolk quantity, which is the source of nutrition for the embryos for the following eight days of development, did not vary significantly between the periphery and centre of an egg clutch (Fig. 2b, Table 1). Measures of yolk and egg size varied significantly between clutches (Table 1). Embryo's from clutch 4 did not vary in size between the centre and the periphery (Fig. 2a, b).

Size at hatching

Initial embryonic size differences were increased throughout embryonic development. Newly hatched fish (*hatching*) sampled from the outside of the clutch were significantly smaller than fishes sampled from the centre of the egg clutch. Specifically, body length, body area (excluding fins), eye diameter and head depth were 7, 11, 4, and 8 % smaller on larvae from the outer edge of all clutches sampled (Figs 3a, b, c, Table 1). The proportional size differences in propagules between the periphery and centre of a clutch increased from day 1 (range 2-3%) to hatching (4-11%).

Oxygen consumption

Rate of oxygen consumption of embryos at peripheries and centres of clutches reflected patterns in embryonic and larval morphometrics across clutches. Embryos on the very edge of egg clutches ('far right' and 'far left', Fig. 1) had 63 % lower rates of oxygen consumption throughout development

compared to embryos from the centre of clutches, standardised for the initial average egg length difference (Fig. 4, Table 2). Half a centimetre from the periphery of the clutch, the rate of respiration approximated the respiration rates

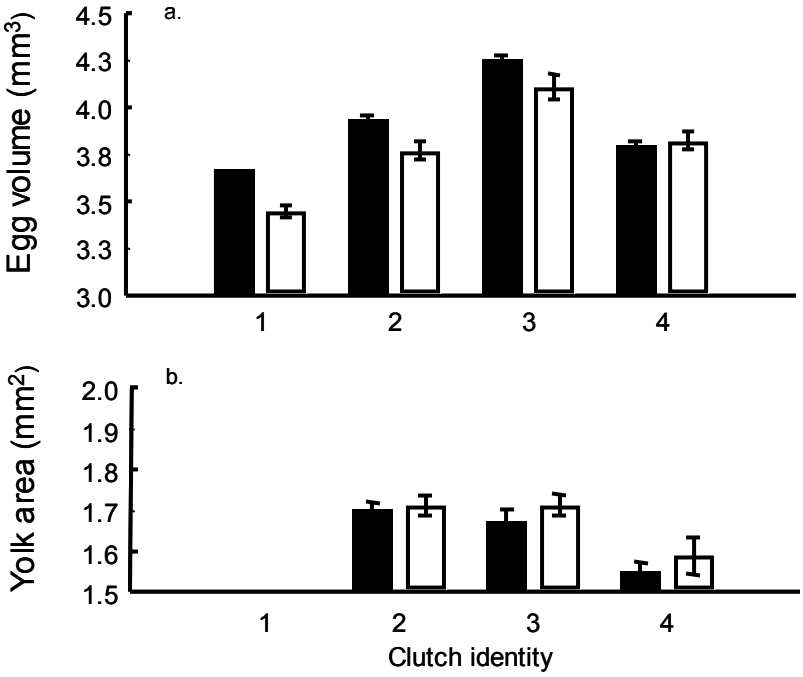

Fig. 2. Comparison of embryo morphology between clutch centre (black bars) and periphery (white bars) for 4 separate clutches sampled on the day the eggs were laid. a. mean egg volume (mm^3); b. mean yolk area (mm^2), (three clutches sampled only). Error bars are standard errors.

for all non-edge embryos, (Fig. 4). Differences in respiration between the centre and periphery were maintained throughout development.

Density of egg clutches ranged from 1.9 embryos mm^{-2} to 6.0 embryos mm^{-2} and the average density was 3.6 embryos mm^{-2}. When respiration rate was standardised to embryo density, central embryos had a higher respiration rate than peripheral embryos, 4.31x 10^{-5} $\mu molO_2$ egg^{-1} sec^{-1} cf. 2.89 x10^{-5} µmol O_2 egg^{-1} sec^{-1}. Similarly, when respiration rate was standardised to egg mass at the clutch centre and periphery, respiration at the clutch centre was 30% higher than at the periphery, 4.42 x10^{-5}, vs 3.38 x10^{-5} µmol O_2 mg^{-1} sec^{-1}. Embryos from the periphery and centre of the clutch hatched at the same time, suggesting that neither initial size nor differential micro-environmental conditions influenced developmental rate.

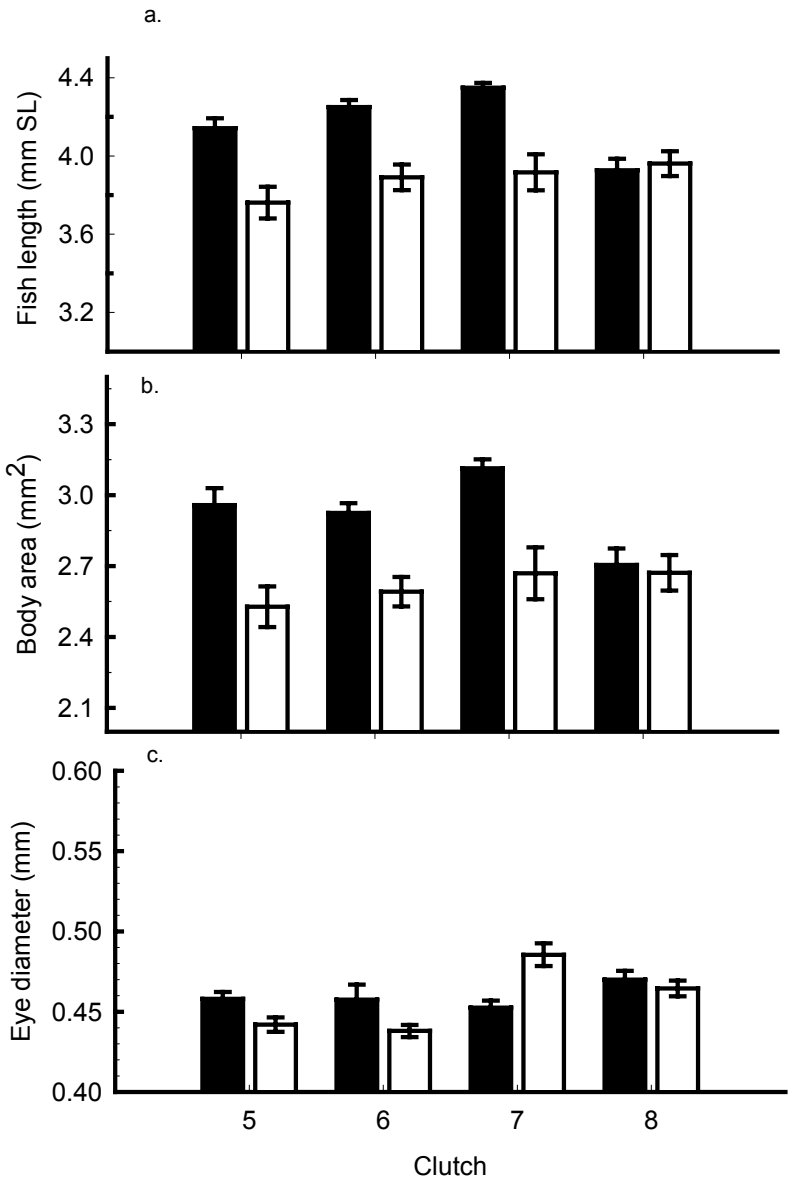

Fig. 3. Comparison of larval morphology sampled immediately after hatching between fish originating from centre (black bars) or periphery (open bars) of the clutch, for 4 separate clutches. a. mean fish standard length (SL); b. mean fish body area (without the fins) (mm^2), c. mean eye diameter (mm). Error bars are standard errors.

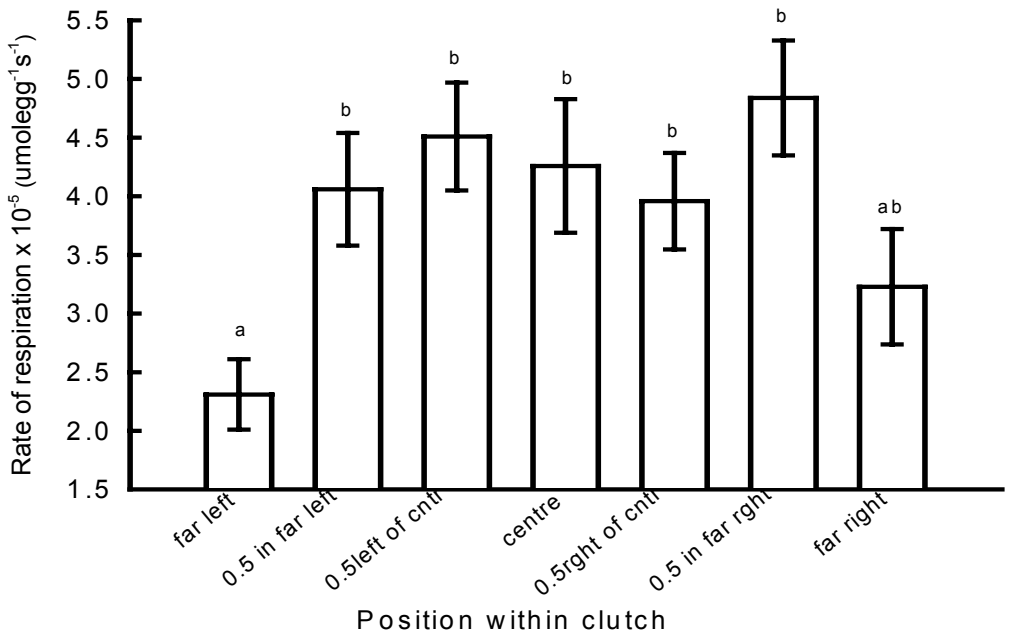

Fig. 4. Comparison of mean rate of oxygen consumption of A. melanopus embryos within an egg clutch (n=5), measured at 7 points within the clutch throughout development, perpendicular to the flow rate. All values are standardised for embryo density within each clutch. Superscripts denote significant differences (Tukey's HSD). Rght = right, cntr = centre.

Discussion

Much work on the ecological importance of growth has centred on growth equations, modelling juvenile to adult size differences and incorporating assumptions such as initial size (size at hatching) is constant (see Stelzer 2002) for discussion). The present study clearly shows that size at hatching is not uniform and the identified size differences at hatching may have been determined by parental investment in the embryo, enhanced by influences from the embryonic microenvironment, and particularly nest design.

Embryogenesis and variation in morphology or performance derived from this period are too often overlooked in ecological theory on size differentiation and growth modelling, particularly in fishes. Embryonic environment can impact on the developmental trajectory, modifying the adult phenotype (Burggren 1999) and in particular affecting body size (Stelzer 2002). If size at hatching in fishes determines the magnitude of future recruitment episodes (see Vigliola & Meekan 2002) then understanding the sources and possible causes of variation

in larval size at hatching, such as that found in this study, is crucial to understanding the nature of early life histories of marine fishes.

Maternally derived propagule variation

Variation in the size of propagules within a clutch from the benthic spawning *Amphiprion melanopus* are initially maternally derived. The initial small differences in egg size between the periphery and centre of the clutch suggests either females invested more into central eggs than peripheral eggs, or the female nest design favoured growth in embryos on the centre of the clutch and this advantage is apparent in egg size within hours of being laid. While these size differences were small they provided a growth advantage to central embryos that is increased throughout development. Maternally derived propagule size variation is ubiquitous in natural populations (Roff 1992, Bernado 1996), with 35-71% variation in egg sizes maternally derived in three species of fishes (Chambers & Leggett 1996). Possible mechanisms for differential investment include: 1) that maternal resources are limited; 2) that risk of predation upon embryos within a clutch under parental care is not evenly distributed across the clutch; or alternatively 3) that variation in propagule size enhances survival as propagules of different sizes will not directly compete with each other for resources (McGinley et al. 1987). The first mechanism assumes that propagule variation is undesirable while final two suggests it is an adaptation to optimising lifetime reproductive fitness.

Limited female resources often create size differences in embryos, however the relationship between embryo size and maternal resources varies and is not predictable. In some cases the female lays largest eggs first (e.g. (Takahashi & Iwasawa 1988) and runs out of resources to distribute to the last eggs, whilst in other cases females partition resources and lay the largest eggs last (e.g. Potti 1993). As *A. melanopus* females commence laying their clutches at one end of the clutch and zigzag across the clutch, finishing at the opposite end (Green unpublished data), the size of the eggs has no relationship to the temporal pattern of laying. The distribution of smaller embryos around the entire periphery suggests that resource limitation is an unlikely mechanism for the observed size variation.

Alternatively risk of predation on a parentally protected egg clutch may be higher for peripheral eggs. Benthic spawning fishes spend much time and

energy defending their nests against egg predators, such as wrasses and butterflyfishes (Tyler 1995). In colonial nesting fishes, nests at the periphery of a colony reduce predation risk from egg predators for central nests (Dominey 1981, Foster 1989) in a system known as the selfish herd (Hamilton 1971). It follows that in a near circular benthic clutch, nest-guarding parents have a higher likelihood of protecting central eggs from egg predators at the expense of peripheral eggs. Females could adapt to this biased risk by reducing investment in embryos at higher risk of predation by producing smaller eggs on the periphery of an egg clutch. While *A. melanopus* females produced smaller eggs on the clutch periphery, there was no difference in the yolk quantity in embryos from centre to periphery, which reduces the power of the selfish herd theory to explain the initial size differences. Whichever mechanism is driving the differences in initial propagule size, the effects are increased throughout development and these size differences have ramifications for larval survival and recruitment success (Vigliola & Meekan 2002).

Variation in egg and larval size between clutches were significant in this study, and one clutch from early and late development showed little differences in size between periphery and centre of the clutch. Size variation between clutches is generally a product of female condition, age, experience or spawning season. Larger females commonly produce larger eggs and larvae (Chambers & Leggett 1996, Benoit & Pepin 1999). Similarly, females in better condition produce bigger eggs (e.g. Chambers & Waiwood 1996, Marteinsdottir & Steinarsson 1998) with more yolk (e.g. Chambers et al. 1989, Kerrigan 1997). While female size and condition were not measured in this study, they are the most likely source of variation between clutches as each clutch measured for morphometrics was from a different female. The differences in eye diameter between centre and periphery of the clutch were less consistent than the other morphometrics used. This is not unexpected, as the eye size generally does not respond to environmental variation (Fuiman et al. 1998).

Variation in size at hatching

Fishes from the centre of an egg clutch hatched out larger than fishes from the periphery. Size differences from day 1 were increased throughout development by the effects of the position within a clutch. As size specific oxygen consumption was higher in the centre of the clutch compared to the

periphery it is apparent that position *per se* had an influence on oxygen consumption, regardless of the size difference. Therefore clutch micro-environment appears to contribute in part to growth rates throughout embryonic development.

The size variation at hatching relative to position within the clutch in this study has implications for the survival and future recruitment potential of these reef fish. Marine fish recruitment depends on larvae surviving the pelagic period and having sufficient ability and resources to locate, settle to and survive in the highly competitive reef environment. As most larvae do not survive the pelagic phase (estimated 99.9 % mortality, Ferron & Leggett 1994) any advantages, such as a size advantage, will enhance the likelihood of survival. As size is a cumulative trait, an early size advantage will be augmented (Vigliola & Meekan 2002), as bigger fish are more competitive for resources, benefiting growth and further increasing their competitive advantage (Chambers & Leggett 1996).

Importance of clutch micro-environment

Variable pattern in growth and development within an egg clutch or mass is a common response to variation in oxygen supply within that mass. The laws of diffusion and the metabolic requirements of the clutch often govern the constraints on the size and shape of an egg clutch. The structure and density of amphibian and marine invertebrate nests was critical in delivering oxygen to the innermost eggs e.g., barnacles (Lucas & Crisp 1987), gastropods (Lee & Strathmann 1998, Lardies 2002). Peripheral individuals develop faster in three-dimensional gelatinous eggs mass of gastropods (Chaffee & Strathmann 1984), some marine fishes (Giorgi & Congleton 1984) and frogs (Salthe & Mecahm 1974) as oxygen diffusion is restricted to central embryos, retarding their development. Egg position was important to normal development and survival of squid embryos laid in strands and developing with no parental care (Steer et al. 2002). *A. melanopus* egg clutches are single layered, and position in the clutch was important to growth and oxygen consumption rate regardless of clutch density, however in the reverse pattern to 3-dimensional clutches. The mechanism driving this was unclear and maybe a response to gas exchange around the clutch, metabolites exchange between embryos, or enhancement of initial size differences through size-related metabolism. It was clear though, that

within the clutch microenvironment, a size advantage was gained for embryos in the clutch centre.

Embryos from the periphery and centre of the clutch hatched at the same time suggesting that neither initial size nor differential micro-environmental conditions influenced developmental rate, as has been demonstrated in organisms in three-dimensional egg masses. Oxygen consumption was lower in embryos at the periphery of an egg clutch. Slower metabolism (indicated by oxygen consumption) would further enhance initial embryonic size differences as growth is constrained by metabolic rate.

Relevance of laboratory measures to fields conditions

The laminar flow system employed for these measurements are quantitatively different to the conditions demersal eggs would experience in wild conditions, and more so in *A. melanopus* where the parental egg tending would create a turbulent flow regime. Further, the relatively uniform and benign conditions of the laboratory may mask or filter ecological selection pressures on maternal investment in egg size. However the physiological ecology under test was whether embryology was a source of size variation and what the relationship of this was to nest design and oxygen consumption. The artificial nests and flow regime allow these factors to be partitioned out in a controlled environment. The slow experimental flow regime reflect flows likely to be experienced in the wild, as flow regimes are relatively stagnant in the semi-cryptic habitat that some cnidarians prefer (Patterson et al. 1991). Anemone fishes show a high degree of parental care and nest-tending, oxygenating the eggs with fanning of the pectoral fins. Chapter 3 investigates whether there is a response in parental fanning intensity relative to the identified developmental requirements of the embryos.

Conclusion

Examination of growth trajectories in demersally spawning marine fishes should no longer be restricted to the larval or adult phase. As this paper demonstrates, size differences emerge from a third major part of the developmental ecology, the embryo. Size variation within and between clutches of fish may originate from maternal endowment interacting with the environmental conditions during embryogenesis. Growth is cumulative within a

fish's life and no phase is independent of the rest (Vigliola & Meekan 2002).

Almost 100 years of empirical research on the larval period in marine fishes (e.g. Hjort 1914, Cushing 1972, Vigliola & Meekan 2002) have culminated in the recognition of distinct critical periods or days in larval development, on which response to environmental variation can influence the quality and magnitude of annual recruitment. The remaining variation may arise from the pre-larval stage where embryos cannot actively select their preferred environment, or physiologically adapt to their assigned environment. Therefore, embryogenesis should it be considered a critical period in the development of demersally spawned marine fish. The implications of maternal contribution interacting with clutch microenvironment require investigations to identify further links between development, environment and subsequent growth and recruitment.

Table 1. Results of two-way ANOVA's comparing the effects of clutch and position within a clutch on each morphometric measured for eggs 'Day 1' and larvae 'hatching'. Significant results are in bold type.

Factor		Source of variation	df	F	p
Day 1:	Egg volume	Position	1	10.7	**0.001**
		Clutch	3	21.2	**<0.001**
		Position x clutch	3	2.3	0.080
		Error	232		
	Yolk area	Position	1	1.6	0.208
		Clutch	2	14.7	**<0.001**
		Position x clutch	2	0.2	0.798
		Error	153		
	Egg area	Position	1	22.3	**<0.001**
		Clutch	3	141.5	**<0.001**
		Position x clutch	3	1.9	0.125
		Error	232		
Hatching:	Fish length	Position		57.5	**<0.001**
		Clutch		4.3	**0.016**
		Position x clutch		0.2	0.84
		Error	114		
	Body area	Position		46.0	**<0.001**
		Clutch		2.5	**<0.001**
		Position x clutch		0.3	0.71
		Error	114		
	Eye diameter	Position		22.3	**<0.001**
		Clutch		7.8	**.001**
		Position x clutch		1.1	0.32
		Error	114		
	Head depth	Position		32.4	**<0.001**
		Clutch		0.3	**<0.001**
		Position x clutch		0.22	0.805
		Error	113		

Table 2. Summary of a two-way ANOVA testing the effect of egg age and position within a clutch on the rate of oxygen consumption, standardised for embryo density within a clutch and egg size on day 1. Data were transformed (square root) to achieve homogenous variances. Significant results are in bold type.

