Larval Clownfish *Amphiprion ocellaris* Predatory Success and Selectivity when Preying on the Calanoid Copepod *Parvocalanus crassirostris*

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By
James M. Jackson

Thesis Committee:

Daniel K. Hartline, Chairperson
Petra H. Lenz
Julie H. Bailey-Brock
Clyde S. Tamaru

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Abstract

Larvae of the clownfish *Amphiprion ocellaris* and the evasive copepod prey *Parvocalanus crassirostris* were used as a model system for the study of larval reef fish predatory behavior during the planktonic larval phase. Little is known about the feeding behavior of coral reef fish larvae during this critical phase. The use of a model larval reef fish predator and natural prey allowed for the investigation of feeding behaviors such as prey selectivity and capture success from hatching to settling. Filming techniques were used to examine larval fish predatory behavior when presented with multiple copepod life-stages in mixed and single prey-type assemblages. *A. ocellaris* growth characteristics were measured and compared to prey size. *A. ocellaris* predatory ability improved with age. Larvae hatched with the ability to capture *P. crassirostris* nauplii, but not copepodites. Capture of copepodites occurred on day 3 post-hatch with an initial success rate of 22%. Capture of adult copepods was first observed on day 8 post-hatch, with an initial success of 4%. Early in development, *A. ocellaris* exhibited preference for nauplii (Manly-Chesson index value of 0.68 at day 1 post-hatch) and avoidance of adults (Manly-Chesson index value of 0.05 at day 1 post-hatch). Selectivity between nauplii and adults decreased as fish larvae aged and *A. ocellaris* aged 10-14 days post-hatch demonstrated no selectivity for any prey type. The common assumption that larval fish mouth gape size relative to prey size is a good predictor for prey preference was not supported. *A. ocellaris* mouth gape measurements relative to prey size measurements would have predicted consumption of all *P. crassirostris* life-stages by day 2 post-hatch. This study provides novel insight into feeding behavior during the planktonic larval phase of a model larval reef fish preying on multiple life-stages of a natural evasive prey.

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Introduction

Prior to joining the adult population, most reef fishes must complete a planktonic larval phase during which they undergo rapid growth and acquire abilities necessary to survive in the reef environment (Sale 2004, Carrasou and Ponton 2009). The time spent in the planktonic larval phase begins at hatching and concludes days to weeks later when fish settle to adopt a benthic existence on the reef (Wellington and Victor 1989). This settlement results in the addition of new individuals to the adult breeding population, i.e. recruitment (Williams 1983, Jones 1990). Variability in recruitment has been shown to be capable of explaining over 90% of the spatial variation in abundance of some reef fish populations (Doherty and Fowler 1994). Reef fish spawn hundreds to thousands of eggs, of which ≥98% do not survive to settlement (Bailey and Houde 1989). The planktonic larval phase is the most critical period of growth and development in the life cycle of all reef fish, yet it is also the least well understood part of the life cycle (Pepin and Myers 1991, Hoey and McCormick 2004). In the thesis presented herein, a model larval reef fish predator and copepod prey were used to investigate changes in allometry, capture success and prey selectivity across the complete planktonic larval phase.

Some characteristics of the reef fish planktonic larval phase that have been established are: a relative lack of morphological differentiation, high risk of mortality, rapid growth and voracious appetite (Houde 1989, Leggett and Deblois 1994). It is well documented that planktonic reef fish larvae feed on zooplankton (Hamner et al. 1988, Sampey et al. 2007, Carassou et al. 2009), which consists largely of copepods and other small crustacean larvae (Emery 1968, Hamner and Carleton 1979). A major factor

influencing survival during the planktonic larval phase is the amount of available prey (Hartmann 1983, Houde 1987) and the ability of larvae to consume them (McCormick and Molony 1992).

Recently a detailed study of larval reef fish gut contents was performed by Sampey et al. (2007) in which they primarily examined larvae that were in the post-flexion stage (i.e. beginning with formation of the caudal fin where the hypural elements are vertical), which is the final sub-stage of development before metamorphosis to the juvenile stage. This study provides an excellent assessment of prey taxa that are consumed by various larval reef fish families during their planktonic larval phase. Copepods and their nauplii were shown to be the primary prey for the majority of larval fish examined within the study. However, gut content analysis has its limitations, such as the tendency for soft bodied organisms to be underrepresented (due to the relatively rapid rate at which they are digested).

Another way to approach the study of the planktonic larva phase is through laboratory studies with captive-reared larval fish. Such studies allow investigators to examine the earliest stages of larval development through to metamorphosis and beyond, in detail. Captive-reared larval clownfish have been used to examine swimming and search behavior (Coughlin et al. 1992, Coughlin 1993). However such investigations have one shortcoming: rotifers are usually used as prey during the initial phases of development (Hagiwara et al. 1997). Rotifers are an unnatural, non-evasive prey organism commonly used in aquaculture and are much easier to capture than natural evasive prey e.g. copepods (Sarma et al. 2003). The progression of larval reef fish predatory behavior (feeding success and prey preference in particular) with respect to

natural evasive prey across the complete planktonic larval phase has yet to be explored in detail for any species.

Planktonic larval fish with greater feeding success will grow more rapidly and will be subject to a lower risk of mortality (Hare and Cowen 1997). Larval fish feeding success depends upon the availability of appropriate prey in the environment. The identification of suitable prey is crucial for one to accurately evaluate prey availability in the field. A measure of presumed prey suitability often used by investigators is prey size relative to the predator's mouth gape (Schmitt and Holbrook 1984, Bremigan and Stein 1994, DeVries et al. 1998, Gill 2003). However, when highly evasive zooplankton prey are considered, size may not be the best predictor. Failure to distinguish between the abundance of zooplankton in the environment and the availability of zooplankton prey-types suitable to individual larvae has compromised previous evaluations of food limitation in the field (Leggett and Deblois 1994). Better understanding of larval reef fish predatory preference and ability with respect to natural, evasive prey will help to inform the evaluation of *in situ* prey availability and thus to clarify the importance of food limitation as a source of pre-settlement mortality.

Due to the small size, fragility and difficulty of species identification for larval reef fish, examination of feeding behavior is presently extremely difficult in the field or in the laboratory with field-collected specimens (Moser and Smith 1993, Sampey et al. 2004). This is therefore best accomplished with use of a model larval reef fish predator in a controlled laboratory setting. The clownfish *Amphiprion ocellaris* served as the model predator in this study as it is one of the few coral reef fish for which captive rearing protocols have been developed (Kumar and Balasubrananian 2002). The planktonic larval

phase of *Amphiprion ocellaris* spans two weeks from hatching, which is representative of a typical reef fish's larval planktonic duration (Wellington and Victor 1989). A model predator requires model prey, and copepods provide an excellent choice as they are a crucial component of the diet of most larval reef fish (Sampey et al. 2007). Small paracalanid copepods often dominate the copepod communities of subtropical waters (Hopcroft et al. 1998, McKinnon and Duggan 2001).

Parvocalanus crassirostris, a typical paracalanid for which laboratory culturing protocols have been developed (McKinnon et al. 2003), served as the model prey species in this study. Copepods are a highly evasive prey and react to threats with very rapid escape responses. For example, *Acartia tonsa* males have been reported to initiate escape responses to a sudden suprathreshold hydrodynamic stimulus with a mean reaction time of 3.6 ms and a mean maximum velocity of 432 mm s^{-1} (Buskey et al. 2002). *Temora longicornis* has been reported to initiate escape responses from the same type of hydrodynamic stimulus with a mean reaction time of 2.8 ms and a mean maximum velocity of 262 mm s^{-1} (Burdick et al. 2007). Other maximum escape velocities reported in the literature include *Calanus finmarchicus* of 800 mm s^{-1} (Lenz et al. 2004) and *Paracalanus parvus* at 318 mm s^{-1} (Waggett and Buskey 2008).

The most important factor in a successful copepod escape is the sensory ability of the copepod, that is the ability to detect an approaching predator. Once initiated, an escape response from fish predators is successful most of the time, and thus a fish predator will usually only capture its copepod prey if that prey does not sense the predator. (Waggett and Buskey 2007a, Waggett and Buskey 2007b, Gemmell 2010). As stated previously, preferred prey for larval fish predators is often considered as a factor of

mouth gape. However, large diversity in the prey preferences of larvae feeding *in situ* is not sufficiently explained by larval fish size (Sampey et al. 2007) which is correlated with mouth gape (Schmitt and Holbrook 1984).