Source of variation	df	F	p
Egg age	6	8.48	**<0.001**
Position within a clutch	6	9.46	**<0.001**
Egg age x position	36	1.22	0.193
Error	230		

Chapter 3: Males are more proactive than females in replenishing oxygen to fish nests.

Publication: Green, B.S. & McCormick, M.I. (in review) Males are more proactive than females in replenishing oxygen to benthic fish nests *Behavioural Ecology*

Synopsis

Parental care through nest maintenance and defence enhances offspring success. In nature, obligate anemone dwelling fishes and their nests of benthic eggs are protected against most predators by their host anemone, thus parental care comprises of nest tending through fanning and cleaning. Fanning is believed to oxygenate the eggs, however a real-time link between fanning and oxygenation is tenuous. This study investigated whether fanning modified the oxygen microenvironment of the embryos, and subsequently, whether fanning was modified according to ambient dissolved oxygen (DO), increasing metabolic demands of developing embryos and water temperature. There was a time lag of 1 second between fanning and increases in the amount of oxygen within the nest, demonstrating that DO is directly affected by parental tending. While there was evidence of biparental care, males invested more time tending nests (40 % initially) than did females (20 – 30 %), and male investment increased to 70 % as embryo development progressed and embryonic metabolic demands increased. Additionally, male fish adjusted fanning effort on a diel cycle as ambient DO fluctuated: time spent fanning was lowest between 10:00 - 14:00h (35 %), when ambient DO was highest, and increased throughout the day, reaching a peak of 70 % between 22:00 – 02:00h, when ambient DO was lowest. Increased water temperature reduced the number of fanning bouts min^{-1} throughout the day, but did not influence any other aspect of fanning behaviour. These results suggest that fish adapt fanning behaviour coincident to changing conditions in the nest, both at a daily level and throughout development of the embryos.

Introduction

Parental fitness is measured by reproductive success, which in part depends on offspring survival. In oviparous species, females can enhance reproductive success by investing in the size, quality and number of eggs. Males, on the other hand, make very little contribution to the initial embryonic aliquot. One life history mechanism that increases parental fitness is actively investing in some form of parental care. Parental care enhances offspring development (Sargent 1988,1997), can affect offspring phenotype (Shine et al. 1997) and increase offspring survival (Sabat 1994). For many marine fishes, parental input to their offspring ceases when gametes are released directly into the ocean currents, however, 14% of marine species attach adhesive eggs to the benthos (Leis 1991) and actively tend them (Clutton-Brock 1991). Brooding eggs on the substratum is the most common form of parental care in fishes (Sargent 1997), and the male is generally the primary tender (Gross 1985, Mazzoldi et al. 2002). Thus, parental tending is a mechanism by which males can invest in their offspring (Bernado 1996).

Brooded eggs remain in the parentally chosen environment, and developing embryos have minimal physiological capacity to adapt to environmental change (Pelster 1999). Variables such as temperature and oxygen availability will influence their metabolism or physiology, and therefore their survival (Hale et al. 2003). In organisms with parental care, adults can compensate for environmental conditions by changing the microclimate around the eggs. Snakes employ shivering thermogenesis which acts to warm their eggs (Shine et al. 1997), amphipods actively ventilate the brood pouch (Dick et al. 1998) and fishes fan to increase water circulation (Coleman 1992, Takegaki & Nakazono 1999). Compensating for natural environmental variation is rarely considered to be a cost in empirical studies of parental care (St Mary et al. 2001), even though environmental variation is ubiquitous. Theoretical models assume that the rate and degree of parental investment is dependent on environmental factors (van Iersal 1953, Perrin 1995), however empirical evidence of this in marine fishes is limited.

Parental care of benthic fish eggs consists of several distinct behaviours: fanning the clutch using the fins serves to move water over the

eggs, thereby removing debris and disturbing the boundary layer (St Mary et al. 2001) and replacing deoxygenated water with oxygenated water (Takegaki & Nakazono 1999); mouthing to remove dead embryos and clean live ones (Keenleyside 1991) using antimicrobial properties within the epidermal mucous (Knouft 2003); and nest guarding, protecting the nest from predators (Keenleyside 1979, Sargent 1997).

Temperature and dissolved oxygen are the most important influences on fish metabolism in early development (Rombough 1988) and oxygen is more likely to be limiting in aquatic than terrestrial habitats (Kramer 1987). Dissolved oxygen (DO) levels fluctuate in coral reef habitats due to both biotic processes (e.g., respiration and photosynthesis of algae and coral) and abiotic processes (e.g., currents, tides and small-scale water circulation) (Kraines et al. 1996). In particular, the cryptic habitats where demersal eggs are often spawned may, by their nature, have poor water circulation. Because oxygen supply to the eggs can be a critical determinant of developmental success (Chaffee & Strathmann 1984, Fernández et al. 2003) some species of fish choose nest sites with reference to the oxygen environment (Lukas & Orth 1995, Jones & Reynolds 1999b, Takegaki 2001), while many species use fanning behaviour to compensate for sub-optimal oxygen conditions (Takegaki & Nakazono 1999). If fanning intensity compensates for environmental oxygen availability then parents should adjust nest tending behaviour to the needs and requirements of their brood, and to the response of the brood to environmental variation (St Mary et al. 2001). However, the complex relationship in marine organisms between the environment (oxygen and temperature); embryo developmental stage; and parental tending has not been examined in detail.

In this study we used the benthic-spawning, coral-reef fish *Amphiprion melanopus* (Pomacentridae) as a model to explore whether adults adjust their nest-tending behaviour to changes in the environment and needs of their offspring. Specifically, we addressed the following questions: (1) Does fanning change the quantity of oxygen at the nest, and (2) do parents alter their tending behaviour according to changing environmental conditions and the increasing metabolic demands of their brood? We predicted that fanning would increase the DO concentration available to the eggs, that tending

behaviour would track changes in ambient O_2 concentrations, and that nest-directed tending would increase with increased embryonic development.

Materials and methods

Study species and maintenance

In their natural environment, pairs of *A. melanopus* attach a monolayer of eggs in a circular clutch (diameter: 40-100 mm) to hard substrata under the margin of the stinging tentacles of their host anemone (Wilkerson 1998). The embryos develop for 8-9 days before hatching at ambient temperature (28 ^{o}C), and are tended by both parents, although males typically tend more than females (Allen 1980).

Pairs of *A. melanopus* were collected from the northern section of the Great Barrier Reef, Australia, adjacent to Cairns (16º8' S, 145º7' E), where they would naturally experience annual temperature fluctuations from 25-31ºC. Pairs were housed in 70-litre circular tanks with constant flowing salt-water at the James Cook University Research Aquarium. Pairs were randomly assigned to either hollow cement blocks (internal dimensions 160x160x190mm) or pieces of PVC pipe (internal diameter 100mm) simulating a protected nest where they laid their eggs. Pairs were fed twice a day on a mixed diet of Wardley's marine flakes (Wardley's Aquarium products, Australia) and chopped pilchards (*Sardinops spp*) and squid (*Loligo spp*).

Experimental design and quantifying nest-tending behaviour

For aquatic organisms, temperature and oxygen are linked, as an increase in temperature causes a decrease in the amount of dissolved oxygen in the water, as well as increasing metabolic rate in exothermic animals. To determine whether variation in temperature affected parental behaviour, nest-tending was filmed on multiple clutches at three temperatures which spanned the range of temperatures that the species experiences during embryogenesis across its geographic range. The saltwater supply to tanks containing breeding pairs was maintained at 28 ºC ± 0.5 ºC. Once eggs were laid, the temperature of water within the tank was either maintained 28 ºC ± 0.5 ºC

(n=11 clutches); elevated to 31 ºC ± 0.5 (by the addition of two 300W submersible water heaters per tank; Rena™)(n=10 clutches); or cooled to 25 ºC ± 0.5 ºC (using a chiller unit; Carrier's heat pump™)(n=2 clutches). Reducing the temperature to 25 ºC reduced or ceased egg production in these treatments resulting in only 2 replicate clutches at 25 ^{o}C.

Nest tending

Following these temperature manipulations, nest-tending effort was recorded throughout embryonic development, commencing 4 hours after the temperature change. Tending behaviour was recorded using waterproof colour CCTV cameras with an in-built infrared light and automatic infrared trigger for filming under low illumination (AVC667, Jaycar, Australia). As fish cannot see in the infrared spectrum (Batty 1983, Higgs & Fuiman 1996), we assumed that the infrared lighting did not affect tending behaviour. Tending behaviour for the 23 experimental clutches was recorded simultaneously from 4 nests, using 4 cameras connected to a 4-channel quad processor and a real-time video recorder (Xpose, model QV3053, Electus Distribution, Silverwater Australia); and viewed on a Sony 54cm monitor.

To sample fish tending behaviour on a diel cycle, footage of the nest was recorded at 8 separate intervals, each of 20 minutes, within each 24hr time period. Intervals were spaced three hours apart, totalling 160 minutes of footage per pair, per day. Each interval was randomly sub-sampled and, to reduce rates of error, the shortest possible time interval for sub-sampling was used (Martin & Bateson 1993). Nest-tending video records were therefore sampled for 1 minute of playback time, (corresponding to 2.5 minutes periods of real time), 5 times, every 3 hours, for the 8 intervals per day. All video observations were recorded by an observer, using a purpose-designed manually operated computer data logger program (written in Access 2000, Microsoft).

Quantifying nest tending

Nest tending was defined as any of two distinct types of behaviour directed at eggs within the nest: (i) nest cleaning - when fish tended the eggs with their mouths; and (ii) fanning - fanning eggs with either left or right pectoral fins or caudal fin. Nest tending behaviour was quantified by recording

the start and end of each tending approach to the eggs, commencing when the parent fish was within one body length of the eggs. Each nest-directed activity within an approach was then recorded by type and frequency, ie. the number of times the mouth or the fins were directed at the eggs. In order to detect subtle differences in behaviour, or trade-offs between length and regularity of tending event (Reebs et al. 1984), three measures of tending behaviour were recorded: proportion of time spent tending; frequency of each behavioural type per minute; and the duration of each tending approach.

Oxygen Consumption Measurements

Oxygen concentration within the egg clutch was measured using a micro-optode (140 µm tip, Presens, Germany) as per Chapter 1. In order to measure the real-time effect of parental fanning on the oxygen available to benthic embryos, the quantity of oxygen (% air saturation) was measured within the egg clutch using a micro-optode while simultaneously recording parental nest-tending behaviour with the CCTV camera equipment. Oxygen measurements were made on two clutches of eggs, referred to as clutch 1 and clutch 2. Clutch 1, laid on an acetate sheet attached to a cement block, was filmed on day 4 of development at 31 ^{o}C. Clutch 2, laid inside PVC pipe, was filmed on days 1-3 of development at 28^{o}C. Oxygen was measured in clutch 1 by pushing the micro-optode needle through the acetate from underneath the eggs so the fibre protruded, sitting flush with the tops of the eggs. Oxygen was measured in clutch 2 by drilling a small hole through the pipe and inserting the needle-probe. In both cases the tending parents damaged the fibre as they cleaned their nests of foreign objects, and so further measurements were aborted. Forty minutes of oxygen readings were collected from clutch 1 before the fibre was damaged, and 30, 59 and 27 minutes were collected on days 1, 2 and 3 respectively, for clutch 2.

Rates of embryonic oxygen consumption throughout development were collected as described in (Green in review, Chapter 1) and have been included here for comparison with fanning regime throughout development. Embryonic development was observed at 6-hourly intervals and related to oxygen consumption of embryos within clutches throughout development, measured using the vertical profiling method (Kuhl et al. 1995).

Ambient water oxygen content

Oxygen content of the aquarium water was measured over 5 nights to determine the natural variability within the breeding tank system, using a Clark-type microelectrode (YSI Inc, USA) attached to a data logger (WP 82, TPS Pty Ltd Australia). The probe was placed in high-flow water stream at the outlet of a brood- stock tank within the flow-through system, where all breeding pairs were housed. % oxygen saturation was recorded every 20 minutes for five 24-hour periods.

Data analysis

We determined the effects of time of day, egg age and temperature on the quantity, frequency and duration of parental fanning behaviour using repeated-measures MANOVA and ANOVA. Some data were missing from the original design, due to anemones obscuring the camera's view of the eggs, or the adult fish bumping the camera out of alignment, so it was not possible to conduct a repeated-measures MANOVA including all factors. Therefore, separate analyses were performed addressing nest-tending behaviour at different times throughout this study. First, we examined the influence of egg age (repeated-measure) and temperature (fixed factor) on time-spent nest-tending, as well as on the duration and frequency of tending episodes. Second, we examined the influence of egg age (repeated measure) on the type of nest-directed tending behaviour. Third, we tested time of day (repeated measure) and temperature (fixed factor) on the proportion of time spent tending and the frequency and length of tending bouts. Finally, the influences of time of day (repeated measure), temperature and developmental stage (divided into early, middle and late development) on total time spent tending the nest were examined. Where ANOVA results did not conform to the assumption of sphericity, Greenhouse-Geisser approximations were used (StatSoft 2002). Tukey's post-hoc comparisons were used to determine the source of significant differences.

The real-time relationship between oxygen and parental tending was examined using autocorrelation function plots (ACP) and partial autocorrelation function plots (PACP) to indicate the degree of time lag that explained most of the variation in the data set. Once the appropriate time lag

was identified, a polynomial distributed-lags ANOVA and regression were examined to identify whether the proposed time lags were significant.

Results

Patterns of DO concurrent to Parental Fanning

Parental fanning behaviour affected the quantity of oxygen in the water adjacent to the eggs. There was an increase in the amount of oxygen (up to 95-100 %) available to the eggs in response to parental fanning, and a decrease to low levels (minimum 67 %) when parents were away from the nest ($p<0.001$, Table 1). A time lag of one second best described the relationship between parental fanning activity and an increase in the quantity of oxygen in the vicinity of the eggs for the 4 clutch/age combinations measured (Fig. 1, Table 1).

Sex-related Tending Behaviour

Parental care of egg clutches was recorded for a total of 96 hours out of 156 days of embryonic development. Females fanned the clutch an average of 20 – 30 % of this time. Overall, male fish spent significantly more time (2-4 times) tending the nest than did females (Fig. 2a, b, c, Table 2). The

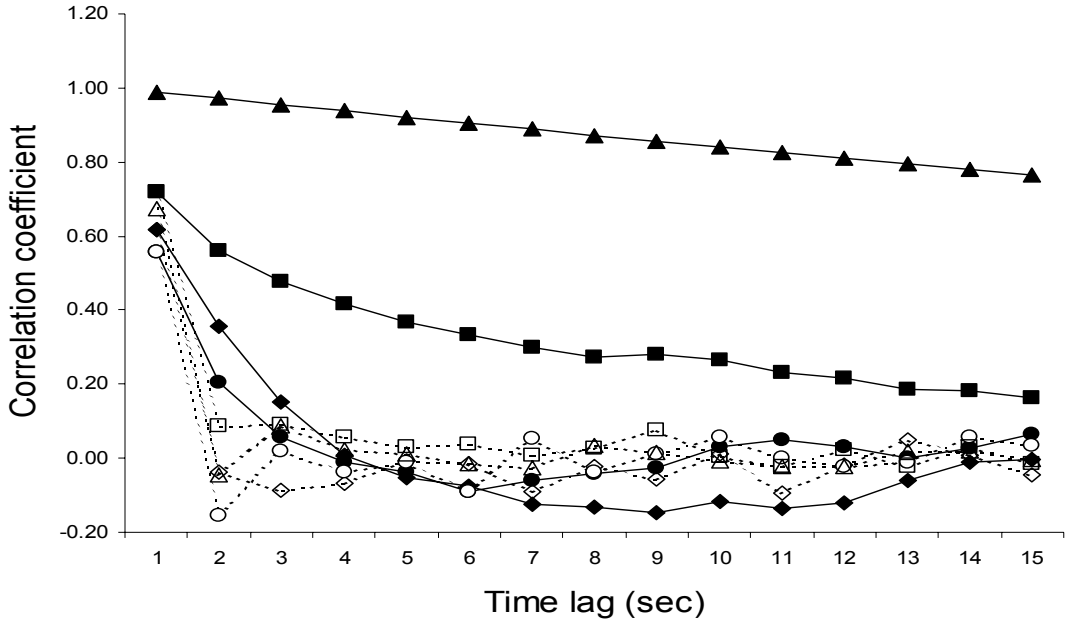

Fig. 1. Plots of autocorrelation (ACP) and partial autocorrelation (PACP) coefficients from time lag analysis of simultaneous parental nest-tending and oxygen measurements. Filled markers are ACP and unfilled markers are PACP plots. ⇦ Clutch 1 day 4, ⬇ Clutch 2 day 1, ⬉ Clutch 2 day 2, ■ Clutch 2 day 3.

Table 1. Real time comparison of parental fanning behaviour and quantity of oxygen within egg clutches, summarised by polynomial distributed lags ANOVA. a. clutch 1 day 4, 31°C; b. clutch 2 day1, 28°C; c. clutch 2 day 2; d. clutch 2 day 3. R=0.56 R^2=0.31 N:606. Significant results are in bold type

		df	MS	F	p
a.	Regression	3	19.86	92.57	**<0.001**
	Residual	603	0.21		
b.	Regression	2	23.84	112.79	**<0.001**
	Residual	484	0.21		
c.	Regression	2	44.69	199.35	**<0.001**
	Residual	1622	0.22		
d.	Regression	2	6.76	52.07	**<0.001**
	Residual	550	0.13		

amount of time tending by males and females did not change significantly with incubation temperature (Fig. 2a, b, c; non-significant 3rd order interaction Table 2b). Further, the female contribution was relatively constant throughout development with the exception of a small increase on the last day of development, corresponding to hatching time for the embryos (Fig. 2a, b, c.). Given the relatively small, unvarying contribution to overall nest tending by females, the rest of the results focus on the male contribution to tending the egg clutch.

Nest-Tending Behaviour

Egg fanning behaviour occurred more frequently than cleaning behaviour (Fig. 3). The average frequency of fanning episodes per minute increased linearly from day 1 (7 min^{-1}) to day 7 (26 min^{-1}) of development, when it reached an asymptote until hatching at day 8 or 9 (Fig. 3, Table 3). Egg fanning occurred considerably more frequently than cleaning in days 4-9 of development (Fig. 3, significant Egg age*Behaviour interaction, Table 3). The observed frequency of egg cleaning behaviour was relatively constant throughout development, occurring approximately 7 times min^{-1} from day 1 to 9 of development.

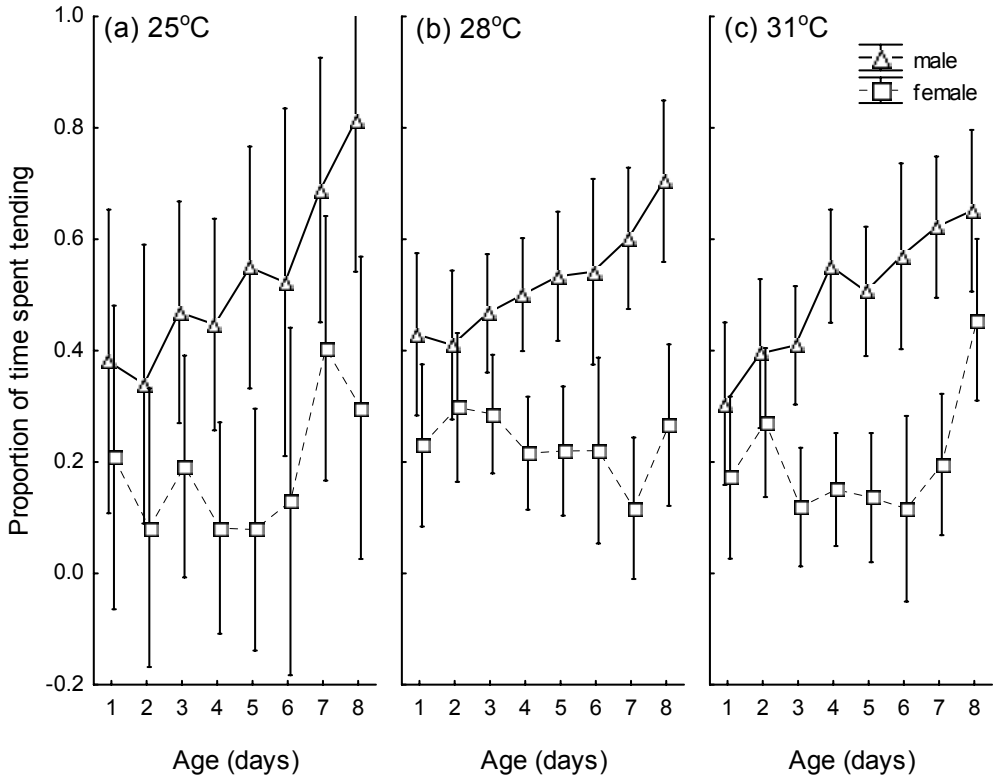

Fig. 2. The average proportions of time females (squares) and males (triangles) spent tending their benthic egg clutches at three water temperatures: (a) 25 °C; (b) 28 °C; (c) 31 °C; for each day throughout embryonic development. Error bars are standard errors.

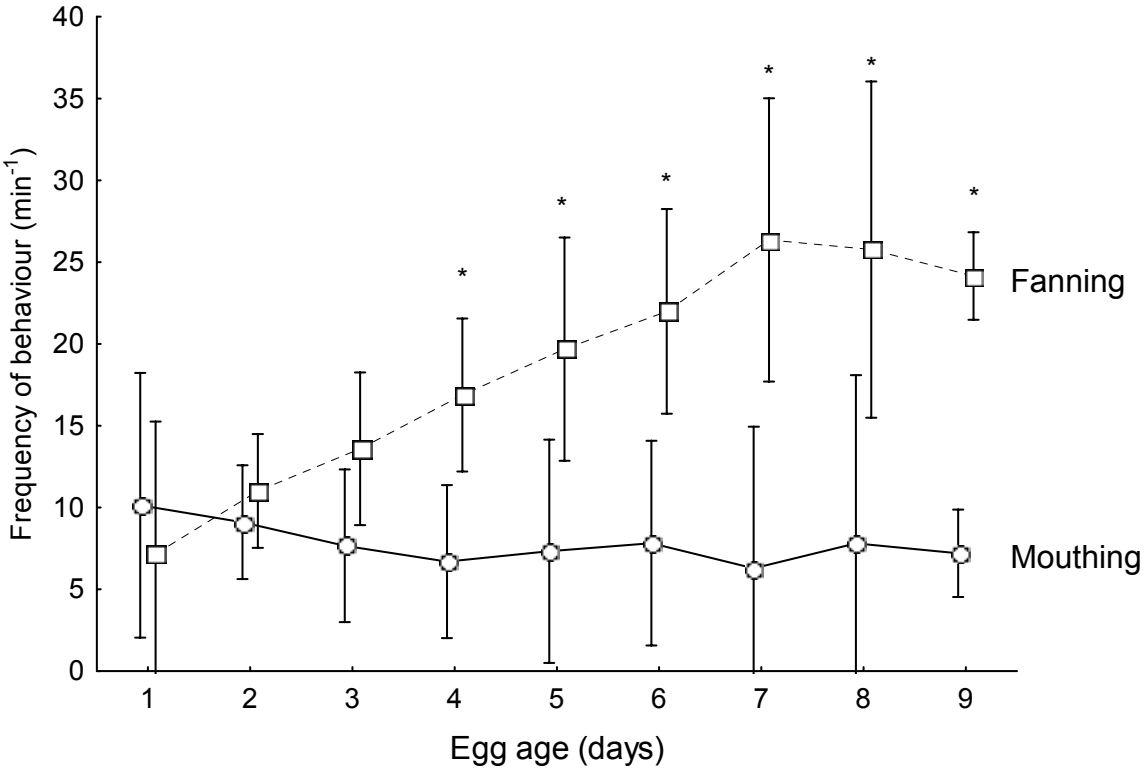

Fig. 3. Frequency of different nest-tending behaviours of males throughout embryology. Squares represent fanning, circles represent cleaning. * denotes significant differences from Tukey's HSD means comparisons (α 0.05). Error bars are 95% confidence intervals.

Changes in Nest-directed Tending Behaviour throughout embryogenesis

Time spent tending

The proportion of time the male fish spent nest tending the egg clutch increased gradually with development of the embryos from day 1 to day 8 (Fig. 4a, Table 2ai), however, water temperature had no effect on the proportion of time spent fanning throughout development (Table 2ai,bi). During early development the time spent tending the eggs was, on average, less than 40 % and increased to close 50 % when eggs reached the mid-stage of development (day 4 - 6). There was a large increase in the overall average amount of time spent tending in the last two days of development, reaching almost 70 % (Fig. 4a). Interestingly, the overall increase in the proportion of time spent tending throughout development followed the general change in embryonic oxygen consumption throughout embryo development (from Chapter 1), except during the peak in oxygen consumption on day 4 of embryo development (Fig. 4a).

Frequency of tending

Egg tending activity was least frequent on the first day of development, and increased sharply from approximately 7 tending episodes min^{-1} on day 1 of development, to 12 min^{-1} on day 2, and then remained around 12 min^{-1} (Fig. 4b); this was averaged over temperature treatments as there was no temperature effect on frequency of tending intervals (Table 2ai, bii).

Duration of tending event

Average duration of each egg tending event increased two-fold throughout development (Fig. 4c) from approximately 15 sec $sequence^{-1}$ (day 1 of egg development) to 30 sec $sequence^{-1}$ (day 8) and was not affected by the experimental temperatures (Table 2aiii, biii).

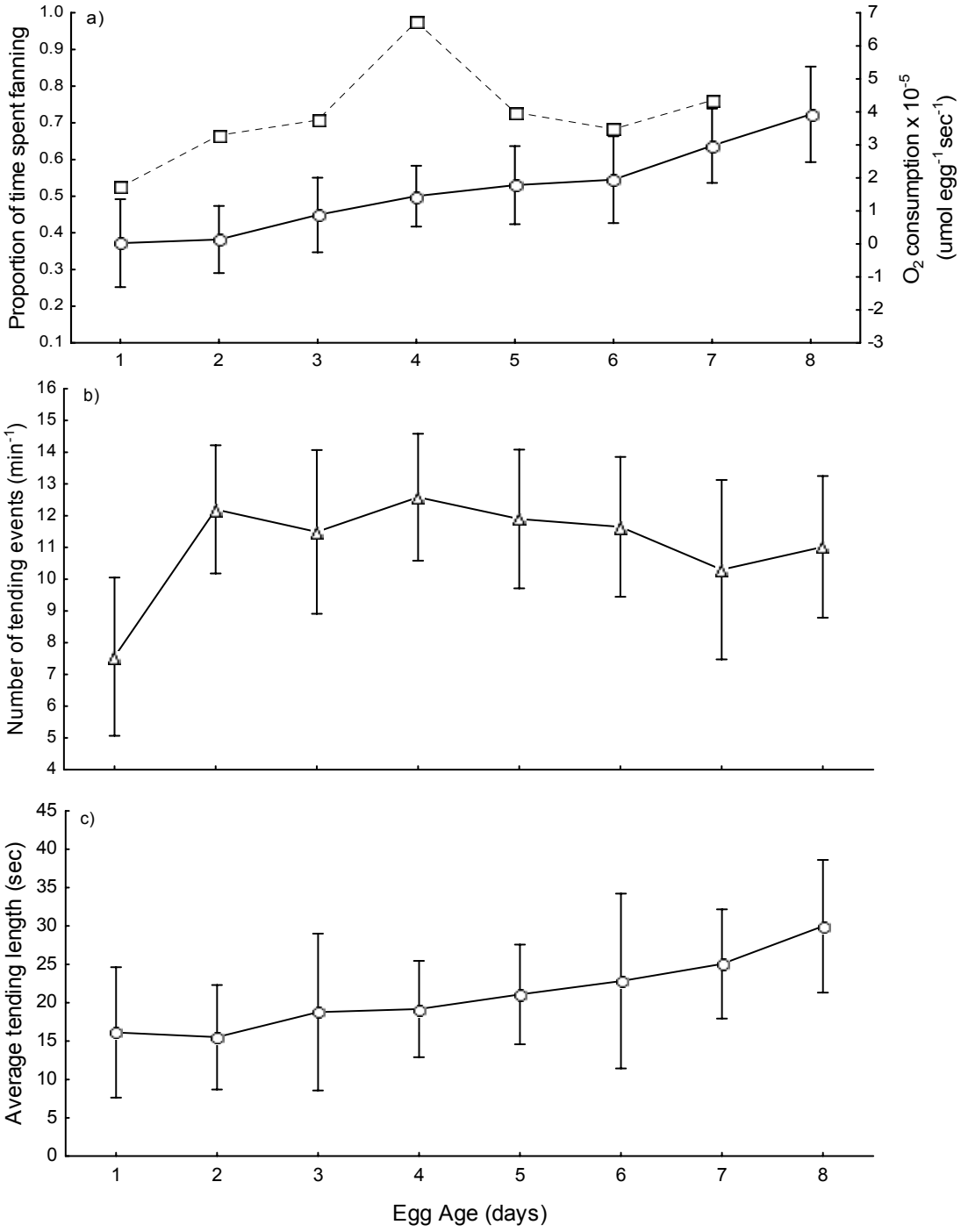

Fig. 4. Nest fanning activity of males throughout development: a) proportion of time spent fanning (solid line), including mean rate of embryo oxygen consumption (broken line); b) mean frequency of tending events per minute; c) average length of tending events. Error bars are 95% confidence intervals.

Diel changes in Tending Behaviour

Time spent tending

Time of day influenced the proportion of time spent tending egg clutches (Table 4ai) with distinct patterns of fanning following a diel cycle and patterns in ambient DO concentration (Fig. 5a). Time spent fanning was lowest during the daylight hours when DO was highest, increased at dusk (1800h) as DO was decreasing, and peaked in the hours around midnight (2200h-0200h) when DO was lowest (Fig. 5a). Experimental manipulation of water temperature did not affect the proportion of time spent fanning throughout the day (Table 4bi).

Frequency of tending

The number of fanning events min^{-1} showed a general decrease throughout the day, and at all temperatures was lowest between 2200 and 0200h (Fig. 5b, Table 4aii, bii). Water temperature affected the frequency of tending events throughout the day (Table 4bii). Fish tending eggs in 25^{o}C water had the highest average number of fanning events for each time period, with approximately 10 more approaches to the eggs min^{-1} than fish in 31^{o}C water. Fish at 28^{o}C, or ambient temperature, had the lowest frequency of tending episodes (Fig. 5b).

Duration of tending event

There was a diel pattern in the average length of egg tending event that was not affected by water temperature (Fig. 5c, Table 4aiii,biii). The average length of tending duration was lowest during the day (approximately 15 sec $sequence^{-1}$) and increased at dusk (1800-2200 h) to peak around midnight and early morning (2200-0200 h) (Fig. 5c).

Trade-offs between frequency and duration

The length of tending sequence showed an inverse relationship to the number of tending events in relation to time of day (Fig. 5b cf. 5c). When the number of tending events was high, the length of tending sequence was low (Fig. 5b cf. 5c). Conversely, average length of tending sequence increased similarly to the proportion of time spent tending (Fig. 5a cf. 5c) such that as the proportion of time spent fanning increases, males spend more time in each fanning bout, reducing the frequency of events, thereby leaving the clutch for other activities less often.

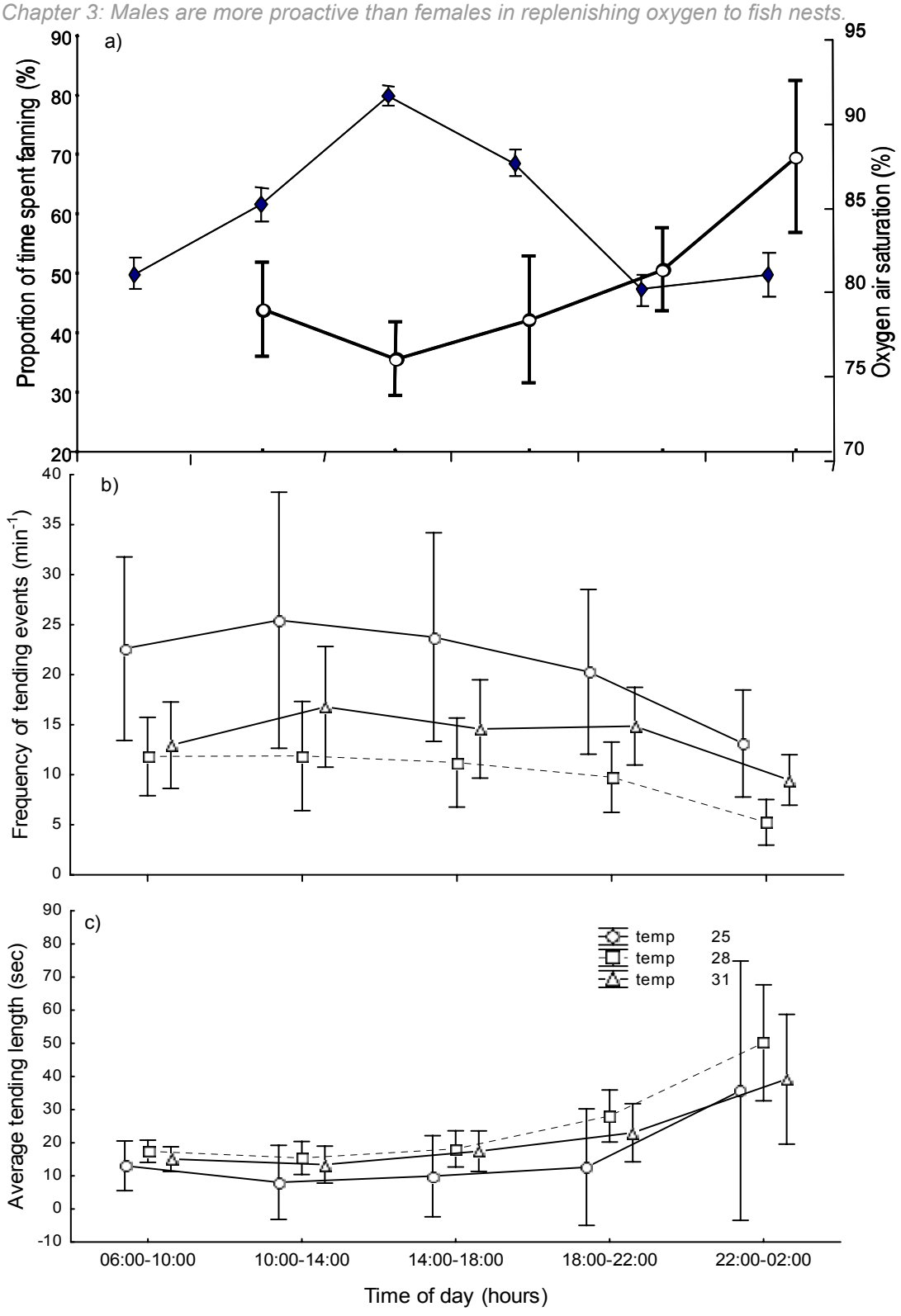

Fig. 5 Diel patterns in nest fanning activity of males summarised for 5 time periods throughout the day at three experimental water temperatures: a) proportion of time spent fanning (○) and ambient DO (⇦); b) average number tending events per minute period; c) average length of tending event. No significant temperature effect in a), so temperature not displayed. Symbols for b) and c): ○ 25°C;□ 28°C; △ 31°C. Error bars are 95% confidence intervals.

Temperature, developmental and diel fluctuation in time spent tending

When the effects of time of day, developmental stage and water temperature on time spent fanning were considered simultaneously, the diel allocation of time spent actively tending the eggs changed in response to the developmental stage of the embryos, but not in response to experimental water temperature (Fig. 6, Table 5). The overall time spent fanning late stage embryos was approximately 10 % higher than for early stage embryos for all times throughout the day (Fig. 6). Time spent fanning was lowest for all developmental stages through the daylight hours (1000-1800 h) (Fig. 6). The proportion of time spent fanning increased at nightfall and peaked between 2200-0200h for all stages of development.

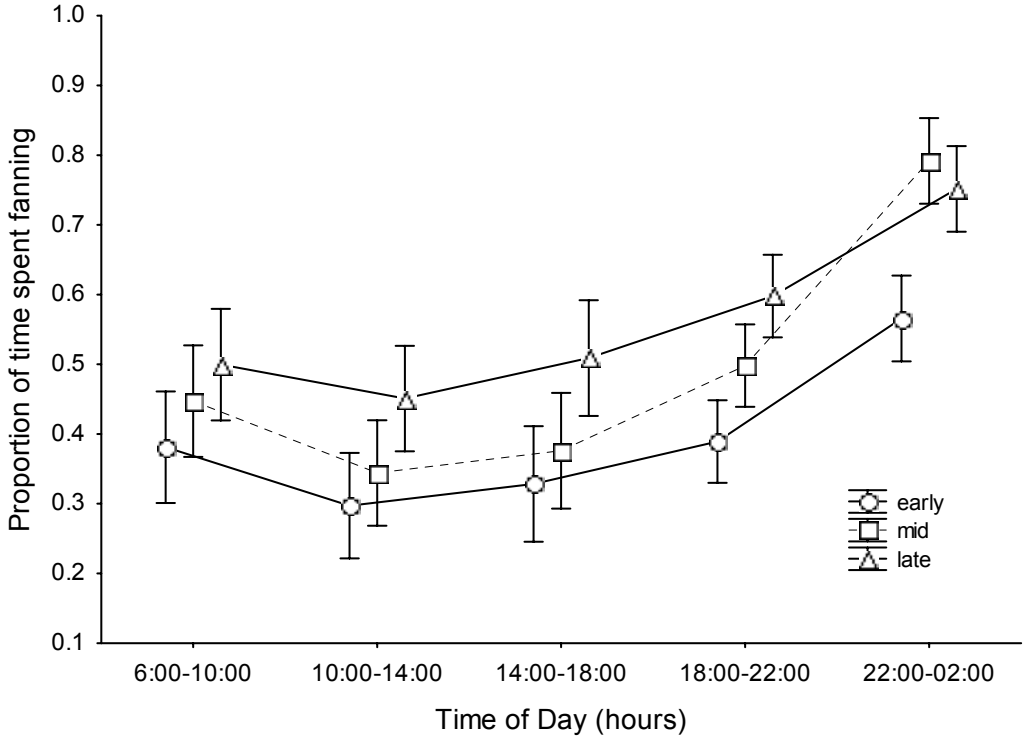

Fig. 6. The proportions of time adult males spent tending their benthic egg clutches averaged over temperature treatments summarised for 5 time periods throughout the day for three stages of development. Early development- days 1, 2, 3 (↓); mid-development- days 3, 4, 5, (♣) and late development- days 7, 8, 9 (↖). Error bars are 95% confidence intervals.

Discussion

Parental fanning increases oxygen to eggs

A comparison of parental fanning behaviour and the simultaneous measurement of the quantity of dissolved oxygen (DO) at the embryos' surfaces showed a significant link between these two events, suggesting that fanning behaviour does indeed replenish oxygen to the nest. We found an increase in the amount of oxygen available to the eggs in response to parental fanning, and a decrease to low levels when parents were away from the nest, so increasing O_2 to the eggs is the likely objective of fanning behaviour. Such behaviour is likely necessary because the semi-cryptic areas where many demersal eggs are laid have poor water circulation and boundary layers rapidly form around eggs, reducing the transfer of oxygen from the surrounding water to the developing embryos (Rombough 1988). Males spent more time than females in active egg tending, which is typical of the genus *Amphiprion* (Allen 1980, Wilkerson 1998) and 58% of other fish families with parental care (Clutton-Brock 1991).