Reef fish larvae must feed successfully on evasive zooplankton prey to grow and survive. What types of prey are they capturing? Do they exhibit prey selection for certain prey types? How does their predation ability change as they develop? The current project, addressed these questions using a model larval reef fish predator and evasive copepod prey ranging in size from 60 μm – 400 μm. Specifically, the changes in allometry, predation ability and prey selectivity across the complete planktonic larval phase were investigated using a clownfish/copepod model. The results of this study provide insight into key factors influencing prey preference and feeding success in the natural environment.

Background

Planktonic larval phase and recruitment

Although analysis of the factors that affect marine fish recruitment is generally very difficult (Miller et al. 1988), it has been suggested that growth and survival during the planktonic larval phase is critical to recruitment success (Takasuka et al. 2004). Some observations have reported that small changes in larval growth and mortality rates can generate an order-of-magnitude or greater difference in annual recruitment (Shepherd and Cushing 1980, Houde 1987). Although the vast majority of fish recruitment studies have

been conducted with commercially valuable temperate species, theoretical work has suggested that considerably different circumstances operate in the tropics e.g. warm water temperatures result in relatively fast larval growth rates (Houde 1989).

Most tropical marine fish recruitment studies have instead focused on larval dispersal as opposed to patterns of growth and survival during the planktonic larval phase (Shenker et al. 1993, Planes et al. 1993, Milicich 1994, Kingsford and Finn 1997, Robertson et al. 1999, Shima and Swearer 2010). These studies have had little success in predicting recruitment success; however, they have demonstrated that reef fish larvae often settle in close proximity to the reef area from which they were spawned. In such a situation, local conditions (e.g. food availability) will be exceedingly relevant to local reef fish recruitment (Cowen 2002). It is in this context that the feeding ecology of larval reef fish is critical for the assessment of local conditions when attempting to predict recruitment outcomes.

Studies of tropical reef fish populations have suggested that recruitment is largely determined by mortality during the pre-settlement stage of life history, whereas post-settlement mortality has a lesser influence (Williams 1980, Doherty 1983, Victor 1986, Doherty and Fowler 1994, Sale 2004). The question of whether predation or starvation has a greater influence on pre-settlement mortality remains an open one, although these two causal factors would not necessarily operate independently, e.g. under-nourished prey would likely exhibit reduced evasive ability. Sufficient growth and development during the planktonic larval phase is critical for post-settlement survival (Carrasou et al. 2009) and feeding success during that time should remain a relevant consideration regardless.

Fish Growth and Feeding Behavior

Much of what is known of planktivorous reef fish predatory behavior comes from studies with mature fish. Waggett and Buskey (2007a) studied predation vulnerability for adult calanoid copepods *Temora turbinata* and *Paracalanus parvus* when exposed to the adult blenny *Acanthemblemaria spinosa.* Differences in the swimming patterns of the two copepod species affected their predator detection susceptibility and thus their vulnerability to predation. In another study, Coughlin and Strickler (1990) examined details of prey capture mechanisms used by the pomacentrid *Chromis viridis* while feeding on adult stages of the calanoid copepod *Eucalanus crassus* and the branchiopod *Artemia*. *C. viridis* employed the more bio-mechanically simple ram-jaw feeding method for the passive *Artemia* and suction feeding for evasive copepod prey. These studies show that mature fish can vary feeding behavior according to the evasiveness of prey and thus demonstrate the importance of predator behavior for feeding success.

Much of what is known about larval reef fish feeding and growth has been reported from aquaculture studies in which behavior has not been considered (Ostrowski and Laidley 2001, Olivotto et al. 2008a). What little is known about feeding behavior during early larval reef fish development rests heavily on studies conducted with clownfish. Using overhead (top-down) filming techniques Coughlin (1993) found that larval Pink Clownfish, *Amphiprion perideraion,* fed rotifers, exhibited an increasing capture success rate over the first week of development. *A. perideraion* larvae were reported to display two search modes: a linear mode used to locate patches of food and a complex searching mode with many directional changes used to exploit those patches

once they were located (Coughlin et al. 1992). Method of prey capture was also examined using early larval *A. perideraion* and rotifers; major improvement in feeding method, from ram to suction feeding, was reported for larvae as early as day 3 post-hatch (Coughlin 1994). Although these studies provided novel insight into previously unexplored behaviors, Coughlin used the non-evasive rotifer as the model prey organism. This leaves questions concerning predatory abilities of larval fish feeding on evasive prey unanswered. The next logical step is to investigate feeding behavior with evasive prey and to study changes across the entire planktonic larval phase from hatching to settlement.

Kumar and Balasubramanian (2002) investigated the effects of providing larval *A. ocellaris* with copepod nauplii in place of, or in combination with, rotifers (followed by *Artemia* at day 7 post-hatch) and reported an increase in growth rate and a decrease in mortality rate for larva fed copepod nauplii during the planktonic larval phase. Similar results have been reported for closely related *Amphiprion clarkii* fed calanoid copepod *Centropages typicus* (Olivotto et al. 2008a) and harpacticoid copepod *Tisbe spp* (Olivotto et al. 2008b). However, these studies sought only to evaluate the effects of feeding copepods, rotifers and *Artemia* on net growth rates and survivorship. No data were collected on feeding behavior.

Mouth Gape

The concept of mouth gape limitation (wherein the optimal size of prey is deduced from a measure of the distance between the jaws at maximum gape) is commonly applied to larval fish in the aquaculture and fisheries fields (Schmitt and Holbrook 1984, DeVries 1998). Using mouth gape as a predictor of prey size (and thus type) is usually successful for artificial systems developed in aquaculture where non-evasive rotifers and *Artemia* are used as prey. In this context, fish larvae are able to consume prey sizes up to but less than their maximum mouth gape (Gill 2003). However, rotifers and A*rtemia* are unnatural, slow-moving prey which are incapable of responding to a predator's attack with an escape response (Samara et al. 2003, Clarke et al. 2005). In the natural environment most reef fish larvae feed on highly evasive copepods (Hamner et al. 1988, Sampey et al. 2007). A larval fish predator's feeding success with copepod prey may actually be limited by the difficulty of capturing such a highly sensitive and evasive prey item even when the prey size relative to the predator's mouth gape would predict otherwise. It is also important to note that not all copepod species have the same sensory and escape abilities. Specifically, differences in stimulus thresholds, response latencies, swim velocities and accelerations have been reported (Lenz and Hartline 1999, Lenz et al. 2000, Viitasalo et al. 1998, Burdick et al. 2007). In light of this variability, gape size would appear to be even less valuable as a predictor for prey size/type when dealing with copepod prey.

Prey Characteristics: Calanoid Copepods

Calanoid copepods are a common component of the zooplankton around tropical reefs and have been shown to be a preferred prey for some planktivorous reef fishes. Mitchell (1991) presented two species of captive-reared planktonic larval pomacentrids with wild-caught zooplankton assemblages (consisting of various copepods and other small crustacean species) and preferential selection for calanoid species was documented. From surveys conducted in the tropical waters of Northwestern Australia, Sampey et al. (2007) reported that copepods dominated the zooplankton community. The diets of most fish examined (via gut content analysis) was composed primarily of copepods while a small number of fish families consumed alternative prey such as mollusk veligers, chaetognaths, appendicularians and protists. Specifically, 85% of the 47 fish families examined consumed some calanoid copepods and for 43% of these families, calanoid copepods comprised >50% of their diet (by prey count in gut). In comparison, 72% of the families fed on oithonid copepods but only in 6% of the cases did oithonid copepods form >50% of the diet. Naupliar stage calanoids composed approximately 30% of zooplankton in the area surveyed and copepod nauplii were the most important dietary component for larvae in 34 of the 50 families studied.