Parental care in *A. melanopus,* consisted of fanning and mouthing the eggs, initially in equal amounts, but as development progressed, fanning increased to 83 % of tending activity while cleaning remained at a constant level. In addition to oxygenation, fanning is thought to remove metabolic wastes (Keenleyside 1991); while mouthing behaviour removes dead larvae and cleans live ones (Keenleyside 1991) with an antimicrobial compound within the parental epidermal mucous (Knouft et al. 2003). Parental care is vital in maintaining the health of a brood, through oxygen replenishment and waste and bacteria removal, which in turn increases the reproductive success by increasing the chances of offspring survival.

Diel patterns in ambient oxygen and tending

Oxygen replenishing parental tending behaviour changed with time of day and ambient dissolved oxygen (DO) levels. Although oxygen levels were not directly manipulated, there was a clear relationship between ambient oxygen availability and tending. As ambient DO decreased after dark, when algal photosynthesis ceases and respiration dominates, parental tending activity increased. Parental tending was reduced from dawn throughout the daylight hours, corresponding to increasing DO. We conclude that fish are

adapting their nest-tending behaviour on a diel basis according to the availability of DO.

Time invested in nest tending is the product of frequency and duration of tending events. Subtle differences in the composition of fanning behaviour were apparent in *A. melanopus,* whereby night bouts were longer and less frequent than day bouts. Similar trade-offs compensating for a DO decrease have been described in *Gasterosteus aculeatus*, the three-spined stickleback (Reebs et al. 1984), where despite the increase in nocturnal bout length, an overall difference in time spent tending was not detected. Diel patterns of fanning behaviour are rarely considered in fishes despite evidence that differences in day and night exist (Reebs et al. 1984, Hinch & Collins 1991). Typically, fanning is observed once a day for a short period (10 minutes) and tending behaviour is quantified from this (Bjelvenmark & Forsgren 2003). Subtle adaptations in behaviour to environmental changes may be overlooked without multiple measures of tending over both day and night.

Nest-directed tending throughout embryonic development

In addition to diel variation in tending behaviour, *A. melanopus* demonstrated a change in tending over the course of embryonic development. Time spent tending the nest was low in the early embryonic stages and then increased as embryogenesis progressed and embryonic oxygen consumption increased (Green in review, Chapter 1), aside from a peak in O_2 consumption midway through development, which coincided with the appearance of haemoglobin within the circulatory system (Green in review, Chapter 1).

Such changes in time spent fanning over development, coupled with the real-time correlation between tending and oxygen level, suggest that males increased their nest tending to compensate for the increased requirements of their developing propagules. Quantity of nest fanning has been negatively correlated to oxygen concentration within fish nests throughout development (van Iersal 1953, Reebs et al. 1984, Torricelli et al. 1985, Jones & Reynolds 1999a, Takegaki & Nakazono 1999). However, this study is the first demonstration of real-time changes in oxygen relative to parental care, and changes in embryonic metabolism and parental care.

Temperature Effects on Tending Behaviour

DO decreases with increasing temperature, while the metabolism of poikilotherms increases with increasing temperature. Considering this, we expected an increase in temperature to increase fanning behaviour, as parents adapt to the increased metabolic needs of their embryos in an environment with less oxygen available. However, our results did not support this prediction. *Amphiprion melanopus* did not modify their nest-directed fanning behaviour in response to a 3 $^{\circ}$C temperature change. Water temperature did not interact significantly with time of day or egg age, however it did result in one trade-off, which was between the number of tending events and the average length of tending event. While we predicted that temperature would influence tending behaviour, *A. melanopus* is not unique in showing little change in parental care with temperature change. The Florida flagfish, *Jordonalla floridae* (St Mary et al. 2001), showed no difference in tending behaviour with similar experimental variations in temperature among treatments, leading the authors to conclude that changes in egg demands were primarily responsible for differences in tending behaviours. Further, the amount of goby nest-tending behaviour was not correlated with water temperature, however there was a diel increase in pectoral fin beat frequency with an increase in temperature (Torricelli et al. 1985). Male fish may have adapted subtle aspects of their tending to temperature changes, such as fin beat frequency, but this was not measured in the present study. Alternatively, the reduction in DO with increased temperature at these tropical temperatures is small (approximate q10: O_2 solubility, for a temperature change of 28 - 31 ºC = 0.15 c.f. 10 – 13 ºC = 0.24). As temperature increases, the relative reduction in DO decreases, i.e. there is an exponential decrease (Broecker & Peng 1974), and so it is possible that the small difference in DO caused by the experimental 6 ºC range among temperature treatments was not enough to influence parental tending. Further, the efficient oxygen uptake mechanisms of fishes may preclude the oxygen change resulting from increased temperature having much impact, except during times of high demand (Fry 1971).

While temperature did not directly influence the daily quantity of parental tending behaviour, a reduced temperature slows embryonic

development, thereby increasing the number of days parental care is required (small mouth bass, Ridgway & Friesen 1992, *A. melanopus*, this study). It is likely then that temperature change can change the cost of parental care through its effects on rate of embryonic development, despite no detection of direct responses to temperature change.

Relevance to field studies

This study examined tending in a laboratory, free from predators, and did not mimic a tending fish's natural environment, where they may be faced with trade-offs between nest fanning and defending their nests from predators and competitors. Fishes of the genus *Amphiprion* all have obligate associations with anemones and lay their eggs on the substrata under the shelter of the anemone's stinging tentacles (Wilkerson 1998). Therefore, they have a natural system of defences against predators, and time allocated to predator defence probably does not differ markedly between the laboratory and field (Sargent 1985). Further, my results are similar to observations on wild nests of a congeneric, where the tending parents were subject to multiple natural stimuli, for which fanning behaviour was found to increase on the last two days of development from 30 % to 87 % of the males time (Allen 1972). Monitoring behaviour and oxygen concentration in the laboratory has allowed me to partition the importance of temperature, oxygen and embryonic development to the level of parental tending, without the confounding influences of irregular defence and foraging excursions. This laboratory study suggests that embryonic developmental stage, time of day and ambient oxygen concentrations are the most important factors in determining the amount of parental nest-tending, while incubation temperature showed only indirect increases to cost through prolonged embryonic development.

Nest-tending Trade-offs

Fanning a nest to provide oxygen to the eggs can be the most demanding part of parental care (van Iersal 1953). Through active nest tending, parent fish are resolving a behavioural conflict between present and future reproductive success. Theory predicts that parents should maximise lifetime reproductive success by allocating tending effort according to the costs and benefits derived from investing in current broods relative to those forfeited for future broods (Sargent & Gross 1993). On one hand, active nest

tending increases the survival of the current brood, while on the other, tending may reduce parental condition by the amount of energy allocated to tending or missed feeding opportunities, and, therefore, can compromise future reproduction (Sargent 1985, Zink 2003). Adapting parental care to the prevailing conditions and to propagule requirements can optimise this trade-off by reducing the costs to the parents while providing sufficient parental care for the offspring.

Conclusions

Adult tending behaviour modifies the oxygen environment of the eggs as concurrent measures of oxygen and fanning illustrated. Parental tending behaviour increased with decreasing ambient DO levels, and with the developmental stage of the embryo, supporting my predictions. The trends in fanning behaviour and DO suggest that parents are adjusting their fanning rates in response to oxygen requirements and availability, minimising their own costs in nest tending. Previous studies have documented that fish larvae (Breitburg 1992), juveniles and adults (Kramer 1987) modify their behaviour and avoid water with sub-optimal quantities of dissolved oxygen. It appears that fish have some mechanism for detecting subtle DO concentration gradients and a feedback mechanism that directs them to modify their behaviour. My study suggests that parental tending of benthic clutches actively replenishes oxygen supply next to the eggs. Being able to detect and respond to subtle differences in oxygen concentration will have obvious fitness advantages to the parents if it leads to greater survival of offspring while optimising parental input.

Table 2. Result summary table for a repeated measures ANOVA testing for the effect of temperature (Temp) and egg age on: (i) sex and the proportion of time spent fanning; (ii) frequency of male tending events; (iii) average length of male tending sequence. a. Assumption of sphericity and compound symmetry was violated, therefore the Greenhouse & Geisser approximations were used to compensate. b. Between subjects summary. Significant results are in bold type

a.	Source	G-G Epsilon	G-G Adj. df1	G-G Adj. df2	G-G Adj. p
i.	Egg age	0.63	4.42	115.09	**<0.001**
	Egg age *sex	0.63	4.42	115.09	**0.019**
	Egg age *temp	0.63	8.85	115.09	0.205
	Egg age *sex*temp	0.63	8.85	115.09	0.457
ii.	Egg age	0.54	3.76	52.63	**0.033**
	Egg age *temp	0.54	7.51	52.63	0.866
iii.	Egg age	0.39	2.76	35.85	**0.041**
	Egg age *temp	0.39	5.51	35.85	0.599
b.		df	MS	F	p
i.	Sex	1	4.43	48.94	**<0.001**
	Temp	2	0.02	0.22	0.802
	Sex*temp	2	0.01	0.09	0.916
	Error	26	0.09		
ii.	Temp	2	102.44	2.59	0.110
	Error	14	39.53		
iii.	Temp	2	751.21	0.96	0.409
	Error	13	783.57		

Table 3. Repeated measures ANOVA testing the effect of egg age on frequency type of tending behaviour. Significant results are in bold type.

Source	df	MS	F	p
Behaviour	1	1553.88	18.07	**0.013**
Error	4	85.97		
Egg Age	8	53.19	6.29	**<0.001**
Egg Age*behaviour	8	90.36	10.68	**<0.001**
Error	32	8.46		

Table 4. Repeated Measures MANOVA (a) and ANOVA (b) results summary tables for the effect of temperature and time of day on: (i) the proportion of time male fish spent tending their eggs; (ii) frequency of male tending events (per minute) with time of day and temperature; (iii) average length of a tending event. Pillai's trace statistic is used for MANOVA. Significant results are in bold type.

a.	Source	Effect df	Error df	Pillai's trace	F	p
i	Time of day	4	13	0.79	12.28	**<0.001**
	Time of day *temp	8	28	0.45	1.03	0.437
ii	Time of day	4	16	0.57	5.35	**0.006**
	Time of day *temp	8	34	0.32	0.80	0.604
iii	Time of day	4	14	0.57	4.71	**0.013**
	Time of day *temp	8	30	0.24	0.51	0.842
b.		df		MS	F	p
i	Temp	2		0.03	0.38	0.684
	Error	16		0.07		
ii	Temp	2		573.38	3.86	**0.039**
	Error	19		148.41		
iii	Temp	2		492.95	1.18	0.329
	Error	17		415.71		

Table 5. Repeated measures MANOVA (a) and ANOVA (b) results for the combined effects of temperature, time of day and developmental stage on the proportion of time male fish spent tending their eggs. MANOVA results represent the multivariate test using Pillai's trace. Significant results are denoted in bold type.

a. Source	Effect df	Error df	Pillai's trace	F	p
Time of day	4	15	0.91	40.13	**<0.001**
Time of day *stage	8	32	0.53	1.46	0.211
Time of day *temp	8	32	0.80	2.67	**0.023**
Time of day *stage*temp	16	72	0.77	1.08	0.392
b.	df		MS	F	P
Stage	2		0.32	21.03	**<0.001**
Temp	2		0.04	2.46	0.114
Stage*Temp	4		0.01	0.71	0.593
Error	18		0.01		

Chapter 4: Temperature influences swimming speed, growth & larval duration

Publication: Green BS, Fisher R (2004) Temperature influences swimming speed, growth and larval duration in coral reef fish larvae J. Exp. Mar.Biol. Ecol 299:115-132.

Synopsis

The effects of temperature on growth, pelagic larval duration (PLD) and maximum swimming speed were compared in the tropical fish marine species *Amphiprion melanopus,* to determine how temperature change affects these three factors critical to survival in larvae. The effects of rearing temperature (25 °C and 28 °C) on the length of the larval period and growth were examined in conjunction with the effects of swimming temperature (reared at 25 °C, swum at 25 and 28 °C, reared at 28 °C, swum at 25 and 28 °C) on critical swimming speed (U-crit). Larvae reared at 25 °C had a 25 % longer pelagic larval duration (PLD) than larvae reared at 28 °C, 12.3 (± 0.3) days compared with 9 (±0.6) days at 25 °C. To offset this effect of reduced developmental rate, growth and U-crit were measured in larvae reared at 28 °C and 25 °C at the same absolute age (7days after hatching (dah) and same developmental age (7dah at 28 °C c.f. 11dah at 25 °C), corresponding to the day before metamorphosis. Larvae reared at 25 °C were smaller than larvae reared at 28 °C at the same absolute age (7dah at 25 °C c.f. 7dah at 28 °C), yet larger at similar developmental age (11dah at 25 °C c.f. 7dah at 28 °C) when weight and standard length were compared. This stage specific size increase did not result in better performance in larvae at the same developmental age, as there was no difference in U-crit in pre-metamorphic larvae reared at either temperature (7dah at 28 °C c.f 11dah at 25 °C). However, U-crit was considerably slower in 7-day old larvae reared at 25 °C than larvae of the same absolute age (7dah) reared at 28 °C. Swimming temperature controls demonstrated that a change in temperature immediately prior to swimming

tests did not affect swimming performance for larvae reared at either temperature.

Decreased rearing temperature resulted in longer larval durations, reduced growth rates and slower swimming development in larvae. However, the magnitude of the response of each of these traits varied considerably. As such, larvae reared at the lower temperature were a larger size at metamorphosis but had poorer relative swimming capabilities. This study highlights the importance of measuring a range of ecologically relevant traits in developing larvae to properly characterize their relative condition and performance in response to environmental change.

Introduction

All organisms have lethal limits to their temperature range (e.g. Hokanson 1977) and yet within this range they also have optimal temperatures for development of structure and function (Rombough 1997). Within an ectotherms tolerance limits, variation in temperature will influence metabolism (see Rombough 1997) and therefore related physiological processes, affecting growth (Nicieza & Metcalfe 1997), development (Koumoundouros et al. 2001), and performance - encompassing physiological and behavioural capabilities (Fuiman & Higgs 1997, Koumoundouros 2002). Growth is the most commonly measured response in ectothermic animals and is often measured in isolation, as indicative of response to temperature (e.g. McMullen & Middaugh 1985, Zhang & Runham 1992). However temperature influences a range of characters, and early development such as the larval phase is especially susceptible to temperature change (Rombough 1997).

Ontogeny is a complex collection of steps and intervals, and is dependent on the timing of developmental processes (Kovac 2002). The timing of these ontogenetic steps and intervals in many marine ectotherms is plastic (Koumoundouros et al. 2001) and environmental change can cause shifts in the rate of ontogenetic change. Temperature in particular causes variation in rates of fish development in the embryonic (Heath et al. 1993), larval (Hunt von Herbing et al. 1996, Björnsson 2001) and juvenile stages

(Beacham & Murray 1990, Benoit & Pepin 1999). A decrease in the rate of ontogeny caused by a change in temperature results in a longer larval duration and increases exposure to the high-risk pelagic larval environment (Atkinson 1996). Moreover, through varying rates of development, temperature can influence the size of the organism at which ontogenetic transformations occur.

Performance is an expression of the physiological and behavioural capabilities of an organism (Fuiman & Higgs 1997) and can exhibit effects of a changed environment. Swimming speed and behaviour are often used as measures of the performance capabilities of a fish as they are important to dispersal (Leis 2002), prey capture (Hunt von Herbing et al. 2001), predator avoidance (Rice et al. 1987, Fuiman 1993) and avoiding advection away from suitable habitat (Armsworth 2001). Temperate marine fish show large responses to temperature change through swimming performance at all stages of development. Incubation temperature affects swimming escape velocity (Johnston et al. 2001), thermoclines determine vertical distribution in larvae (Batty 1994) and swimming temperature affects critical swimming speed (Koumoundouros 2002) and spontaneous swimming activity (Fuiman & Ottey 1993) in juveniles. Further, temperature induced morphological changes may have indirect effects on the development of swimming performance, as the development of functional structures such as muscle, gills and biochemical pathways are retarded (Taylor 1997).

Growth, larval development and swimming performance in temperate fish species have distinctive responses to temperature change. The relative importance of temperature change in the tropics has been alluded to (Rombough 1997, Hunt von Herbing 2002), but rarely tested. Tropical latitudes generally have little temperature fluctuation relative to temperate environments, due to the large ocean surfaces and absence of a cold season (McGregor & Nieuwolt 1998). For example, the Great Barrier Reef, Australia sea surface temperature fluctuates from 4-6°C seasonally, and 1°C diurnally (McGregor & Nieuwolt 1998). Accordingly, temperature variation of only a few degrees represents a proportionally large change for organisms that are adapted to this relatively stable thermal environment, as physiologically

expensive adaptations to temperature change are not often maintained in relatively stable systems (Feder 1978, Relyea 2002). As a consequence, small changes in temperature could have a disproportionately greater impact on development of tropical fish larvae than larvae in temperate systems with naturally large temperature variation.

The objective of this study was to examine the combined changes in developmental rate, growth rate (size) and swimming performance (in terms of critical swimming speed) to a change in environmental temperature in a tropical marine fish *Amphiprion melanopus*. The results are compared to other studies to determine the magnitude of response in each trait for this tropical species per degree of temperate change compared to temperate fish larvae.

Materials and methods

Larval rearing and experimental protocol

The study species is an anemonefish, *Amphiprion melanopus,* from the family Pomacentridae and occurs from Indonesia in the north and along the 7 degrees of latitude spanned by the Great Barrier Reef, Australia. They lay benthic eggs and have a short larval duration, which lends them to experimental manipulation. Broodstock were collected from the northern section of the Great Barrier Reef, adjacent to Cairns (16°8' S, 145°7' E), and would naturally experience temperature fluctuations from 25-30 °C annually. *A. melanopus* larvae were reared in an indoor laboratory at the James Cook University aquarium facility following the methods of Green and McCormick (1999). Eggs were obtained from adult broodstock maintained at 28 °C and conditioned to lay eggs onto cement blocks lined with acetate sheeting. On the night the eggs were due to hatch they were transferred indoors where larvae were hatched into a 70-litre glass aquarium held at 28 °C. Upon hatching 10 larvae were sampled, preserved in 70% ethanol and later used for measurements of standard length and wet weight. Immediately after hatching between 300 and 400 larvae were transferred at random into ten 70-litre glass aquaria held at 28 °C. Temperature in five tanks was gradually adjusted to 25 °C overnight. Through the remainder of the larval phase, 5

tanks were maintained at 28 °C and 5 tanks at 25 °C. This was repeated for three clutches of larvae from three separate pairs of broodstock. Larvae were reared using the 'green water' method described by Daintitch (1993), where *Nannochloropsis* sp. algal culture is added to tanks each morning. Tanks were lit by fluorescent lights simulating a 14H light:10H dark summer light cycle, and maintained as a semi-closed system, flushed nightly with temperature controlled water (25°C and 28°C) when the lights were off. Larvae were fed rotifers (*Brachionus* sp.) at a density of approximately 5 ind.ml^{-1} for 1-3 days after hatching, and on day 3 after hatching *Artemia* nauplii were added at 1-2 ind.ml^{-1}.

Quantifying the effects of temperature

Length of larval duration was used to determine the effects of temperature on the ontogenetic rate of *A. melanopus*, and metamorphosis was used to mark the end of the larval period. Metamorphosis in marine fish from pelagic larvae to demersal juvenile can entail a shift in habitat, appearance or structure of the fish and is species specific (McCormick 2002). In *A. melanopus* the stage when the post-orbital stripe becomes pigmented coincides with a shift in habit and is a more discrete measure than full body pigmentation (Green & McCormick 1999). In the current study, fishes within individual tanks were grouped to determine the number of days until metamorphosis, otherwise described as the pelagic larval duration (PLD), and a tank was considered metamorphosed when greater than two-thirds of all the fish in each tank had a visible post-orbital stripe. Developmental rate was calculated following (Fuiman et al. 1998) as R_{dev} =1/age, where R_{dev} is the developmental rate and age is the numbers of days since hatching.