Copepods develop through six distinct naupliar stages followed by six distinct copepodite stages all separated by molts. Naupliar stages commonly numerically dominate zooplankton communities, and have been reported to outnumber adult copepods by orders of magnitude (Durbin et al. 2003). All copepod stages are capable of rapid escape responses (Titelman and Kiørboe 2003). Although, naupliar stages exhibit

less capable sensory abilities (Titelman and Kiørboe 2003) and slower escape responses (Titelman, 2001, Green et al. 2003) relative to the more mature copepodite stages. Evasive abilities improve as the copepod passes through each developmental stage (Landry 1978, Jonsson et al. 2009). Not much is known about the sensory capabilities of nauplii, but differences in morphology between the naupliar (NI-NVI) and copepodite-adult (CI-CVI) stages suggest a sensory advantage for the copepodites and adults. Naupliar stages are less capable of detecting predators (Buskey 1994) and even when an approaching predator is detected, the escape of a young copepod is often much less effective than an adult's (Titelman 2001). Nauplii rely on their paired cephalic appendages (1st antennae, 2nd antennae and mandibular palps) for both normal swimming and escape jumps, which may hinder their sensory perception of planktonic predators (Bradley 2009). The boundary layers surrounding the setae on these appendages are larger than the space between them and it has been suggested that this would impede water flow through the setae and further hinder the transmission of mechanosensory signals (Paffenhöfer 1998). In contrast, the copepodite and adult stages possess stationary 1st antennae that are deployed into the surrounding water and are covered with mechano- and chemo- sensors (Lenz et al. 2000). These antennae spread sensory structures across a larger area and allow for better detection and localization of potential predatory threats than the naupliar sensory structures (Yen et al. 1992, Weatherby and Lenz 2000). In adults and copepodites, the 1st and 2nd antennae contribute little to the escape movement as the antennae become folded against the body making the copepod more streamlined (Lenz et al. 2004). Adult and copepodite stages also possess more powerful pereiopods that allow for an escape with a higher velocity and greater travel distance (Landry 1978).

Prey Characteristics: *Parvocalanus crassirostris*

In this study, the calanoid copepod *Parvocalanus crassirostris* was used as the model prey species due to its stability in culture, relevance to *A. ocellaris* as an appropriate natural prey and the fact that its escape behavior has been studied previously (Bradley 2009). *P. crassirostris* has also been recommended as a first feed for fish larvae by McKinnon et al. (2003) based on key characteristics such as essential fatty acid composition, small naupliar size (<100μm), susceptibility of nauplii to predation and the absence of cannibalism. *P. crassirostris* is an evasive prey with myelinated axons (Lenz et al. 2000) and has demonstrated escape jump distances of over 3 mm for all developmental stages with maximum speeds of 200-500 body-lengths per second (Bradley 2009). *P. crassirostris* is common in subtropical marine ecosystems across the globe and it is likely that *A. ocellaris* and other reef fish larvae would encounter this species in their natural environment (Leis 1982).

Clownfish Life-History and Biogeography

Reef fish of the genera *Amphiprion* and *Premnas* (family *Pomacentridae*) constitute the group of species commonly known as Clownfish. Coral reefs throughout the Indian and Pacific Oceans provide the habitats for the 29 species of clownfish presently recognized. The Great Barrier Reef, Ryukyu Island, the Indo-Malaysian region, southeast Asia and the Red Sea offer particularly ideal clownfish habitat. However, clownfish are not native to the Hawaiian Archipelago.

All clownfish species live in social groups with a size-based dominance hierarchy. As protandrous hermaphrodites, they characteristically change sex from male to female at some point in their life history. For clownfishes there is also an additional influence on when that takes place as in a group, the largest clownfish is usually female and the others are males. When the dominant female is removed, the next largest male fish becomes female to take its place (Fricke 1979, Buston 2003).

Clownfish will pair up for each spawning event. The female attaches her eggs to a hard substrate and the male then fertilizes them. The clownfish pair "brood" the eggs until hatching, which involves guarding the eggs from predators and fanning water over the eggs to ensure adequate gas exchange. After hatching, the clownfish larvae pass through a planktonic larval stage spanning two weeks and then undergo metamorphosis to the juvenile stage at which time settlement onto the reef benthos occurs. Soon after settlement clownfish typically form a strong mutualism with a sea anemone, with each clownfish species being specific to only a few different species of anemone. Adult clownfish consume algae, mollusks and crustacea (mostly isopods, mysids and copepods). Larval clownfish primarily feed on copepods as well as small amounts of suspended algae (Allen 1980, Green and McCormick 2001).

Amphiprion ocellaris

Of the approximately 1,400 marine fish currently sold in the aquarium trade, fewer than 50 have been successfully reared in captivity. *Amphiprion ocellaris* is the most popular species in the marine aquarium hobby and has helped lead the way in the

development of the marine ornamental aquaculture industry (Bunting and Meyers 2002, Alencastro 2004).

A. ocellaris is commonly referred to as the False Percula because it exhibits a similar morphology and is closely related to the True Percula Clownfish, *Amphiprion percula*. *A. ocellaris* occurs in a wide range in the Indo-Pacific region including: Australia, the East Indies, Melanesia, the Philippines and the Ryukyus Islands (Allen 1980). Adult *A. ocellaris* are orange in color with three white bars and black markings on the fins. Adults reach about 8 cm total length and often live in symbiotic mutualisms with the sea anemones *Heteractis magnifica*, *Stichodactyla gigantea* or *Stichodactyla mertensii* (Rainer and Pauly 2008).

In captivity, mated pairs of *A. ocellaris* typically spawn every 10-14 days and the eggs hatch in about 7-8 days. The average nest size for *A. ocellaris* is about 250 eggs. *A. ocellaris* eggs are attached to a hard substrate by the female and the parents brood the eggs until hatching, which typically occurs after nightfall. Larvae are around 3-4 mm at hatching and are ready to feed the following morning. Yolk sacs are fully absorbed and stomachs are fully formed by day 3 (Gordon and Hecht 2002). Metamorphosis to juvenile and settlement occurs around day 14 post-hatch (Madhu et al. 2006).

The role of fatty acids in larval clownfish nutrition has been examined (Blazer and Wolke 1983, Avella et al. 2007) and calanoid copepods have been shown to contain adequate fatty acid profiles for nourishing larval clownfish. The biochemical and molecular effects of a calanoid copepod diet on larval clownfish has also been examined and these effects have been shown to be significantly more beneficial than rotifer and *Artemia* diets (Olivotto et al. 2009). These studies along with the growth and survivorship

studies mentioned previously suggest that calanoid copepods are the most nutritious live food available. However, rotifer and *Artemia* are still commonly used due to the relative simplicity of culturing these organisms and that their nutritional profile can be enhanced to meet the requirements of many marine fish larvae. Captive reared *A. ocellaris* are traditionally fed rotifers at hatching followed by *Artemia sp.* nauplii at day 7 post-hatch. Following metamorphosis around day 14, they are transitioned to dry pellet or flake feed (Green and McCormick 2001).

Materials and Methods

Culturing protocols

Phytoplankton, copepods and fish larvae were maintained in seawater, provided constant aeration and kept under a 12:12 hour light:dark cycle. Seawater was obtained from a deepwater well operated by the Waikiki Aquarium and sterilized before use with an Ultraviolet Sterilizer (Lifegard Aquatics, Cerritos, CA; model QL-25). Salinity was maintained at 35 ppt with the use of a portable salinity refractometer (Extech, Torrence, CA, USA; RHS-10ATC). Aeration was provided by a Sweetwater® air pump (Aquatic Eco-systems Inc., Apopka, FL, USA; SL24) connected to silica air stones (4 x 1.3 cm, Aquatic Eco-systems Inc., Apopka, FL, USA; AS1) with Tygon® plastic tubing (0.3 cm inner diameter, Saint-Gobain Corporation, Aurora, OH, USA; T18) and Sweetwater® gang valves (Aquatic Eco-systems Inc., Apopka, FL, USA; VG5). Lighting was provided

with 20 watt fluorescent lights (T12/Daylight). Cultures were maintained at ambient room temperature which ranged from 21-26°C.

Isochrysis galbana

I. galbana was cultured in 3 L Erlenmeyer flasks filled to a volume of no more than 1.6 L. After approximately 2 weeks of growth, 150 ml of this "mature" culture was sieved through 20 µm Nitex® mesh (Nitex Corporation, Miami, FL, USA) and used to seed new cultures. Nutrient supplementation was provided by Pro Culture (Kent Marine, Franklin, WI, USA; catalog no. 415, 417) added at a ratio of 0.5 ml Pro Culture : 1 L algal culture.

I. galbana was maintained in *A. ocellaris* rearing containers at a density of $1x10^{3}$ cells ml^{-1}. A mature (i.e. started 2-4 weeks prior) *I. galbana* algal culture was selected and algal cell density was estimated via cell counts performed on 2-3 $1x10^{-4}$ ml aliquots. Larger 1 ml aliquots were initially drawn from the algal culture and $1x10^{-4}$ ml of each aliquot was placed underneath a coverslip on a Bright Line hemacytometer (Hausser Scientific, Horsham, PA, USA). Each $1x10^{-4}$ ml aliquot was viewed at 250x magnification with an inverted stereomicroscope (Olympus Corporation, Shinjuku, Tokyo, Japan; IM). The number of algal cells present in three 0.25 mm squares were counted and the mean of those counts was multiplied by 25 (the total number of squares present) to find the number of cells present in the $1x10^{-4}$ ml volume. If the density estimates of the two aliquots disagreed by more than 10%, a third aliquot was taken and densities were calculated from the mean of the three aliquots. The same protocol was

used to determine *I. galbana* concentration in the *A. ocellaris* rearing containers. From the two algal density assessments, the volume of *I. galbana* culture that would be needed to bring the algal density in the *A. ocellaris* rearing container to a density of $1x10^3$ cells ml^{-1} was calculated and the necessary volume added. Algal density assessments were made and algae added to the *A. ocellaris* rearing container daily after water changes.

Parvocalanus crassirostris

The female reproductive rate of the subtropical calanoid copepod, *Bestiolina similis,* which is closely related to *P. crassirostris*, has an inverse relationship with stocking densities and with the age of the culture (VanderLugt and Lenz 2008, VanderLugt et al. 2009). *P. crassirostris* behave in a very similar manner (personal observation), thus, dilution and algal feeding practices described by VanderLugt and Lenz (2008) allowed for culturing of *P. crassirostris* at high densities (up to 10 individuals ml^{-1}) in 21 L containers (29 x 27 x 37 cm, Rubbermaid, Sandy Springs, Georgia, USA).

In *A. ocellaris* rearing containers, a full range of *P. crassirostris* life-stages was provided. *P. crassirostris* densities in rearing containers were assessed by counting the total number of nauplii (NI-NVI) and copepodites (CI-CVI) present in 2-3 25 ml aliquot samples. The rearing container was gently stirred to ensure even distribution of *P. crassirostris* and then two 25 ml aliquots of water were removed from the container. The copepods present in the aliquots were concentrated into a smaller volume (~10 ml) by sieving the whole 25 ml volume through 35 µm mesh and rinsing the mesh with

approximately 10 ml of seawater. The individual nauplii and copepodites present in that volume were then counted with a stereomicroscope (Olympus Corporation, Shinjuku, Tokyo, Japan; SZX-ILLB2) at 50x magnification. Densities were calculated from the mean of the two aliquots. If the density estimates of the two aliquots disagreed by more than 10%, a third aliquot was taken and densities were calculated from the mean of the three aliquots.

After the assessment of *P. crassirostris* densities in the rearing container, one of the most visibly dense *P. crassirostris* cultures was selected and an assessment of the density of individual copepods in that culture was performed in exactly the same manner as described above for copepod densities in the *A. ocellaris* rearing container.

From these two density estimates, the volume of *P. crassirostris* culture that was required to obtain the necessary number of nauplii and copepodites to bring copepod densities in the *A. ocellaris* rearing container to 5 individuals ml^{-1} at a ratio of approximately 1:1 nauplii:copepodites was calculated. This volume was removed from the *P. crassirostris* culture, concentrated (sorted by sized with appropriate sieves as needed) and added to the rearing container.

P. crassirostris introduced to the filming containers, described in Feeding Experiments (pg. 23), were handled differently. Life-stages were restricted for each of the three prey-type groupings such that only the NI-NIII naupliar stages, CI-CIII copepodite stages and CVI final copepodite (i.e. adult) stage were presented. I will refer to these 3 life-stage groupings as “prey types” throughout. This method of life-stage grouping allowed for easy and accurate identification of prey types in the mixed prey assemblage feeding trials.

To accomplish this life-stage sorting of *P. crassirostris,* life-stage groupings were isolated from *P. crassirostris* culture populations based on size ranges of NI-NIII: 60-110 μm, CI-CIII: 201-292 μm, and CVI: 308-403 μm, as estimated from copepodite measurements made by McKinnon et al. (2003) and personal measurements of nauplii and adults (which agreed with McKinnon to within 5%).

To obtain these size ranges, *P. crassirostris* were removed from culture and sorted with the use of in-house built sieves constructed of 35-123 μm Nitex® mesh mounted in cylindrical PVC tubing with 8 cm diameter and 15 cm length. NI-NIII stages were obtained by filtering *P. crassirostris* from culture through a 35 μm sieve and gently rinsing the animals retained (i.e. all life-stages) from the sieve into a 500 ml beaker filled with approximately 25 ml of seawater (to concentrate the animals). The copepods in this seawater were then poured through a 100 μm sieve (through which only NI-NIII naupliar stages would pass) into a 250 ml beaker containing 25 ml of seawater. Those copepods retained by the 100 μm sieve (i.e. life-stages NIV and larger) were rinsed into a 250 ml beaker containing 25 ml of seawater so that the copepodite prey type (CI-CIII) and adult prey type (CVI) could be extracted. The density of NI-NIII life-stage nauplii in the 250 ml beaker were then estimated by counts of copepods present in 2-3 10 ml aliquots with the use of a stereomicroscope (Olympus Corporation, Shinjuku, Tokyo, Japan; SZX-ILLB2) at 50x magnification. This procedure was repeated for copepodites (CI-CIII) using first 200 μm and then 275 μm sieves and the procedure was repeated for adults (CVI) using first 323 μm and then 400 μm sieves.

The efficacy of this size-fractioning protocol was validated by counting the number of life-stages present in each of the three size fractioned groupings with a

stereomicroscope (Olympus Corporation, Shinjuku, Tokyo, Japan; SZX-ILLB2) at 50x magnification. Stages were determined based on morphological identification methods from Lawson and Grice (1972). Less than 10% of counted copepod stages were outside of the desired life-stage range.

After sieving, *P. crassirostris* densities were determined for each 250 ml beaker containing one of the three life-stage groupings and the volume of seawater containing *P. crassirostris* necessary to yield a concentration in the filming container of 1 individual ml^{-1} (for experiment I) and 0.33 individuals ml^{-1} (for experiment II) was calculated. This volume was then added to the observation container after the 15 minute acclimation period.

Size measurements were completed for nauplii and adult *P. crassirostris* (n=100) with the same Wild (model M5A) dissecting microscope and reticle using 100x magnification. Mean naupliar and adult lengths (85 and 360 µm respectively) agreed to within 5 and 4 µm of those previously reported (80 and 356 µm) by McKinnon et al. (2003). The mean length of copepodite stages (248 µm) was obtained directly from McKinnon et al. (2003).

Amphiprion ocellaris

Amphiprion ocellaris eggs were obtained from a commercial breeder. The eggs were affixed, by the mother, to the inside of a 15 cm diameter clay flower pot. The evening prior to hatching the eggs, the pot was transferred to a clear plastic container (21.5 x 21.5 x 23 cm, Cambro Co., Huntington Beach, CA, USA) containing 7 L of 35ppt

seawater and transported from the breeder's location to the Békésy laboratory. Aeration was provided such that a steady flow of small (<1 mm) air bubbles continuously passed over the eggs. The following morning, the clay pot with any un-hatched eggs was removed, leaving the newly hatched larvae (150-200 individuals), and a 50% water change was performed. *P. crassirostris* were added at a density of 5 individuals ml^{-1} and a ratio of approximately 1:1 nauplii:copepodite stages (obtained by sieving). This density was maintained for the duration of the study by twice daily copepod addition performed according to the density assessment and adjustment procedures described above.