Critical swimming speed (U-crit, following Brett 1964) was used to determine the effects of rearing temperature on the functional swimming capabilities of larvae. U-crit is a measure of the maximum sustainable swimming speeds of larvae. As temperature treatment significantly affected the length of the pelagic larval duration, we measured the critical swimming speed of larvae both at a similar age in days after hatching (dah), as well as on the day before metamorphosis for both temperature treatments. Larvae were swum 7 dah for both temperature treatments and at 11 dah for fish

reared at 25 °C, an *a priori* sampling decision to coincide with one day prior to the average time taken to metamorphosis for 28 °C and 25 °C treatments respectively (Green unpublished data). This allowed for a comparison of critical swimming speed between fishes of similar absolute age as well as similar developmental age (c.f. Job and Bellwood 2000). Two fish were swum from each of the five replicate tanks from each temperature treatment (25 °C and 28 °C), making a total of 10 larvae per clutch swum at each temperature. To control for the effects of temperature *per se* on critical swimming speed, within each clutch, 6 fish randomly selected from the 28 °C treatments were swum at 25 °C, and 6 fish randomly selected from the 25 °C treatments were swum at 28 °C, (Fig. 1). Fish were acclimatised to the new swimming temperatures over 4 hours prior to swimming. This experimental protocol resulted in five rearing/swimming temperature and age combinations with between 6 and 10 individual larvae used for each treatment (see Fig. 1).

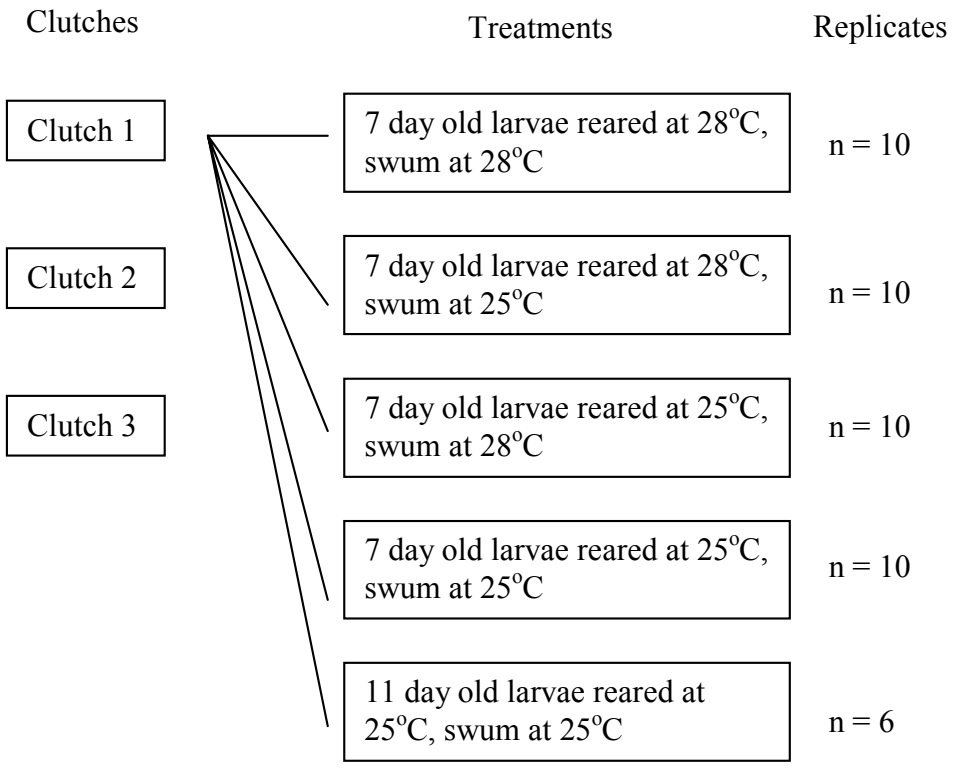

Fig. 1. Experimental design tree showing the five different treatments and levels of replication.

Swimming experiments

Swimming experiments were carried out using a three channel experimental swimming flume (c.f. Stobutzki & Bellwood 1997). This apparatus consisted of a perspex chamber divided into 3 channels, with flow straighteners at the mouth of each channel to produce laminar flow. A 270 lpm 2.4 Kw Onga™ pump circulated water through the system and a gate valve calibrated with a protractor controlled the volume (and therefore current speed). Calibration was carried out by recording the volume of water passing through the chamber over a set time period for different angles on the protractor. Recorded volumes were divided by the sum of the cross sectional area of the each channel to determine speed.

The maximum swimming speed of larvae was determined following the methods of Bellwood & Fisher (2001). Two fish were placed in each channel and allowed to acclimatise for several minutes before the start of the experiment. The speed was then increased by 3 body lengths per second (2.0 cms^{-1}) every 2 minutes until the fish could no longer maintain position in the swimming channel. The speed and time spent in the last interval was recorded. The critical swimming speed (U-crit) of larvae is calculated as: U-crit = $U + (t / t_i * U_i)$, where U is the penultimate speed, U_i is the velocity increment (2 cms^{-1}), t is the time swum in the final velocity increment and t_i is the set time interval for each velocity increment (2 minutes).

Fish from all swimming treatments were retained after each experiment and preserved in 70% ethanol. These samples were used to determine standard length and wet weight of larvae from each treatment for each clutch. All specimens were preserved for 2 months and treated in the same manner so any shrinkage due to ethanol storage would be proportional. As growth of *A. melanopus* is linear during the larval phase (plotted from Green and McCormick 2001) growth rates were estimated according to the formula: $R_g = (L_s - L_h)/T_s$, where R_g is the rate of growth in mm/day, L_s is the length (mm) at sampling time, L_h is the length (mm) at hatching and T_s is the time (days) from hatching to sampling.

Comparison to other studies

To compare the fish's response to temperature change from this study with temperate studies the changes in rates of growth (mm/d), PLD (developmental rate) and swimming performance (bl/sec) from the available literature were expressed as Q_{10} values. Q_{10} is a thermodynamic expression of temperature effects and is commonly employed to standardise a rate of change in response to temperature to a common index. It describes the response of biological processes to a change in temperature of 10°C (Schmidt-Nielsen 1997) and can be calculated by the following equation

$Q_{10}=[R_2/R_1]^{10/(T2-T1)}$, where T_1 and T_2 are the temperatures over which the change was recorded, R_1 is the rate of a process at T_1 and R_2 is the rate of the process at T_2. Like many rate processes Q_{10} varies exponentially with temperature. Where there was a range of experimental temperature changes and related responses in the rate measured, Q_{10} was calculated between each temperature increment and the mean and standard error were calculated.

Data analysis

As there was no difference in the pelagic larval duration among the five tanks within each treatment for each clutch, a paired t-test (Zar 1984) was used to test if temperature significantly increased pelagic larval duration within each clutch. A two way factorial MANOVA (following Tabachnick & Fidell 1996) was used to compare the size (total length and weight) of larvae from the five rearing/swimming temperature and age combinations for each clutch (Fig. 1). A second two way factorial MANOVA was used to compare the absolute swimming speed (cms^{-1}) and speed in body lengths per second (bls^{-1}) of larvae from the five treatments (Fig. 1). Both MANOVA's were performed using the statistical package SPSS. The models tested in both cases were: treatment + clutch + treatment*clutch + error (as per experimental design illustrated in Fig. 1). The assumptions of homogeneity of variance and normality were tested using levene's test and graphically using residual and qq plots and it was found that these assumptions were not violated. Pillai's trace was used as the multivariate test of significance. Significant effects were

explored using univariate ANOVA's for each variable and Tukey's post hoc analysis (Zar 1999).

Results

Temperature effects on development rate

A lower rearing temperature significantly increased the pelagic larval duration of *A. melanopus* larvae, slowing down developmental rate such that larvae reared at 25 °C required 25 % more time to reach metamorphosis. *A. melanopus* larvae reared at 28 °C metamorphosed at 9 dah (± 0.6 days) compared with 12.3 dah (± 0.3) at 25 °C ($t = 9.75$, d.f. = 2, $p < 0.05$).

Temperature effects on size

Different rearing temperatures also caused a significant difference in size of larvae between temperature treatments (Table 1). When larvae from the same clutch were reared at two temperatures, larvae cultured at the higher temperature were bigger than their siblings at the same absolute age. Seven-day-old (pre-metamorphic) larvae reared at 28 °C were significantly bigger than 7-day-old larvae reared at 25 °C, both in total length and weight (Fig. 2). Rearing temperature also affected size of larvae of similar developmental age, with larvae reared at a lower temperature attaining larger size for developmental age than similarly developed siblings. Pre-metamorphic larvae reared at 25 °C (11 dah) were significantly larger than pre-metamorphic reared at 28 °C (7 days after hatching; Fig. 2). Within each rearing temperature, larvae swum at the two different temperatures were of similar size (Fig. 2).

Temperature effects on swimming speed

Temperature significantly affected the swimming performance of larvae among the five rearing/swimming temperature and age combinations identified in Fig. 1 (Table 2). At 7dah, larvae reared at 28 °C were capable of maintaining significantly faster speeds (cm s^{-1}) than 7-day-old larvae reared at 25 °C, regardless of the temperature of the swimming experiment (Fig. 3a). However, when comparing larvae of similar developmental stage, ie. pre-metamorphic larvae (7 days after hatching for 28 °C and 11 days after

hatching for 25 °C) there was no significant difference in the U-crit swimming speeds achieved by larvae from each rearing temperature (Fig. 3a).

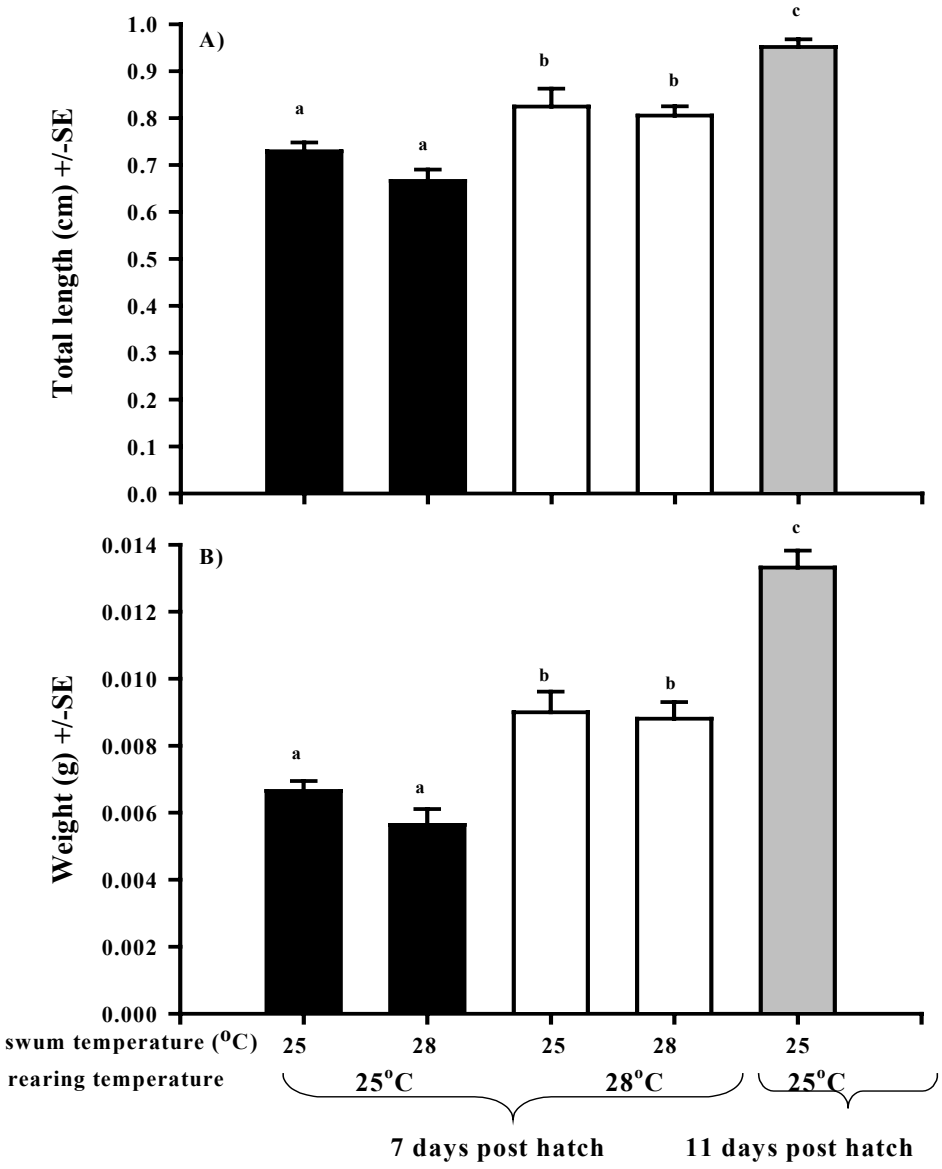

Fig. 2. Average total length (A) and weight (B) of 7 day old fish reared at 25 °C (black bars) and 28 °C (white bars) and 11 day old fish reared at 25°C (grey bar). Letters above bars indicate significant subgroups. For larvae reared at 28°C, 7 day old fish represent 1 day prior to metamorphosis. For larvae reared at 25°C, 11 day old larvae represent 1 day prior to metamorphosis.

When U-crit is standardised for larval length (body lengths per second, bl s^{-1}), larvae aged 7- and 11-dah reared at 25 °C achieved slower critical

swimming speeds than larvae 7 dah reared at 28 °C (Fig. 3b). While U-crit measured in absolute terms (ie. cm s^{-1}) of premetamorphic larvae reared at 25 °C were similar to premetamorphic larvae reared at 28 °C, relative to their size their critical swimming speed was slower.

When larvae of the same age (7 dah) and from the same rearing temperature were exposed to short-term temperature change, there was no difference in their maximum sustained swimming speed (U-crit), for either absolute (cm s^{-1}) or relative (bl s^{-1}) speed (Fig. 3). Larvae reared at 28 °C and then exposed to 25 °C for 4 hours and tested at this temperature did not achieve different u-crit than larvae maintained and tested at 28 °C (Fig. 3). Similarly, larvae cultured at 25 °C and then exposed to 28 °C for 4 hours did not achieve different U-crit than larvae maintained and test at 25 °C (Fig. 3).

Short-term temperature change had no immediate effect on U-crit, although long-term (rearing) temperature differences did.

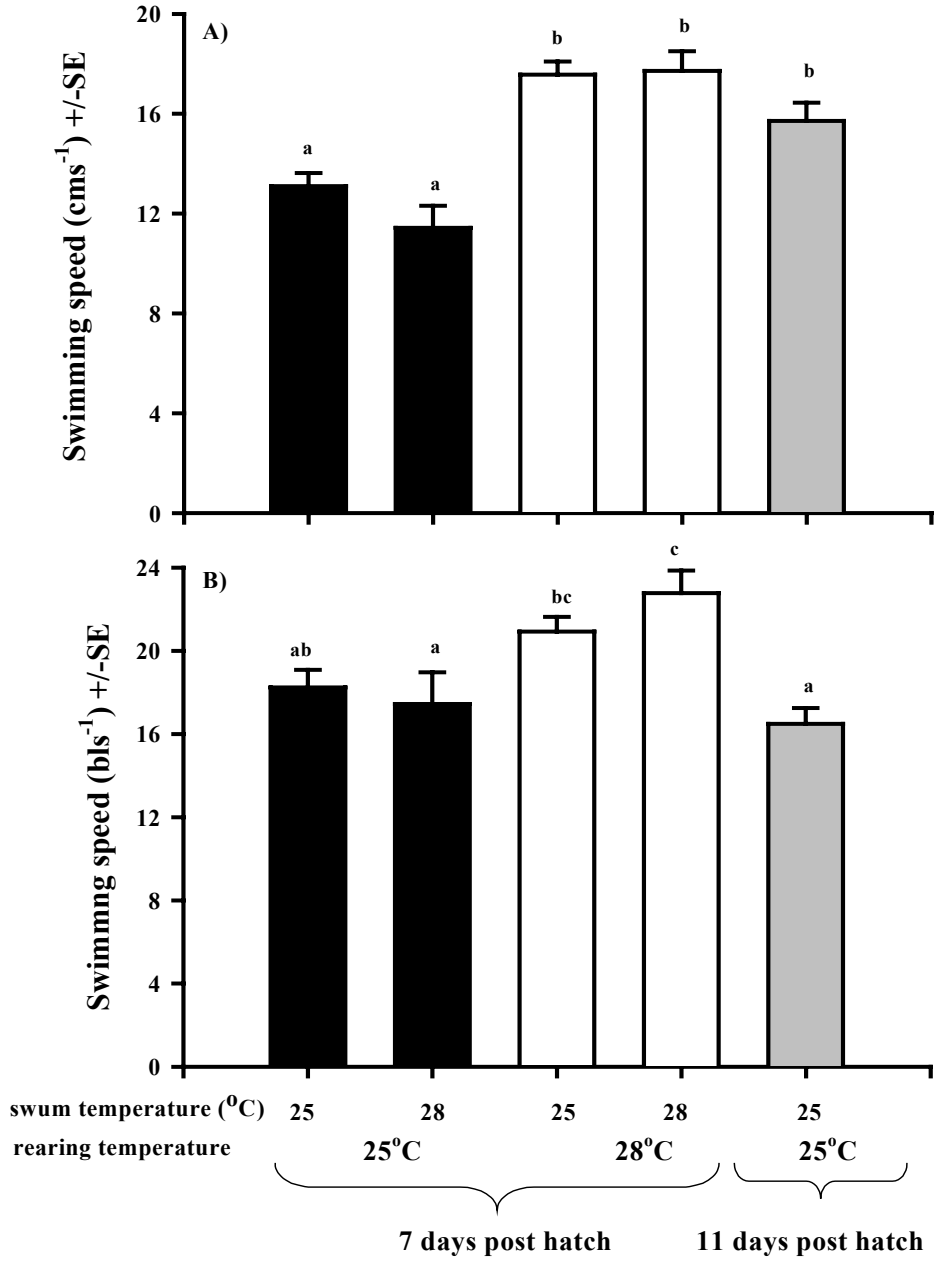

Fig. 3. Average swimming speed in centimetres per second (cms^{-1}, A) and body lengths per second (bls^{-1}, B) of 7 day old fish reared at 25 °C (black bars) and 28 °C (white bars) and 11 day old fish reared at 25 °C (grey bar). Letters above bars indicate significant subgroups. For larvae reared at 28 °C, 7 day old fish represent 1 day prior to metamorphosis. For larvae reared at 25oC, 11 day old larvae represent 1 day prior to metamorphosis.

Discussion

In the tropical reef fish species, *A. melanopus*, a small variation in temperature resulted in a large variation in growth, development and swimming performance. These factors individually are central to survival and dispersal for pelagic marine larvae, and in combination they are fundamental to replenishment of reef fish populations. A 3ºC reduction in rearing temperature decreased the growth, developmental rate and swimming speed in *A. melanopus* larvae. However, when multiple effects of decreased temperature were combined and developmental rate was factored in, reduced temperature resulted in pre-settlement larvae that were larger and capable of similar swimming speeds to larvae reared at 28 ºC. While the differences in these three traits recorded at temperatures only 3 ºC apart demonstrate a relatively large degree of plasticity in the larval phase of these fish in response to environmental variation, they also illustrate the importance of choosing the most appropriate endpoint for comparison. If this experiment only compared pre-settlement fish, then little effect of temperature on growth and swimming would be found. Conversely, if only size- and swimming- at absolute age were compared then the most parsimonious conclusion would be that lower temperature results in smaller, slower fish.

Ecological implications of response to temperature change

My findings of an extended PLD coupled with decreased maximum swimming speed at lower temperature indicate that even small changes in temperature may substantially decrease the chance of survival for these reef fish larvae. It is during development in the pelagic environment that fish are the most susceptible to changes in temperature (Rombough 1997). This stage also has the highest risk of mortality through predation or starvation (Bailey & Houde 1989, Ferron & Leggett 1994). Increased PLD increases the length of exposure of fish larvae to the high-risk pelagic environment, indirectly reducing probability of survival. Reduced swimming speed increases rates of predation (Miller et al. 1988) and influences the extent to which larvae can use active behaviour to modify their dispersal patterns, actively self recruit or enhance recruitment success (Armsworth 2001,Sponaugle 2002).