Two 20 watt fluorescent bulbs, placed 25 cm above the *A. ocellaris* rearing container, provided 12:12hr light:dark lighting. The rearing container was kept in a water-bath maintained at 26°C with the use of a 25 watt Marineland water heater (Spectrum Brands Inc., Madison, Wisconsin, USA; ML90438). 50% water changes were performed daily during the first week after hatching and every other day during the second week. The bottom of the larval rearing container was cleaned of dead larvae and excrement 1-2 times daily by siphoning. *A. ocellaris* larvae were transferred to a clean rearing container on approximately every 5^{th} day via gentle scooping with either a 12 ml plastic ladle or a 100 ml glass beaker. At 5:00 pm each day, 6 larvae were removed from the rearing container for fixation.

Larval mortality was high during the first 2 days post-hatch (reaching 25-40% cumulative mortality by the 2^{nd} day post-hatch). Mortality was greatly reduced from day 3 post-hatch on and did not exceed 50% of the remaining fish by day 14 post-hatch. Total mortality observed by the commercial breeder providing the eggs, is commonly in the

50% range as are instances of high mortality during the first 2 days post-hatch (Karen Brittain, personal communication).

Total Length and Mouth Gape Measurements

A. ocellaris were transferred via pipette into a solution of 0.06 g/ml ethyl 3-aminobenzoate methanesulfonate salt (MS222) (Sigma-Aldrich Inc., Saint Louis, MO, USA; catalog no. A5040-25G). After 3 minutes, to allow for the anesthetic to have its effect, the 6 larvae were transferred to a 25 ml polystyrene tube (Fisher Scientific Co., Pittsburg, PA; catalog no.14-956-6D) containing a solution of 5% formalin in seawater. The tubes were then sealed and stored at room temperature. Both total length and mouth gape measurements were obtained for each larva within one week of fixation.

Total length, a metric commonly employed for clownfish, is defined as the greatest length of the whole body between the most anterior point of the body and the most posterior point, in a straight line (Frakes and Hoff 1982, Arvedlund et al. 2000). Standard length, another measure commonly used for adult fishes, is measured from the tip of the snout to the posterior end of the last vertebra or to the posterior end of the mid-lateral portion of the hypural plate. Standard length excludes the length of the caudal fin and thus is shorter measurement than total length. Total length measurements were obtained using a Wild (model M5A) dissecting microscope at 12x magnification. Total lengths of fish larvae were measured using a reticle calibrated to a 2.0 mm microscope micrometer.

Mouth gape measurements were obtained using the same Wild dissecting microscope and reticle using 100x magnification. Upper jaw lengths were measured from the anterior-most point of the premaxilla to the posterior edge of the maxilla. Lower jaw lengths were measured from anterior-most part of the mandible to it posterior edge. These measurements were used to calculate total mouth gape according to the formula:

$$S_G = \sqrt{(L_{UJ}{}^2 + L_{LJ}{}^2)}$$

presented in Shirota (1970) and later modified by Guma'a (1978). Where S_G= total mouth gape, L_{UJ}= length of the upper jaw and L_{LJ}= length of the lower jaw. The formula is based on the assumption that the angle at the articulation of the two jaws is 90° at maximum mouth gape. The lengths of the jaws can thus be understood to represent two sides of a right triangle with the hypotenuse represented by the mouth gape.

Feeding Experiments

Two separate feeding experiments were conducted: experiment I examined *A. ocellaris* capture success rates when feeding on copepod prey on 3 separate days of the fish's planktonic larval phase while experiment II examined predator selectivity and capture success across the full 2 weeks of the fish's planktonic larval phase. Key differences as well as features common to both experiments are shown in Table 1.

Experiment I assessed capture success rates for each of the three "prey types" (i.e. nauplii NI-NIII, copepodite CI-CIII and adult NVI) presented one at a time on days 1, 3

and 10 post-hatch. In this experiment the predators were given only one choice of prey type so that the influence of prey selectivity and any other interference factors inherent in a mixed prey assemblage was precluded and thus capture success rates reflect the ability of the predator to capture each prey type when no other choices of prey are present. Comparing the results of this experiment with those of the second experiment determines whether or not the absence of additional prey types affects capture success rates.

Experiment II tested predatory ability and prey selectivity by assessing capture success rates and the Manly-Chesson Index of selectivity when all three prey types were presented together at days 1 through 14 post-hatch. In this way, I was able to investigate the difference in capture success rates and prey selectivity for each prey type on each day post-hatch.

Table 1. Design and specifications of feeding experiments. Experiment I was designed to examine feeding success of *A. ocellaris* presented with single prey-type assemblages on days 1, 3 and 10 post-hatch. Experiment II was designed to examine feeding success and prey selectivity of *A. ocellaris* presented with mixed prey-type assemblages on days 1-14 post-hatch.

	Experiment I	**Experiment II**
Days Filmed (post-hatch)	1, 3 & 10	1 – 14
Prey assemblages	single	mixed
Quantitative Analyses	capture success	capture success prey selectivity
Prey Densities		
NI-NIII	1 ml^{-1}	
CI-CIII	1 ml^{-1}	
CVI	1 ml^{-1}	
NI-NIII+CI-CIII+CVI		0.33+0.33+0.33 ml^{-1}
# fish larvae	10	10
# of cohorts	2	5
# of 60min trials	29	72
time of day	10am-1pm	10am-4pm
Observation Vessel		
Volume	3 L	
Dimensions	18x18x10 cm	
field of view	2 cm x 2cm	
Filming conditions		
Light	1,900 lumens/m^2	
Frame-rate	30 fps	
Temp	23-25°C	
Salinity	35 ppt	

Experiment I: single prey type

This experiment was designed to investigate the limitations of *A. ocellaris* predatory ability when the larvae are allowed to feed only on specific copepod life-stages. On days 1, 3 and 10 post-hatch, 10 *A. ocellaris* larvae were removed from their rearing container via a 12 ml plastic ladle and placed into a transparent acrylic rectangular container (18 x 18 x 10 cm) filled with 3 L of GFC filtered seawater water at 27°C. The larvae were allowed to acclimate for a 15 minute period and then *P. crassirostris* were presented in single prey type assemblages (i.e. nauplii, copepodites or adults only) at densities of 1 individual ml^{-1}. Predator-prey interactions were filmed at 30 fps for a period of 60 minutes. Table 2 shows the number of 60 minute trials conducted. Two separate cohorts of *A. ocellaris* obtained from the same breeding pair were used for this experiment.

Table 2. Number of 60min trials conducted to obtain 100 attacks per prey type during Experiment I. Combined numbers from two cohorts. *low attack rate prevented observation of 100 attacks

Prey Type (developmental stage)	Larva Age (day post-hatch)		
	1	**3**	**10**
NI – NIII	3	3	2
CI – CIII	5	4	4
CVI	1*	5	3

Footage was reviewed, a total of 100 attacks with clearly determinable outcomes (taken in aggregate fro the two cohorts) were identified and outcomes were tabulated for each of the 3 prey-type assemblages at each of the 3 days post-hatch. Low attack rates for adult copepods prevented 100 recorded attacks from being obtained on days 1 and 3 post-hatch. A logistic regression analysis was performed on these data with attack outcome (successful/unsuccessful) as the dichotomous dependent variable and larval *A. ocellaris* age (days 1, 3 and 10 post-hatch) and prey type (naupliar, copepodite and adult stages) as independent variables. Capture success rates were obtained by dividing the number of successful attacks by the total number of attacks.

Experiment II: mixed prey type assemblages

This experiment examined the development of predatory ability and prey selectivity on each day of the first two weeks of *A. ocellaris* development. Daily records of predator behavior were kept. Capture success and prey selectivity were calculated in the presence of mixed prey type assemblages.

On days 1 through 14 post-hatch, 10 *A. ocellaris* larvae were transferred to the observation chamber as in experiment I. After a 15 minute acclimation period, *P. crassirostris* were presented in mixed prey type assemblages (i.e. nauplii, copepodites or adults in equal proportion) at aggregate densities of 1 individual ml^{-1}. The camera was turned on and left to record for a period of 1 hour. Predator-prey interactions were extracted post-hoc.