Replenishment of adult populations in many marine fishes occurs through a pelagic larval phase and reef fish larvae can potentially modify their patterns of dispersal during this phase using active swimming behaviour (Leis 2002). Swimming is also central in hunting and prey capture (Hunt von Herbing et al. 2001). The overall length of the larval phase is important to dispersal and the degree of connectivity among populations of marine fishes (Doherty et al. 1994, Riginos & Victor 2001). Clearly, the diminished swimming performance and increased developmental time of larval *A. melanopus* in response to a decrease in temperature suggests that on coral reefs, small changes in temperature may have critical impacts on dispersal, recruitment and replenishment.

Temperature effects on swimming speed

The substantial effect of larval rearing temperature on maximum on swimming speed (U-crit) in *A. melanopus* larvae contrasts with another key finding from this study, that temperature change in the immediate swimming environment did not significantly alter U-crit. Temperature change (excluding the viscous change normally associated with temperature) of the swimming test environment also did not affect the on spontaneous, or routine swimming activity in small larvae (9.6 $\pm$ 0.39 mm) of Atlantic herring (Fuiman & Batty 1997). Any changes in water viscosity normally associated with decreased temperature had little effect on the swimming capability of these *A. melanopus* larvae (c.f. Batty 1984, Fuiman & Batty 1997), although this change in viscosity may be negligible for tropical fish (Hunt von Herbing 2002). Like most ectotherms, a fish's body temperature equilibrates rapidly to its thermal environment (Taylor 1997). Temperature-specific changes affect an ectotherm at the molecular and cellular level, but are associated with the whole organism adapting its energy consumption to prevailing conditions in the natural habitat (Wieser 1973). That *A. melanopus* larvae achieved similar maximum swimming speeds (U-crit) in different swimming test water temperatures suggest that any short-term equilibration posed by the changed swimming temperature did not significantly override the effects of the larval rearing environment. Short-term temperature change is moderated by immediate physiological or biochemical compensation of the animals to thermal change,

within a thermal tolerance level (Schmidt-Nielsen 1997). So while a long term thermal difference during development affects the functional and developmental physiology of the animal, *A. melanopus* was able to compensate for short-term change and maintain the standard of its critical swimming performance.

The measured effects of rearing temperature on maximum sustained swimming speed of *A. melanopus* larvae are likely due to physiological or biochemical costs involved with adapting to the prevailing environmental conditions that were not measured in this study. Temperature changes can affect many of the structures that enable swimming in a developing larva. Decreased temperature can affect muscle development through reducing the rate of muscle growth and net food conversion efficiency (Hanel et al. 1996). Temperature directly influences myogenesis of the swimming muscles through a trade-off between hypertrophy and hyperplasia (Hanel et al. 1996) and reduces the rate of myofibril synthesis (Johnston et al. 2001) and number of myotomes (Hempel & Blaxter 1961). Further, structures related to respiration develop more slowly in larvae raised at a lower temperature (Hunt von Herbing et al. 1996) and the rate of respiration, or metabolism is slower in lower temperatures (Wieser & Kaufmann 1998). Any or all of these factors could be responsible for the reduced swimming speeds reached by larvae reared at lower temperatures.

Response relative to non-tropical fishes

While it has been identified that there are differences in the response of tropical and temperate species to temperature changes throughout their development (Rombough 1997, Hunt von Herbing 2002) the magnitude of the change has not been previously described. Critical swimming speed was proportionally affected more per degree of temperature in *A. melanopus* than in many temperate species, however growth did not show a corresponding magnitude of change and PLD was affected less compared to temperate species (Table 3). Q_{10} values of the change in rate of swimming speed in response to temperature for temperate marine and freshwater fishes suggest that organisms from colder climates showed a smaller magnitude of change as indicated by smaller values of Q_{10} relative to my tropical species (Table 3; Hunt von Herbing 2002). This response was predicted in a recent review on

the importance of the physical properties of water to physiological function of the fishes, whereby increased temperatures may increase swimming efficiency due to a reduction in the kinematic viscosity in warm water (Hunt von Herbing 2002).

Growth is a function of cellular activities, which are dictated by general physical laws. Therefore when temperature induced changes in growth are standardised for the degree of temperature change, similar patterns could occur across thermal ranges. The change in growth measured in *A. melanopus* (Q_{10} = 2.4) was within the range found in other studies (Table 3), suggesting that the effect of temperature on growth rate is not greater in this tropical fish species compared to temperate regions. Growth and swimming speed show different magnitudes of response to temperature change relative to other organisms and to each other.

Bigger is not necessarily better

The theoretical framework for development of fish larvae, based on temperate studies, suggests size is one of the central factors in survival and can manifest profound differences in success of larvae for recruitment (Miller et al. 1988) and survival (Houde 1989a). The 'bigger is better' and 'growth-mortality' hypotheses encapsulate the importance of size, whereby bigger larvae increase their chance at survival through increased ability to capture food and escape predation, (Anderson 1988, Miller et al. 1988). For *A. melanopus,* decreased temperature reduced the absolute rate of growth and development. This resulted in smaller fish for absolute age although larger fish at settlement. These larger fish did not have better maximum sustained swimming speed, therefore would probably not have the predicted increased ability to capture food and escape predation (see Anderson 1988, Miller et al 1988). Size may infer better survival in an environment where competition is high (Coates 1980, Booth 1995b) but 'bigger is better' theory becomes complicated when performance capabilities are examined. My results suggest that under changed environmental conditions, bigger was not necessarily better as larvae raised at a lower temperature were bigger at metamorphosis but did not perform better in trials measuring critical swimming speed. Therefore the competitive advantage afforded to bigger larvae under stable conditions (e.g. Coates 1980, Miller et al. 1988) is compromised under

changed temperature conditions through decreased swimming performance. Decreased temperate also increased the time until metamorphosis in *A. melanopus*, which increases their time in the high-risk pelagic environment, further compromising the chances of survival for larvae raised at colder temperature, despite the fact they are bigger.

Given the decreased growth and development rates in response to lower temperature, it appears counter-intuitive for larvae from the lower temperature treatment to be larger at metamorphosis. However, the reduction in absolute growth results in an increase in stage-specific growth because the time between intervals was increased (Atkinson 1996). Similar relationships between the responses of growth and development to temperature change have been identified in organisms from 9 phyla within four kingdoms (Atkinson 1996).

Conclusions

Temperature has been considered as an influence on growth and survival of fishes for three decades (Houde 1974) however the implications for performance, development and plasticity are only recently being realised (Koumoundouros 2002, Yamahira 2002). Response to environmental changes must be considered within the context of an organisms normal environment, as thermal sensitivity is generally a reflection of field temperature and levels of local adaptation (eg. van Berkum 1986, Conover & Schultz 1997). To date, many of the predictions regarding the biology and ecology of marine fish larvae come from models of temperate systems. These systems are characterised by large diurnal and seasonal fluctuations, and organisms that are presumably physiologically adapted to these local thermal regimes. The magnitude of the functional response in this tropical reef example suggests that using temperate models to predict thermally induced recruitment could seriously underestimate the effect of small changes in the environment.

This research is a significant first step in understanding how temperature changes manifest themselves on the development of tropical reef fish larvae. We concur with Conover and Schultz (1997) that bigger is not necessarily better, as size does not confer an advantage when performance is considered as well as growth in response to a change in temperature. My

results imply that temperature changes manifest themselves in a variety of ways in the pelagic larvae phase, and the different magnitude of response in growth, development and maximum achieved swimming speed emphasises the value of using measurements appropriate to the ecology of the organism. Future studies should consider performance and developmental attributes such as PLD and swimming speed and not just growth in measuring response to environmental change.

Table 1: Two-way MANOVA comparing total length and weight across the three different clutches and among the five treatments. Between subject effects are only shown for significant multivariate tests. Bold type denotes significant differences <0.05.

Multivariate tests (pillai's trace)	F	df	P
Clutch	11.654	4, 166	**<0.001**
Treatment	13.4999	8, 166	**<0.001**
Treatment x clutch	1.591	16,166	0.076
Between subject effects (total length)	F	df	P
Clutch	32.400	2, 83	**<0.001**
Treatment	46.993	4, 83	**<0.001**
Between subject effects (weight)	F	df	P
Clutch	8.773	2, 83	**<0.001**
Treatment	49.386	4, 83	**<0.001**

Table 2: Two-way MANOVA comparing absolute swimming speed (cm s^{-1}) and swimming speed in body lengths per second (bl s^{-1}) across the three different clutches and among the five treatments. Bold type denotes significant differences <0.05.

Multivariate tests (pillai's trace)	F	df	P
Clutch	67.784	4, 200	**<0.001**
Treatment	42.487	8, 200	**<0.001**
Treatment x clutch	9.661	16, 200	**<0.001**
Between subject effects (cms^{-1}) F		df	P
Clutch	15.768	4, 100	**<0.001**
Treatment	16.906	2, 100	**<0.001**
Treatment x clutch	1.304	8, 100	0.250
Between subject effects (bls^{-1})	F	df	P
Clutch	29.504	4, 100	**<0.001**
Treatment	12.246	2, 100	**<0.001**
Treatment x clutch	1.924	8, 100	0.064

Table 3. Q_{10} values for three traits from the early life history of fishes reflecting the effect of a 10°C increase in temperature on the rate of a given process. * denote Q_{10} values that were published in the cited reference. All other values were calculated following $Q_{10}=[R_2/R_1]^{10/(T2-T1)}$, where T_1 and T_2 are the temperatures over which the change was recorded, R_1 is the rate of a process at T_1 and R_2 is the rate of the process at T_2. ([1] Corrected for viscosity)

Species	Climate	Temperature change (°C)	Q_{10}	Reference
Developmental rate				
Amphiprion melanopus	trop	25-28	0.36	This study
Pseudopleuronectes americanus	temp	2-8	5.1	Laurence 1975
Gadus morhua	temp	5-10	1.5	Hunt von Herbing *et al* 1996
Brevoortia tyrannus	temp	15-20	1.83	
Brevoortia tyrannus	temp	20-25	1	Fitzhugh and Nixon 1997
Critical swimming speed (bls^{-1})				
Amphiprion melanopus	trop	25-28	2.74	This study
Clupea harengus U_{max}	temp	5-12	1.62	Johnston *et al* 2001,
Dicentrarchus labrax	temp	15-28	1.07±0.13	Koumoudouros *et al* 2002
Oncorhynchus nerka	temp fw	5-27	0.75±0.26	Brett 1967
Routine swimming				
Clupeaharengus (TL18.2±1.8mm)	temp	6-13	2.6* (2.2)[1]	Fuiman & Batty 1997
Clupeaharengus (TL18.2±1.8mm)	temp	7-14	1.9* (1.4)[1]	Fuiman & Batty 1997
Growth (mmd^{-1})				
Amphiprion melanopus	trop	25-28	2.4	This study
Channa striatus	trop/sub-trop	21.7-27	1.91	Qin & Fast 1998
Achirus lineatus	sub-trop	24-28	1.66	Houde 1974
Anchoa mitchilli	sub-trop	28-30	0.10	Houde1974
Anchoa mitchilli	sub-trop	24-28	1.67	Houde 1974
Archosargus rhomboidalis	sub-trop	26-30	0.99	Houde 1974
Hippoglossus hippoglossus	temp	5-8	5.3	Galloway et al 1998
Clupea harengus	temp	10-8	0.8	McGurk 1984
Clupea harengus	temp	8-6	1.25	McGurk 1984
Menidia menidia	temp	17-28	4.03	Yamahira & Conover 2002
Menidia menidia	temp	17-28	4.37	Yamahira & Conover 2002
Anarhichas minor (eggs only)	temp	6-8	2.38	Hansen & Falk-Petersen 2001
Anarhichas minor (eggs only)	temp	4-6	5.15	Hansen & Falk-Petersen 2001
Morone americana	temp fw	17-21	2.68	Margulies 1989
Morone americana	temp fw	13-17	6.42	Margulies 1989
Perca fluviatilis	temp fw	15-20	4.79	Wang and Eckmann 1994
hybrid Lepomis cyanellus x L. macrochirus	temp fw	21-24	2.17	Mischke et al 2001
hybrid Lepomis cyanellus x L. macrochirus	temp fw	19-21	0.95	Mischke et al 2001

Chapter 5: Parental influences determine size, growth and performance

Publication: Green, BS, McCormick MI (Submitted) Maternal and paternal influences determine size, growth and performance in larvae of a tropical reef fish. Mar. Ecol. Prog. Ser.

Synopsis

Larval mortality in marine fishes is strongly linked to individual life history traits, such as size and growth, but the processes that influence variability in these traits are poorly understood. We explore the relative importance of maternal, paternal influences and water temperature on the larval growth and performance characteristics of the tropical clownfish *Amphiprion melanopus* (Pomacentridae). Larvae were reared from a 4 male x 4 female diallel breeding cross at two temperatures (25 and 28 ºC). Maternal size was correlated to the number of eggs within a clutch ($r = 0.68$, $p < 0.05$) and number of hatchlings ($r = 0.61$, $p < 0.05$). Paternity interacted with maternity and affected traits immediately prior to- and after- hatching. Egg characteristics (length, width and area) and size of 1-day old larvae were significantly affected by specific combinations of males and females. Size of larvae at metamorphosis was primarily affected by maternal and paternal influences, but not rearing temperature. Paternity explained 52 % of the variance in growth rates to metamorphosis, while the combination of paternity, maternity and temperature explained 30 %. This strong paternal influence may be due to the extensive roles males play in nest tending, coupled with the relatively long embryonic duration of the species. The importance of these parental effects was further emphasised by the negative relationship between larval growth rate and mortality within a tank.

Introduction

The population dynamics of marine fishes are characterised by high larval mortality (Ferron & Leggett 1994), which is predominantly driven by size- and growth- selective processes (Meekan & Fortier 1996, Vigliola & Meekan 2002, Hoey & McCormick in press). Size at hatching and pre-settlement growth strongly influence survival before and after settlement (Bergenius et al. 2002, Shima & Findlay 2002, McCormick & Hoey In press), however, the seminal causes of variation in larval size and growth are poorly understood. Intrinsically, variation in pre-settlement growth, and the body characteristics that co-vary with growth, are derived from two sources: parental influences and environmental fluctuations (Chambers & Waiwood 1996). The interaction between these factors is also likely to be important for larval growth, but has rarely been examined (but see Benoit & Pepin 1999).

Environmental factors encompass the physical and biotic processes acting on developing eggs and larvae. Fluctuations in food (Green & McCormick 1999b), temperature (Green & Fisher 2004), salinity (Sponaugle & Pinkard 2003), turbidity (Cobcroft 2001), and turbulence (Utne-Palm & Stiansen 2002) influence condition and growth in fish larvae. The oxygen and temperature environment into which eggs are laid may influence early developmental rates and size at hatching (e.g. Collins & Nelson 1993, Cancino 2003, Green et al. in review). As the larva exhausts its yolk-sac reserves and begins exogenous feeding, water temperature (Houde 1990, Houde and Zastrow 1993) and the timing of prey production cycles (Cushing 1990) become particularly important determinants of survival. However, in wild fish populations, environmental factors explain less than 40 % of variation in larval growth (Wilson & Meekan 2002, Caldarone et al. 2003), suggesting that other processes must account for a substantial amount of variability in growth.

Whilst the importance of parentage is widely acknowledged, maternal contributions are generally more important than the paternal contributions due to nutritional provisioning of the embryo (Bernado 1996). When paternal effects have been identified in fishes, (Heath et al. 1993, Herbinger et al. 1995, Hoie et al. 1999a, Rakitin et al. 2001, Yamamoto 2003, Rideout et al.

2004), they are not apparent in all traits (Hoie et al. 1999b). In contrast, maternal effects are common in terrestrial and aquatic systems and have a strong influence on characteristics of offspring (Bernardo 1996, Chambers & Leggett 1996). In fishes, embryo and larval characteristics such as egg size, developmental rate, metabolism, growth, and viability are affected by the body condition and genotype of parents (Chambers et al. 1989, Chambers & Leggett 1996, Kerrigan 1997, Marteinsdottir & Steinarsson 1998). Non-genetic maternal contributions take many forms that directly influence survival probabilities of larvae, including nutritional reserves (Kerrigan 1997), levels of developmental and metabolic hormones (Brown et al. 1988, McCormick 1998, 1999), and parental care (Bernado 1996).

Research on maternal effects in marine fishes has focussed on commercially important species, which generally spawn pelagic eggs or benthic eggs without parental care. In species that lay benthic eggs, males are generally responsible for nest-tending and nest-site selection (Clutton-Brock 1991). Care enhances offspring development (Sargent 1997, 1988), and is therefore a mechanism whereby males can invest in the survival potential of their offspring. It is therefore likely that variation in the size and condition of offspring from nested eggs will reflect the males contribution (Bernado 1996).

The present study experimentally examined the relative importance of paternal, maternal and temperature influences on early life history traits from conception to metamorphosis in a marine fish with paternal egg care. Temperature was selected since it strongly influences metabolism and growth in ectotherms, and the incubation and larval environments can differ markedly in temperature. We employed a diallel breeding design as it allows the variance attributed to the maternal, paternal and temperature components to be partitioned (Heath & Blouw 1998). Larvae were reared through to metamorphosis under experimental manipulations of parentage and water temperature and their growth and performance were assessed.

Materials and methods

Study species and brood stock

This study focuses on anemonefish, *Amphiprion melanopus* (Pomacentridae) a common damselfish in the Indo-Pacific. This species is an ideal model for this study as it lays benthic eggs with a relatively long embryonic duration (8d) and exhibits parental egg tending. While nest care involves both parents, males invest more time (40 – 70 %) into tending embryos than females (20 – 30 %) (Green & McCormick in review-b, Chapter 3).

Brood stock pairs of *A. melanopus* were collected from the northern section of the Great Barrier Reef, adjacent to Cairns (16°8' S, 145°7' E), where they would naturally experience annual temperature fluctuations from 25-30 °C. Pairs were maintained in 100-litre round flow-through tanks at 28 °C at the James Cook University research aquarium. At the commencement of the experiment, adult fish were anaesthetised with clove oil, weighed for wet weight and measured for length, body depth, and body area prior to commencement of the experiments. A measure of adult condition was estimated by Fulton's K condition factor (weight (g)/SL(mm)^3 x 100).

Partitioning maternal, paternal and environmental effects

A diallel breeding design is powerful in estimating the variation in offspring phenotype due to parental and environmental determinants (Heath & Blouw 1998). Within a diallel cross design each female is mated with each male, creating a series of full and half-sibling crosses, which replicates clutches produced by each female and by each male. We employed a 4 female x 4 male diallel cross producing 16 clutches from 16 unique parent combinations. Samples were lost when a freezer broke down, so only 14 clutches are included in the analysis. At the commencement of this experiment brood stock were in established breeding pairs, so to avoid effects of pairwise breeding history, new pairs were formed for the first cross of the experiment. Females remained in the same tank and the males were moved for each new mating. Low numbers of sparsely distributed eggs were generally laid in the first clutch from a newly matched pair of *A. melanopus* (pers obs), therefore, only the second brood from every new mating was

sampled.

Each clutch of eggs resulting from the diallel cross was left with the parents until the night of hatching. Prior to dusk on the night of hatching clutches were taken into a temperature-controlled laboratory, and hatched at dusk into a 70-litre tank.

Temperature manipulation

Immediately after hatching, between 300 and 700 larvae, representing all the hatched larvae from each clutch, were transferred at random into ten 70-litre glass aquaria held at 28 °C. Temperature in five of these tanks was gradually adjusted to 25 °C overnight. Through the remainder of the larval phase, 5 tanks were maintained at 28 °C and 5 tanks at 25 °C for each clutch. Each of the 14 clutches of larvae resulting from the 4 x 4 diallel cross design were divided into two rearing temperatures with 5 replicates tanks of each in this manner. Differences in larval density within tanks were assumed to be neglible, as based on hatchling weight of 0.001 g, the density difference equated to a maximum initial mass difference of 0.006 g l^{-1}.

Larval rearing

Larvae were reared in 'green water' in an indoor laboratory following the methods of Green and McCormick (1999), where *Nannochloropsis* sp. algal culture is added to tanks each morning. Tanks were lit by fluorescent lights simulating a 14H light:10H dark summer light cycle, and maintained as a semi-closed system, flushed nightly with temperature controlled water (25 °C and 28 °C) when the lights were off. Larvae were fed rotifers (*Brachionus* sp.) at a density of approximately 5 individuals ml^{-1} for 1 - 3 days after hatching, and on day 3 after hatching *Artemia* nauplii were added at 1 - 2 individuals ml^{-1}.

Sampling and morphometric measurement

Clutch size and density

Parental influence on clutch dimensions and densities per diallel combination were measured prior to temperature manipulation. All egg clutches were photographed prior to hatching, and clutch dimensions were later measured using UTHSCSA Image Tool graphics package (University of Texas, San Antonio). Egg clutch area, perimeter and number of eggs within

the clutch were measured. Egg density was calculated by: Egg density = number of eggs/clutch area.

Egg size

To compare mean egg size between diallel clutches prior to temperature manipulation, ten eggs from each clutch were sampled immediately prior to hatching and preserved in 70% alcohol. Images of the preserved eggs were captured using a digital camera (Olympus DP12) connected to a stereo dissector microscope and egg length, width and area was measured using UTHSCSA Image Tool.

Post-hatching larval size

To compare maternal and paternal influences on mean size of newly-hatched larvae between diallel clutches prior to temperature manipulation, ten larvae were left in the hatching tank and maintained at 28 ºC overnight. They were sampled the following morning (1 day after hatching) and preserved in 70 % ethanol. Newly hatched larvae were measured for standard length and total length on a dissecting microscope with a graduated eyepiece, and wet weight was measured to three decimal places.

Size at metamorphosis

Temperature manipulated rearing experiments were terminated and all fish were sampled at metamorphosis. Metamorphosis was defined as when more than 70 % of individuals from a tank had their post-orbital white strip and settled to the bottom of the tank. Fish were preserved and measured for standard length and total length on a dissecting microscope with a graduated eyepiece. Fish were blotted to remove excess water and wet weights measured to three decimal places. As growth of *Amphiprion melanopus* is linear during the larval phase (plotted from Green and McCormick 2001) growth rates were estimated according to the formula: $R_g = (L_s - L_h)/T_s$, where R_g is the rate of growth in mm day^{-1}, L_s is the length (mm) at sampling time, L_h is the length (mm) at hatching and T_s is the time (days) from hatching to sampling.

Percentage metamorphosed represents the number of fish that had metamorphosed when a tank was sampled. All specimens were preserved in ethanol and treated in the same manner so any shrinkage due to ethanol storage would be consistent among samples.

Quantifying swimming performance

Critical swimming speed (U-crit, following Brett 1964) was used to determine the effects of rearing temperature and parentage on the functional swimming capabilities of larvae on eight clutches of fish from the diallel design. U-crit is a measure of the maximum sustainable swimming speeds of larvae. As temperature affected developmental rate, the effect of temperature treatment on swimming on absolute versus developmental age is addressed in Green & Fisher (2004). For the purpose of the current study, swimming measurements were taken from fish at the same developmental age, reared at 28 °C on 7 days after hatching (dah) and at 11 dah for fish reared at 25 °C. This was an *a priori* sampling decision to coincide with one day prior to the average time taken to metamorphosis for 28 °C and 25 °C treatments respectively (Green unpublished data). Two fish were swum from each of the five replicate tanks from each temperature treatment (25 °C and 28 °C), making a total of 10 larvae per clutch swum at each temperature. Green & Fisher (2004, Chapter 4) describes the results that control for the effects of temperature *per se* on critical swimming speed, and details of all swimming trials follow their methods.

Results

Parental influences on clutch size and density

Female total length and Fulton's K were the only measured parental characteristics that correlated to clutch attributes immediately before and after hatching. Female total length was significantly correlated to the number of eggs in a clutch and the number of hatchlings, but not clutch size or dimensions, and Fulton's K was negatively correlated to egg count (Table 1). Male size, and combined female and male size were not correlated to any of these clutch characteristics.

Parental influences in egg size

A multivariate analysis of variance (MANOVA) exploring trends in the egg characteristics (egg length, width and area) simultaneously, showed significant effects of male ($F_{8, 398}$ = 15.65, Pillai's trace, $p < 0.001$), female ($F_{4,198}$ = 15.7, Pillai's trace, $p < 0.001$) and the interaction of male and

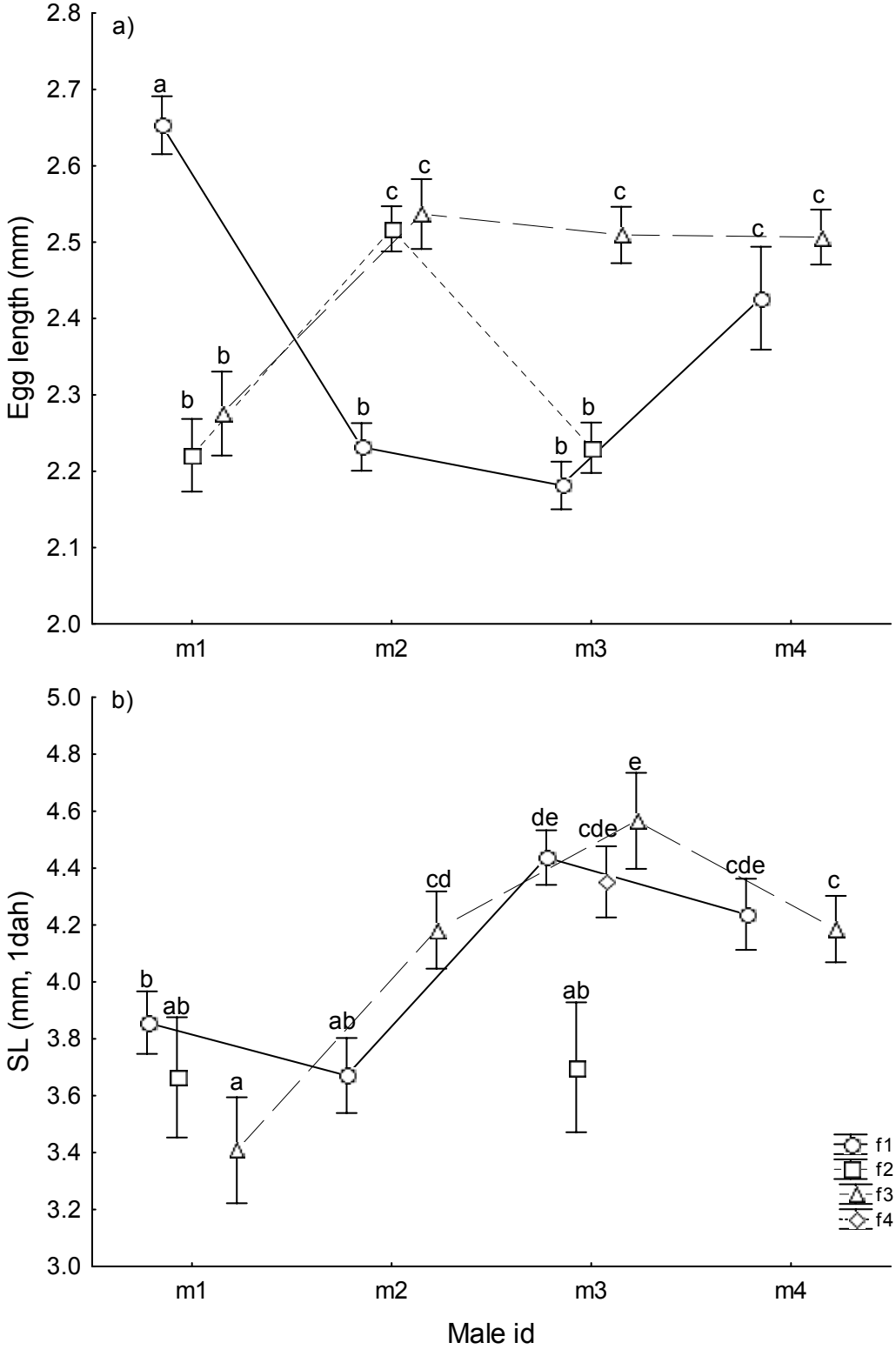

Fig. 1. Combined effects of male and female on offspring size a. pre-hatch egg length, b. Standard length of larvae one day after hatching. Superscripts indicate Tukey's homogenous groups, $p<0.05$. Error bars are 95 % confidence intervals.

female (m*f), ($F_{20,\ 804}$ = 16.3, Pillai's trace, $p < 0.001$) on all three egg characteristics. Females produced clutches that differed in mean egg length when mated with different males. Female f3 had the largest eggs when paired with three of the four males. In contrast, female f4 produced the smallest eggs when paired with two males, but produced some of the largest eggs when she was paired with m1 (Fig. 1a).

Parental influences on larval size

Standard length of larvae one day after hatching differed when females were mated with different males (Table 2, Fig. 1b). Sizes of hatchlings from female f3 were significantly different when she was mated with 3 of the 4 males. Larvae produced by f3 were smallest when sired by m1 and largest with m3. Hatchlings produced by female f1 were significantly different between 3 males, while hatchings produced by f5 showed no difference between males. The influence of the male on his offspring differed depending on the identity of the mated female (Fig. 1b).

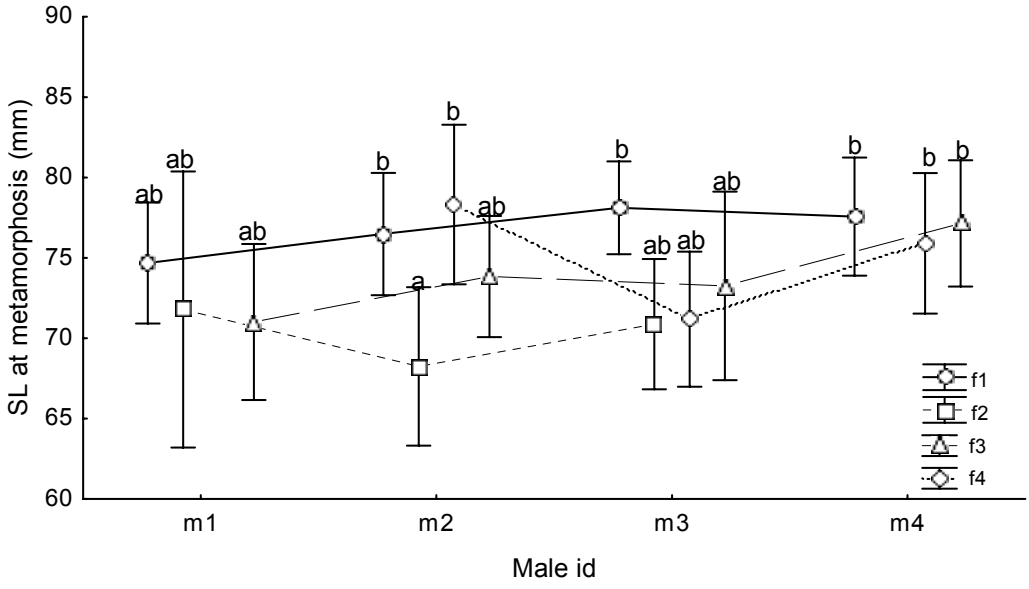

Fig. 2. Standard length (sl) of metamorphosed larvae from a 4 x 4 diallel crosses of males and females. Superscripts indicate Tukey's homogenous groups, $p<0.05$.

Parental and temperature effects on offspring at metamorphosis

Standard length of larvae at metamorphosis differed between females and female by male combinations, while temperature did not have an effect (Table 3, Fig. 2). Larvae from female f1 were significantly larger at metamorphosis from larvae from female f2 when both females were crossed

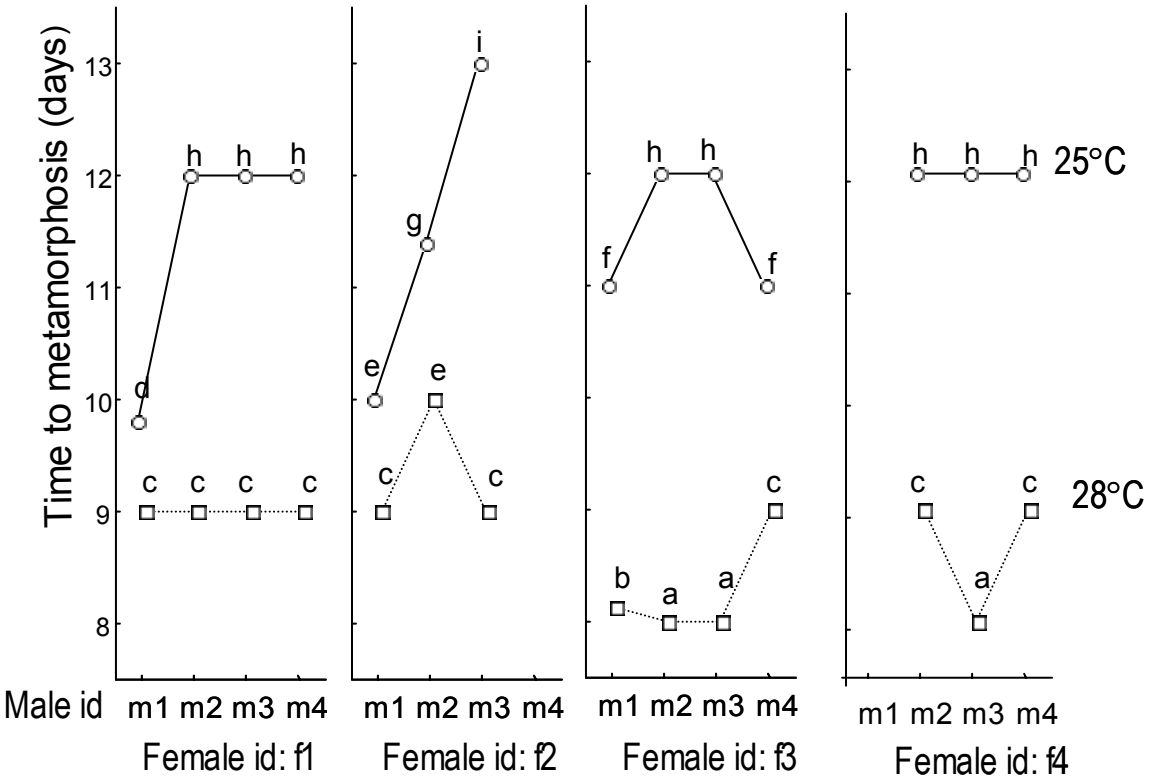

Fig. 3. Mean time to metamorphosis of larvae from 4 female x 4 male diallel crosses raised at 25 ºC (○) and 28 ºC (□). Means are taken from 5 replicate tanks per temperature. 95% confidence intervals are included, but not visible as they are smaller than the markers. Superscripts indicate Tukey's homogenous groups, $p<0.05$.

with m2, but not with the other males. There were no significant differences in size of larvae at metamorphosis in any larvae from females crossed with male m4 (Fig. 2). While fish raised at the lower temperature (25 ºC) metamorphosed at similar size to larvae raised at ambient temperature (28 ºC) (non-significant temperature effects, Table 3), larvae raised at 25 ºC required more days of development to reach metamorphosis (Fig. 3; temp*m*f $F_{7,1348} = 4069$, $p < 0.001$). Time to metamorphosis did not vary in offspring from female f1 at 28 ºC and f4 at 25 ºC with all males, while for female f2, time to metamorphosis varied with all males at 25 ºC, and m1 and m2 at 28 ºC, and f4 varied with multiple males at each temperature (Fig. 3). Although there is a significant interaction between male and female, fish reared at 25 ºC always took longer to reach metamorphosis than fish reared at 28 ºC, mean time to metamorphosis, regardless of parentage was 8.9 ± 0.06 days at 28 ºC and 11.6 ± 0.09 days at 25 ºC.

Individual tanks within temperature treatments and m*f combinations were sampled as units at metamorphosis when at least 70 % of the visible fish had metamorphosed, however the actual number of fish metamorphosed showed more variability. Temperature did not affect the proportion of fish metamorphosed (temp, $F_{1,103} = 2.515$, $p = 0.11$; temp*m*f, $F_{7,103} = 0.931$ $p = 0.48$), however parentage did affect the proportion of fish metamorphosed at the time of sampling (male, $F_{3,103} = 4.348$, $p < 0.05$; female, $F_{3,103} = 3.453$, $p < 0.05$; m*f, $F_{7,103} = 3.42$, $p < 0.05$).

Growth rate and mortality

Paternity and maternity also influenced the growth rate and mortality of fish raised at two temperatures (Table 4, Fig. 4a,b). Growth rate varied significantly at all levels of a factorial analysis of temperature, female and male (Table 4a). The combination of these 3 factors accounted for 30 % of the total variability in larval growth rate, while male identity alone accounted for 52 % (Fig. 4a). Growth rates were generally higher at 28 ºC than 25 ºC.

Mortality rates differed between males, females, temp*females and the combination of the temp*f*m (Table 4b, Fig. 4b). More than 50 % of the variability in mortality was due to differences among replicate tanks, while a further 28 % was attributable to the combination of temp, male and female (Fig. 4b). Mortality rate within a tank was negatively correlated to growth rate averaged for that tank, ($r = -0.30$, $p < 0.005$), and there was little change in this when the effects of temperature were controlled for (partial correlation, $r = -0.29$, $p < 0.05$).

Maternal and temperature effects on swimming performance

Swimming performance, measured as critical swimming ability (U-crit), was affected by rearing temperature and female identity (Table 5, Fig. 5). Differences in swimming ability of larvae from different females were not directly linked to the size of that female, as larvae from the smallest female (f3) achieved the fastest swimming speeds, while larvae from the medium female (f4) achieved the slowest swimming speeds (Fig. 5). Larvae raised and swum at 28 ºC (ambient temperature) were capable of faster speeds than larvae raised and swum at 25 ºC (Fig. 5).

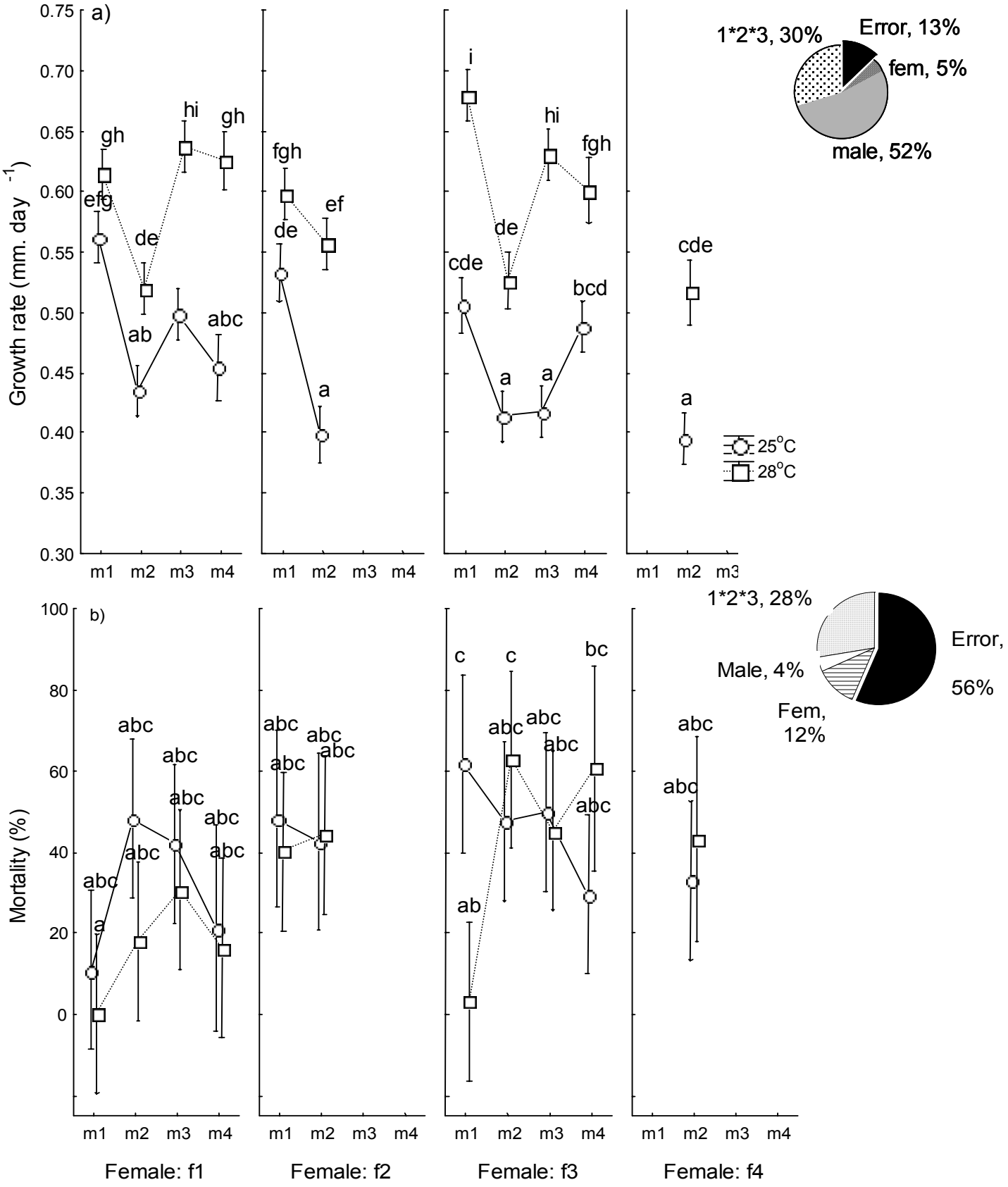

Fig. 4. a) Growth rate (mm.day^{-1}) and b) mortality (%) of larvae raised at two temperatures 25 ºC (O), 28 ºC (□) from a 4 female x 4 male diallel cross, determined at metamorphosis. Error bars are 95 % confidence intervals. Superscripts indicate Tukey's homogenous groups, $p<0.05$. Pie chart represent variance components calculated with time of experiment as a covariate.