The predatory ability of *A. ocellaris* was assessed by capture success rate on each of the three prey types i.e. the number of attacks out of 100 observed on each prey type that resulted in successful prey capture vs. the number that resulted in failure to capture prey, as was done in experiment I. These sets of 100 attacks were obtained in agregate from 5 cohorts of reared *A. ocellaris.*

As footage was reviewed, 100 attacks with determinable outcomes were identified. Attack outcomes were tabulated for each prey type (i.e. the prey type that was the target of the attack) presented at each of the 14 days post-hatch, except where low attack rates prevented 100 recorded attacks from being obtained. A logistic regression analysis (Jansen and Stern 1998) was performed with attack outcome (successful/unsuccessful) as the dichotomous dependent variable and larval *A. ocellaris* age (days 1-14 post-hatch) and prey type (naupliar, copepodite and adult stages) as independent variables. The logistic regression calculates the probability of obtaining the observed results under the logistic model and the probability of obtaining the observed results in a model with no relationship between the independent and dependent variables. It is similar to a linear regression model but is suited to models where the dependent variable (attack outcome here) is dichotomous. Capture success rates were obtained by dividing the number of successful attacks by the total number of attacks.

To determine prey selectivity, the same footage used to determine capture success rates was reviewed again. However, the method of video analysis for the prey selectivity calculation differed from that of the capture success calculation in that the numbers of each prey type attacked were tabulated for a total of 100 attacks on all prey types as opposed to the number of successful vs. unsuccessful attacks per 100 attacks. The 100

attacks were randomly chosen from a subset of all attacks recorded by using a random number generator to select a starting point (to the minute) amongst all of the footage recorded for each day and then reviewing the attacks in continuous order to determine to which prey type the target of each attack belonged. A Pearson chi-square test was performed on these data to test the null hypothesis that attacks on each prey type occurred in proportion to the availability of each prey type in the environment, which was assumed to be 1/3 since prey types were presented in equal proportion.

Prey selectivity, defined here as a pattern of predator preference or avoidance for a prey type that cannot be explained by the probability of encountering that prey type in the environment, is quantified via the Manly-Chesson Selectivity Index. This index accounts for the dynamic probabilities of prey encounter over the course of experimental trials without prey replacement. Use of the index requires the assumption that encounters with prey which do not result in consumption do not affect the predator's subsequent behavior.

Index values were obtained according to the below formula, as presented in Chesson (1983):

$$\alpha_i = \frac{\ln((n_{i_0} - r_i)/n_{i_0})}{\sum_{j=1}^{m} \ln((n_{j_0} - r_j)/n_{j_0})}, i = 1,...,m$$

Where r_i = the number of prey individuals of type *i* attacked, n_{i0} = the number of prey individuals of type *i* present at the beginning of each 60 min trial, m = the total number of prey types present and *j* signifies prey category since each prey category is

added in series. The index represents the ratio of the log of the fraction of animals of a given type that is NOT attacked to the sum of the logs of ALL non-attacked fractions. If no attack preference exists, then, the estimator, α, for each type will be equal to 1/m where m=3 in my experimental design, the number of categories. In the extreme case of selectivity in which only one type is attacked, the non-attacked fractions for the remaining types will each be 1.0 (hence $\ln[(n_{i0}-r_i)/n_{i0}] = 0$), so $\alpha = 1$ ("high" selectivity) for the "preferred" type regardless of the fraction that is actually attacked. As the proportion of non-preferred types that receive attacks increases (their non-attacked fractions decreasing below 1.0), α for the preferred type decreases (and that of the non-preferred increases), even if the proportion of attacks on that type remains unchanged, such that the sum of the indices for all m of the types always equals 1.0.

Filming

A. ocellaris and *P. crassirostris* were recorded at 30 frames per second (fps) with a CCTV video camera (Panasonic Corporation, Kadoma, Osaka, Japan; model WV-BP310) equipped with a Nikkor 50 mm lens (Nikon Corporation, Shinjuku, Tokyo, Japan; model 1433). The camera lens was positioned 0.3 m from the observation container and the lens was focused in a plane in the center of the container such that the field of view was 4 cm^2. The container was lit evenly from above with one 20 watt fluorescent light providing 1,900 lumens of light that uniformly illuminated the filming container. Footage was recorded onto 60 minute high definition mini digital video

cassettes using a digital high definition videocassette recorder (Sony Corporation, Minato, Tokyo, Japan; model GV-HD700).

Filming trials lasted 60 minutes. Larval feeding rates were not sufficient to reduce prey density by more than 25% during the 60 minute period. After filming was completed each day, except during trials where larvae were fixed, the clownfish larvae were returned to a second rearing container designated for larvae that have undergone observation so as to avoid re-sampling.

Footage Analysis

The footage recorded was converted to digital audio video interleave format using Adobe Première CS3 (version 3.0) and transferred to a Freeagent™ 1000 gigabyte external hard drive (Seagate Technology LLC, Scotts Valley, CA, USA; 9NK2AM-510). During the review of footage, attacks were identified as a cessation of swimming followed by the bending of the body into a C-start position and then a rapid acceleration toward the prey item. The frequency of attacks was in the range of 20-125 attacks per 60 minute tape.

All attacks were examined frame-by-frame and categorized into successful, unsuccessful and unknown i.e. the larva captured the prey item, failed to capture the prey item or the outcome was unknown. The color inversion function in Adobe Premiere Pro CS3 was sometimes used to increase the contrast between the prey and background to aid in determining attack outcome. Attacks with unknown outcomes were not included in the final data analysis. An accurate determination of attack outcome was possible for greater

than 70% of attacks. The primary reasons for an attack resulting in an unknown outcome were poor orientation of predator and/or prey to video camera; predator and/or prey moving off screen; or predator and/or prey moving out of the plane of focus.

The identification of prey type was critical for the analysis of footage from experiment II, where mixed prey type assemblages were presented. Owing to the stage grouping technique used for prey presentation (see prey handling for filming below), the identification of prey type was usually a straightforward task. For less than approximately 15% of attacks resolution, clarity or contrast was insufficient to accurately determine prey type and these attacks were excluded from the final dataset.

Statistical Software

Statistical analyses were performed with the use of IBM SPSS version 19.0 (SPSS 2010).

Results

Predatory Behavior

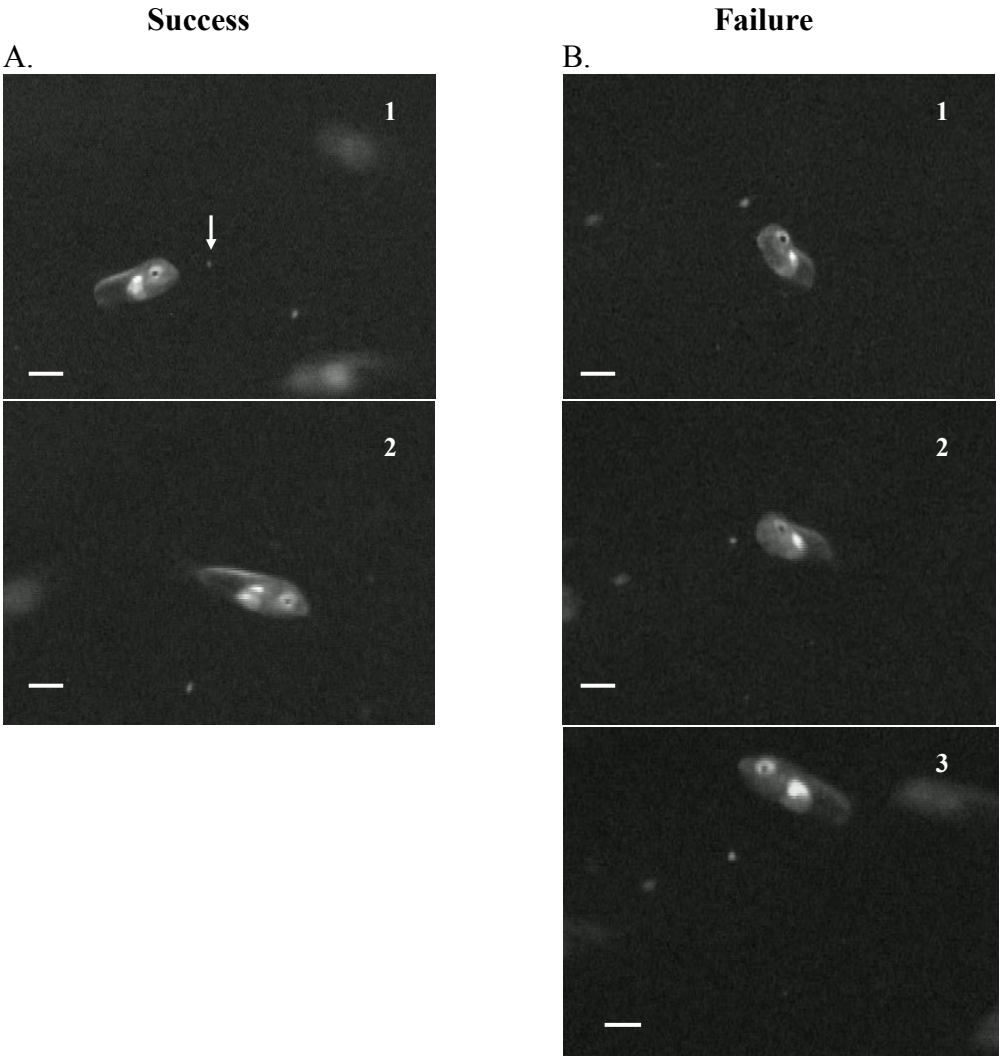
The predatory behavior of *A. ocellaris* larvae includes an initial search, followed by a stealthy approach and finally ending in an attack. While searching for prey, larvae swam around the container at a relatively constant speed. When a prey item was fixed upon the attacking larva stopped forward swimming and approached the prey very slowly

until an appropriate attack distance was reached. For example, mean attack distances for age 1-10 day post-hatch *A. ocellaris* preying on nauplii ranged from 0.8 mm to 1.04 mm. The larvae then adopted a C-start position followed by a rapid lunge toward the prey and simultaneous opening of the mouth. Successful attacks were characterized by no reaction from the prey while unsuccessful attacks were characterized by the prey initiating an escape response just prior to or at the moment of the predator's lunge. Typical attacks spanned a period of 1-3 seconds from the time a predator targeted a specific prey to the time of either capture or escape.

Filming

Footage recorded during feeding experiments allowed for clear identification and interpretation of attacks when *A. ocellaris* and *P. crassirostris* were centered on the plane of focus within the depth of focus, of the camera. *A. ocellaris* larvae were recorded with sharpness that allowed for the viewing of fin movements, body position and orientation to be distinguished with clarity. High contrast between the dark black background and copepods, which reflected white light, made individual prey items highly visible (Figure 1). In mixed prey assemblages, nauplii were clearly identifiable as white spherical objects against the black background. Early copepodites were easily distinguished from nauplii by their characteristic body outline and larger size. Adults were distinguished from copepodites by the prominence of their antennae, complete differentiation of prosome and urosome body segments, and larger size.

Figure 1 shows four sequences of larval *A. ocellaris* in early and late stages of development feeding on *P. crassirostris*. Successful attacks are shown where a larva targets a prey in the first frame and consumes the prey in later frames. Unsuccessful attacks are shown where a larva targets and attacks a prey but fails and the prey escapes.



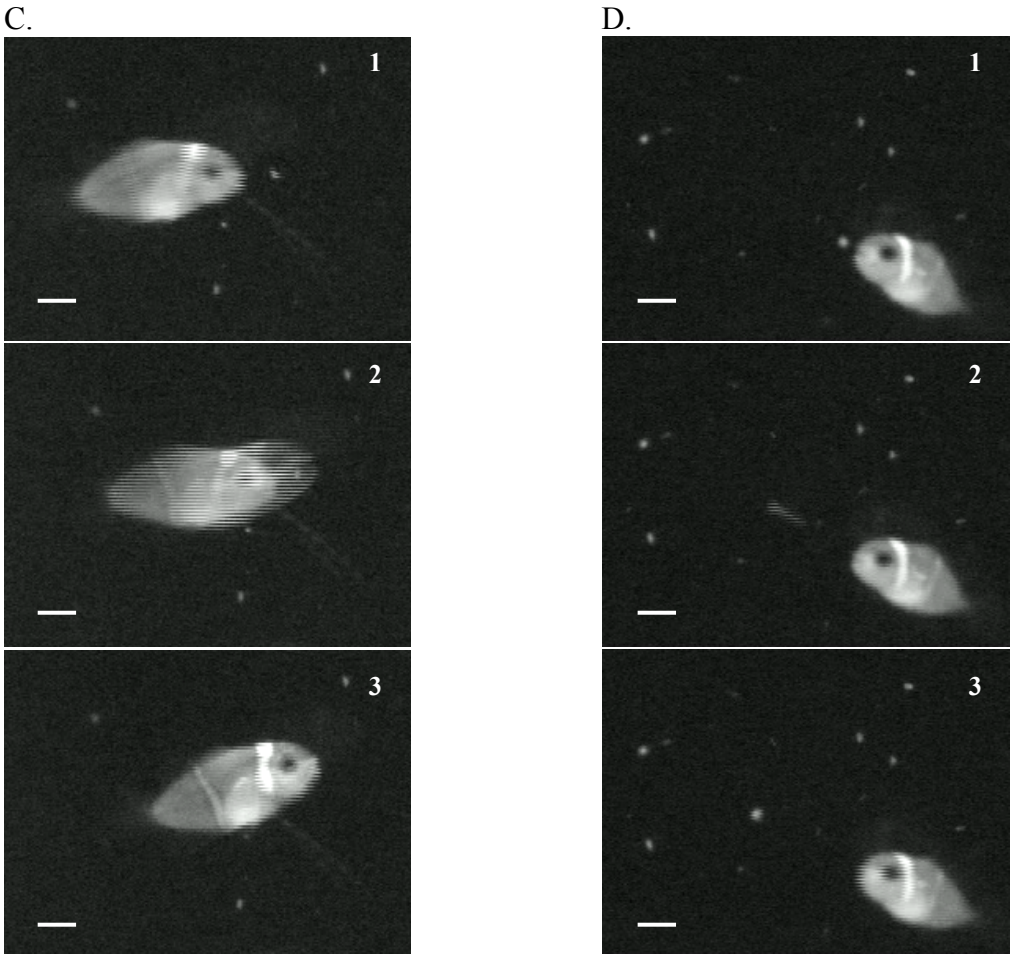


Figure 1. Successful and unsuccessful attacks by *A. ocellaris* larvae on *P. crassirostris* prey cropped from successive frames recorded at 30 *fps*. A. Day 3 post-hatch *A. ocellaris* captures *P. crassirostris* nauplius Arrow indicates location of nauplius. B. Day 3 post-hatch *A. ocellaris* fails to capture *P. crassirostris* copepodite. C. Day 14 post-hatch *A. ocellaris* captures *P. crassirostris* copepodite. D. Day 14 post-hatch *A. ocellaris* fails to capture *P. crassirostris* adult. Scale bars represent 1 mm. Numbers indicate frame order with 0.067 seconds between consecutive frames.

Growth

A. ocellaris growth during the first 2 weeks post-hatching is shown in Figure 2. The larvae almost double their size during this time, growing from 4.1 mm total length at day 1 post-hatch to 7.9 mm total length at day 14 post-hatch. Coefficients of variation for total lengths remained low (<5%) during the first week of development. Coefficients of variation were higher (5-10%) during the second week of development.