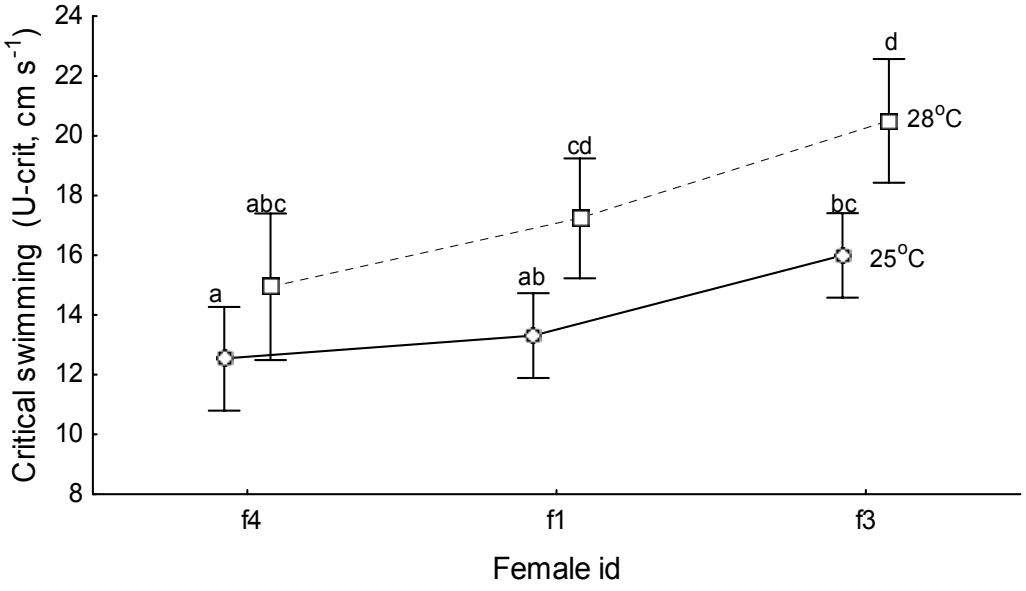

Fig. 5. Critical swimming performance (U-crit, cm s^{-1}) of larvae raised at two temperatures from three females measured 1 day prior to settlement. Error bars are 95 % confidence intervals. Superscripts indicate Tukey's homogenous groups, $p < 0.05$. O 25 ºC; □ 28 ºC

Discussion

Maternal and paternal influences on clutch, egg and larval size

Maternal and paternal effects explain a large proportion of variation in offspring traits in *A. melanopus,* from conception to metamorphosis. Maternal influences were apparent in clutch traits prior to hatching and maternal and paternal traits were important immediately after hatching through to metamorphosis. Within the 14 clutches resulting from the 4 x 4 diallel cross of *A. melanopus* males and females, clutch size and dimensions were related to characteristics of the females but not the males, however, males influenced the pre-hatch trait of egg size. Interestingly, after hatching, the male by female interaction became apparent in all of the larval fish traits measured. This is a remarkable result, as most studies show that maternal heritabilities are higher than paternal heritabilities in early life (Heath & Blouw 1998, Shaw & Byers 1998), if the sire component is characterised at all (Heyer & Rice 2001). However, a strong paternal component is expected in organisms with a male contribution to nest care (Bernado 1996).

The important male contribution to early life history traits can be interpreted in the light of the differential parental contributions from conception to hatching in *A. melanopus*. The territorial male prepares a nest site where

the female lays a monolayer of between 300 and 700 eggs to substratum (Allen 1980) and the male follows, fertilising the eggs, contributing 50 % of the genetic material, but little else to the initial egg aliquot. *A. melanopus* have a long embryonic duration compared to many tropical species (8 days compared to 4 days for other Pomacentrids (Job & Bellwood 2000), and 24 hours for a pelagic spawning Serranid (Masuma et al. 1993) and therefore an extended dependence on maternally-derived yolk supply. Consequently, the maternal investment in the endogenous resources of the larvae is high, as demonstrated by the correlations between female traits and clutch characteristics. While male defence and maintenance of the eggs are critical for their survival (Tyler 1995), his nest-care had no detectable effect on clutch size, but may be responsible for the paternal effect apparent in pre-hatch egg size.

Paternal influences were detected in eggs immediately prior to hatching and in newly hatched larvae. As the embryos develop, incorporating the male genetic contribution, the male actively tends them, fanning to oxygenate the eggs, and mouthing to remove dead eggs. The male dominates maintenance of the embryonic environment, by keeping the eggs well oxygenated in a cryptic habitat that typically has poor water flows. He initially spends 40 % of his time tending, increasing to 70 % towards hatching, compared with only 20 - 30 % time tending from females (Green & McCormick submitted, Chapter 3). Parental care enhances offspring development (Sargent 1988,1997), and increases the chance of survival in offspring in fishes (Sabat 1994, Kuwamura 1997) amongst other organisms (Clutton-Brock 1991). Paternal fanning that enhances growth, development and survival, distinguishes *A. melanopus* from many of the fish commonly targeted for the study of parental effects (e.g. Atlantic cod, Chambers & Leggett 1996, Marteinsdottir & Steinarsson 1998, Clemmesen 2003). Most studies addressing parental influences that did not detect a paternal effect investigated fishes without egg guarding, where the opportunity for a significant male contribution was restricted (Chambers & Leggett 1996, Benoit & Pepin 1999, Clemmesen 2003). The predominately male tending, coupled with the long embryonic duration and ensuing dependence on the maternal

aliquot, increases the prospect for parental contributions to the larval characteristics in *A. melanopus*.

Temperature effects on larval traits

Time to metamorphosis

Temperature did not affect size at metamorphosis, but it did affect the time until metamorphosis and larval growth rates. *A. melanopus* larvae raised at 25 ºC (representing the bottom of their temperature range) metamorphosed at similar sizes to larvae raised at ambient temperature (28 ºC), however fish raised at 25 ºC took longer to reach metamorphosis. The strong influence of water temperature on larval duration rather than size suggests that metamorphosis is dependent on a size-related threshold rather than age, similar to starry flounder (Policansky 1983), winter flounder (Chambers & Legget 1987) and a tropical goatfish (McCormick & Molony 1995).

Temperature and parental influences on swimming performance

Rearing temperature and maternity each affected maximum sustained swimming speed, and a paternal component was not identified. As the water temperature within the swim chamber has no detectable influence on swimming speed (Chambers & Leggett 1996, Green & Fisher 2004), the reduction in swimming performance with decreased rearing temperature is not explained by the animal's metabolic response to the immediate water temperature. Swimming performance is most likely related to the larval body condition, which is a sum of its genotype, modified by environmental history (Brett 1967, Angilletta Jr et al. 2003). In young fishes, environmental effects can significantly modulate the maternal effect causing variation in traits at the end of the larval period (Chambers & Leggett, 1996).

Growth rate and mortality

While there were differences in larval growth rate at all levels of the male x female x temperature experimental design, the majority of variance in growth rate was attributable to paternity, followed by the interaction of male, female and temperature. We found some evidence that initial parental influences are maintained until metamorphosis, by following sibling groups, rather than individual growth trajectories. Growth rates were highest in larvae from the females f1 and f3, which also had the largest eggs and larvae at hatching. These growth rates and sizes identify a maternal and paternal

contribution to initial offspring size that is maintained, though weakened, throughout the larval period to metamorphosis. The persistence of parentally derived traits throughout larval development is rare, although egg size has predicted larvae and juvenile size (Reznick 1991, Roff 1992, Chambers & Leggett 1996), and hatching size has predicted juvenile size in wild populations (Vigliola & Meekan 2002). Generally, environmental variation and favourable conditions after hatching can cause parentally derived growth differences to disappear (De March 1991, Hoie et al. 1999a). While these results reflect similar significant patterns in wild populations (Vigliola & Meekan 2002), they should be interpreted carefully due to the small sample size of this diallel design.

The interaction of paternity, maternity and temperature influenced mortality rate, and more importantly, experimental units (tanks) with fast growth had lower mortality than units with slow growth. Such a relationship between growth and survival was coined the 'bigger is better' hypothesis (Miller et al. 1988, Bailey & Houde 1989) and is based on the reduced likelihood of predation for bigger fish. Subsequent support for this hypothesis has shown that fishes that were larger at hatching and had subsequent higher growth, generally survived selective mortality (Anderson 1988, Meekan & Fortier 1996, Vigliola & Meekan 2002). My study suggests that there are parental origins for this relationship that may determine survival chances for the offspring, in the absence of predators.

Contrary to most studies of maternal effects (see Heath & Blouw for discussion), there were minimal tank- or rearing-environment effects in the current study, with the exception of mortality. Although, particular attention was paid to maintaining identical conditions within all rearing units, more than half of the variability in mortality was attributable to unknown sources among replicate rearing tanks.

This study has addressed the 'within population' variability in offspring traits that have maternal and paternal influences, and found significant effects from both these sources. Maternal effects also exist between populations and between species (Chambers & Leggett 1996), and therefore it is likely that paternal effects do also. To determine the influences of natural variation in recruitment to wild populations, studies of the variation in maternal effects

between populations is important. Identifying that maternity, paternity and their interaction are critical determinants of larval size and performance is a vital first step in looking for sources of variation in populations that has previously been overlooked as ecological noise.

Table 1. Correlations of brood stock and egg clutch attributes. *Indicate significant correlations at p<0.05. n=11.

Variable	No. of hatchlings	clutch perimeter (mm)	clutch area (mm^2)	egg count	egg density (mm^2)
Female weight	0.39	0.06	0.32	0.24	<0.01
Female TL	0.61*	0.23	0.49	0.68*	0.22
Male TL	-0.40	0.33	0.09	-0.19	-0.43
Male weight	-0.31	0.00	-0.09	-0.28	-0.33
Female + Male size	0.20	0.41	0.45	0.40	-0.13
Female Fulton's K	-0.52	-0.25	-0.46	-0.69*	-0.24
Female + Male weight	0.14	0.06	0.22	0.03	-0.22

Table 2. Comparison of the effects of male and female *Amphiprion melanopus* on the mean standard length (SL) of their offspring one day after hatching, from a 4 x 4 diallel breeding design. Type III SS were used. Male is nested within female as there were missing blocks in the full factorial design. Bold type denotes significant differences

Variable	df	MS	F	p
Female id	3	1.19	14.83	**<0.001**
Male id(Female id)	7	2.28	28.43	**<0.001**
Error	180	0.08		

Table 3. Comparison of the effects of male, female and temperature on the standard length (sl) and weight measured at metamorphosis of offspring from a diallel breeding design. Results of a multivariate ANCOVA using date of clutch hatching as the covariate are given. Significant results in bold.

Variable	Pillai's trace	F	Effect df	Error df	p
Temp	0.00	0.57	2	1346	0.567
Female	0.01	2.70	6	2694	**0.012**
Male	0.01	1.22	6	2694	0.290
Temp*Female	0.01	1.17	6	2694	0.319
Temp*Male	0.01	1.57	6	2694	0.151
Female*Male	0.02	1.74	14	2694	**0.042**
Temp*Male*Female	0.01	1.20	14	2694	0.262
Tank(Temp*Male*Female)	0.16	1.13	204	2694	0.109

Table 4. Mortality and mean growth rate of larvae from a full factorial ANOVA of 4 female * 4 male diallel cross raised at two temperatures, measured at metamorphosis. Bold type denotes significant results

	df	MS	F	p
Growth rate				
Temp	1	0.326	582.65	**< 0.001**
Female	3	0.002	3.36	**0.023**
Male	3	0.053	96.10	**< 0.001**
Temp*female	3	0.003	4.56	**< 0.005**
Temp*male	3	0.006	10.63	**< 0.001**
Female *male	4	0.003	4.46	**0.002**
Temp*female *male	4	0.006	11.62	**< 0.001**
Error	77	0.00056		
Mortality				
Temp	1	155.1	0.32	0.572
Female	3	3486.0	7.21	**< 0.001**
Male	3	1775.4	3.67	**0.015**
Temp*Female	3	587.8	1.21	0.310
Temp*Male	3	1550.0	3.20	**0.028**
Female *Male	4	697.4	1.44	0.228
Temp*Female *Male	4	1545.9	3.19	**0.017**
Error	77	483.8		

Table 5. Comparison of the effects of female, male and temperature on mean critical swimming performance. a. results from full factorial cross, b. summary from female and temperature effects only.

Source	df	MS	F	p
a.				
Female	1	343.90	11.34	**<0.001**
Male	1	0.24	0.01	0.929
Temp	0	0		
Female *Male	3	68.98	2.27	0.081
Female *Temp	1	4.99	0.16	0.685
Male*Temp	1	7.41	0.24	0.621
Female*Male*Temp	3	50.18	1.65	0.178
Error	222	30.32		
b.				
Female	2	349.93	11.28	**<0.001**
Temp	1	661.48	21.33	**<0.001**
Female*Temp	2	17.75	0.57	0.565
Error	232	31.01		

General conclusions

General summary

This thesis demonstrates the importance of all parts of the early life history of fishes (egg, larvae and juvenile) in influencing variation in performance, growth and survival of recruits. Additionally, my co-authors and I identified the interaction of parentage and environmental variates as critical sources of variation that contributed to growth, condition and performance in these early life history stages. Through a series of laboratory experiments we was able to partition the variation attributable to the male, female and the environment at different life stages, illustrating the importance the interaction of these factors has to each life stage, and more importantly the relationship between the three stages of the early life history of fishes, and how they are borne into each other. Maternally derived size differences identified in the embryo were borne into the larval stage, and factors acting in the larval stage influenced the quality and performance of recruits. The major source of variation in embryos and larvae was parental, such that parental effects influenced the quality, quantity and persistence of their offspring.

Scope of this study

The limitations in this study are also its advantages, and the confinement of experiments to the laboratory has led to the most salient results of this thesis. That is, while we have examined laboratory reared individuals independently of the selective processes acting on natural populations, we have been able to consider variables that have been identified as key sources of variation in growth and survival in natural populations (e.g temperature (Meekan 2003 et al.), while holding all else constant. In natural populations, the presence of multiple variables can confound results as autocorrelations can suppress key variables. This laboratory study has revealed sources of variation difficult to detect in the field, due to field sampling logistics and limitations of tracking the fate of individuals of known parentage. By examining all of the stages of the early life history of related individuals we were able to trace size advantages from

conception to hatching, and from hatching to successful metamorphosis, a stage which signifies recruitment on the reef.

Summary of key findings

This study highlighted components of initial oviposition that affected the size of hatchlings. The maternal nest design and initial female aliquot to the embryo were sources of significant size differences in larvae at hatching, which originated from oviposition and were enhanced by the clutch micro-environment throughout embryogenesis (Chapter 2).

Males contributed substantially more than females to replenishing oxygen to their benthic egg nests throughout embryological development (Chapter 3). Males spent up to 70% of their time in active nest tending, which was demonstrated to replenish oxygen to the embryos within the nest. Males adapted their tending throughout the day according to the amount of ambient dissolved oxygen, and throughout embryo development, according to the increased metabolic needs of their offspring as described in chapter 2. While previous studies have described nest-tending behaviour, few have experimentally determined that fanning actually increase the amount of oxygen to the nest, or that parental nest tending behaviour is modified to compensate for changes in ambient dissolved oxygen.

This study also demonstrated significant interactions between paternal, maternal and temperature effects on offspring size and performance, which persisted throughout development (Chapter 5). Most notably, we found significant sources of paternal inheritance in larval growth rates, embryo and larval size, and size at recruitment, seldom described for marine fish larvae. While maternal effects are frequently acknowledged as importance sources of variation, strong paternal effects are rarely identified, if they have been considered at all. We speculate that this paternal component is derived from the significant input males had into the nest-tending of their clutches, as described in chapter 3. Such post-oviposition components of parental effects are rarely partitioned out and considered separately, or in conjunction with pre-oviposition effects. As research into sources of size variation in the early life history stages advances, such fine details are receiving more consideration. Most recently, post-oviposition parental effects, such as nest-care, were demonstrated to have a greater impact on offspring size that other

factors incorporated in to the egg prior to oviposition in the red-backed salamander (Crespi & Lessig 2004). Given that size advantages at hatching can persist throughout the larval period, effective fanning may enhance the quality and condition of larvae though their early development. There is huge scope for both field and laboratory studies to further address such sources of variation.

Overall conclusions

Each stage within a reef fish's life history leads into the next stage, and embryogenesis is a critical foundation to the size, growth and performance of larvae. Early size advantages are derived from both male and female, and interact with embryonic and larval environment. This interaction is complex, and not consistent between different male and female combinations, highlighting the amount of variation inherent within and between clutches of larvae prior to selective processes that operate in natural populations. The early size advantages seen in this study resulted in better performance and persisted to metamorphosis. If mortality acts selectively in natural populations, favouring survival in larvae that are bigger and are capable of better performances, then initial size advantages from female aliquot and male inputs- whether through nest tending or the unquantified genetic contribution- will selectively favour survival. Furthermore, the significant parental effects found in this study may explain much of the variation in growth of wild larvae and recruits that cannot be explained by environmental factors.

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Appendix

Publication list from thesis chapters

Green, B.S & Fisher R. (2004) Temperature influences whether bigger is really better? *J. Exp. Mar Biol Ecol* 299: 115-132

Green B.S. Changes in respiration during embryogenesis of a tropical reef fish. *Accepted Comp.Phys. Biochem A.*

Green, B.S., Anthony K.R.N., McCormick, M.I. Variation in size at hatching in marine fishes has maternal and environmental origins. *In review Oecologia*

Green, B.S. & McCormick, M.I. Males are more proactive than females in replenishing oxygen to benthic fish nests *Submitted to Behavioural Ecology*

Green, B.S & McCormick M.I. Maternal effects on development and performance of tropical reef fish larvae. *Submitted Mar. Ecol Prog Ser*

Other publications arising from thesis

Green, B.S., S. Reilly & McCormick, M.I (2002) A cost-effective method of preparing larval fish otoliths for reading using enzyme digestion and staining. *J. Fish Biol*. 61:1600-1605

Green, B.S. & McCormick, M.I. Maternal and paternal investment in spawning, hatching and offspring quality in a benthic spawning tropical reef fish *To be submitted to Animal behaviour after finishing thesis*

Green, B.S & McCormick M.I. Maternal and temperature effects on otolith microstructure in tropical reef fish larvae. *To submit after finishing thesis*