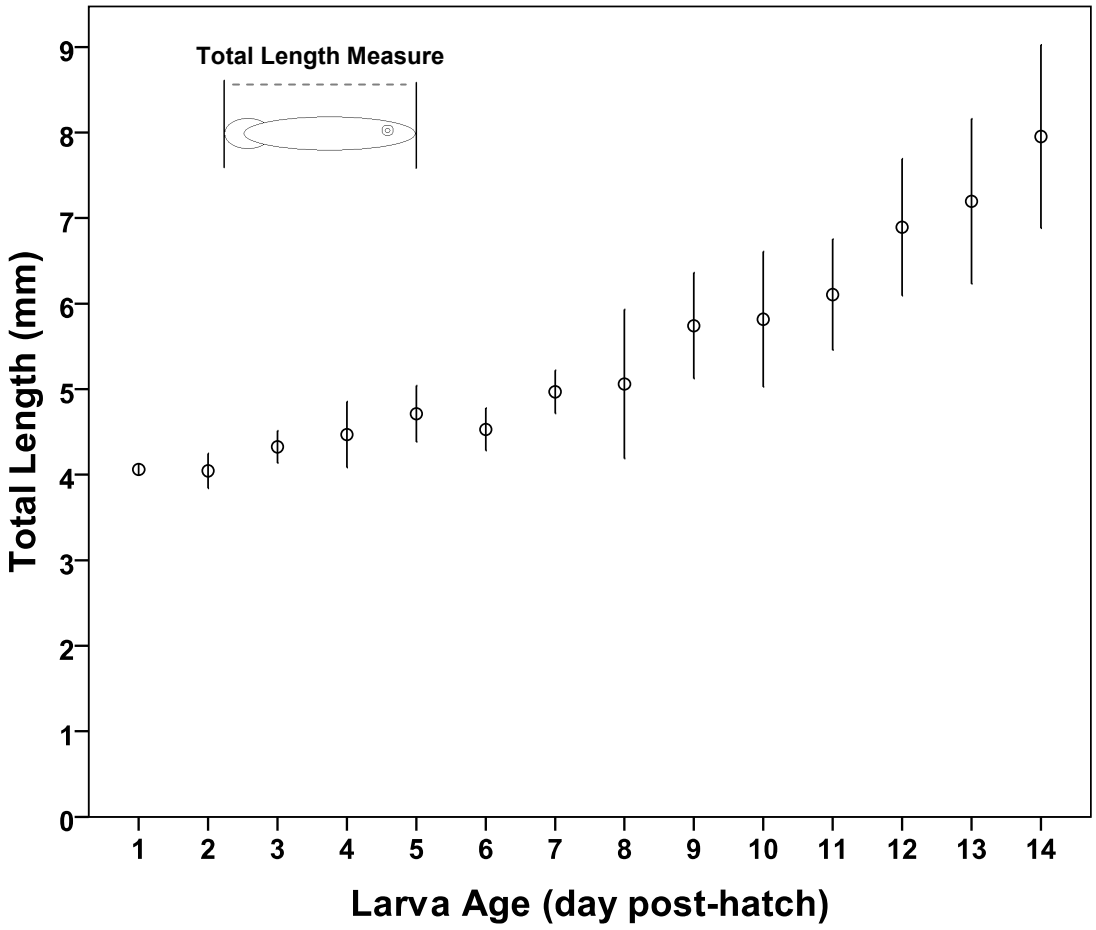


Figure 2. Mean *A. ocellaris* total length during the first two weeks of development. Measurements from 6 individuals were averaged for each age. Error bars indicate +/- 2 standard deviations. Schematic of larval fish shows total length measurement.

The planktonic larval phase of most fish is divided into three sub-stages: pre-flexion, flexion and post-flexion. Flexion is defined as the point in development when the posterior end of the vertebral column begins to bend upwards. This was observed *A. ocellaris* flexion at day 6 post-hatch, which agrees with Liew et al. (2010).

Mouth gape enlargement during the first 2 weeks of *A. ocellaris* development is presented in Figure 3. The mean mouth gape increased from 0.2 mm at day 1 post-hatch to 0.56 mm at day 14 post-hatch, which was a greater increase (proportionally) than the increase in length. Mouth gape measurements show greater variability, with a 10% mean coefficient of variation, compared to total length measurements with a 4.6% coefficient of variation. To show the relationship between predator gape size and prey size, the *P. crassirostris* size ranges are shown in this figure as well. Mean (± 1 S.D.) naupliar lengths (NI-NIII), copepodite prosome lengths (CI-CIII) and adult prosome lengths (C6) are represented by the horizontal gray bars superimposed on the plot of mouth gape data in Figure 3. By day 2, mean mouth gape exceeded adult copepod length.

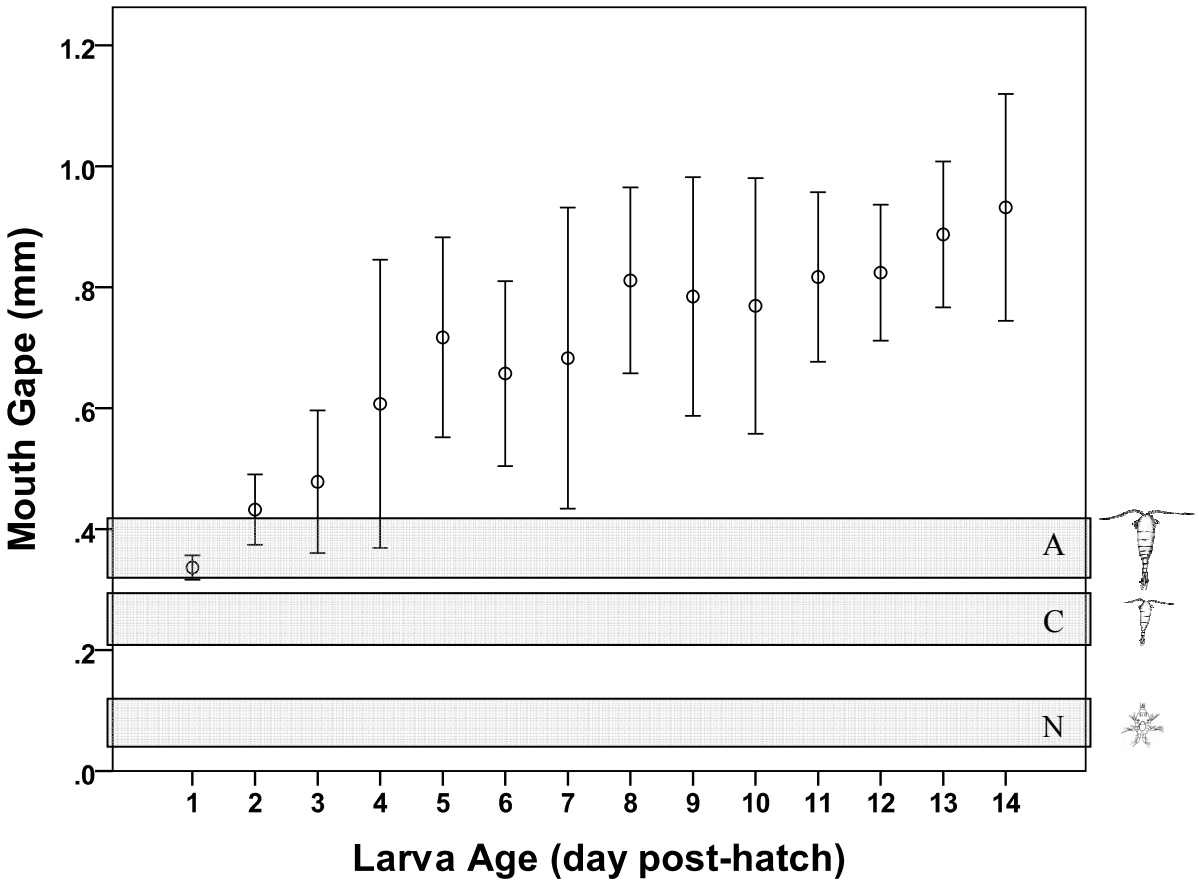


Figure 3. Mean *A. ocellaris* mouth gape as a function of age during the first two weeks of development. Mean gape size was calculated from measurements taken for 6 individuals for each age. Error bars indicate +/- 2 standard deviations. Gray bars show *P. crassirostris* prey size range; N: nauplii (NI-NIII), C: copepodites (CI-CIII) and A: adults (C6).

Mouth gape, plotted as a function of total length during the first two weeks of development, is shown in Figure 4. Mouth gape is less strongly correlated with total length as the larvae age, although this may be because smaller distances measured with the same equipment and methods allowed for less variation. The inflection point in the

scatter plot data occurs at days 7-8 post-hatch. During the first week of development, mouth gape increases in size more rapidly than total length. The inverse is true for week 2, where the total length increases at a more rapid rate than the mouth gape.

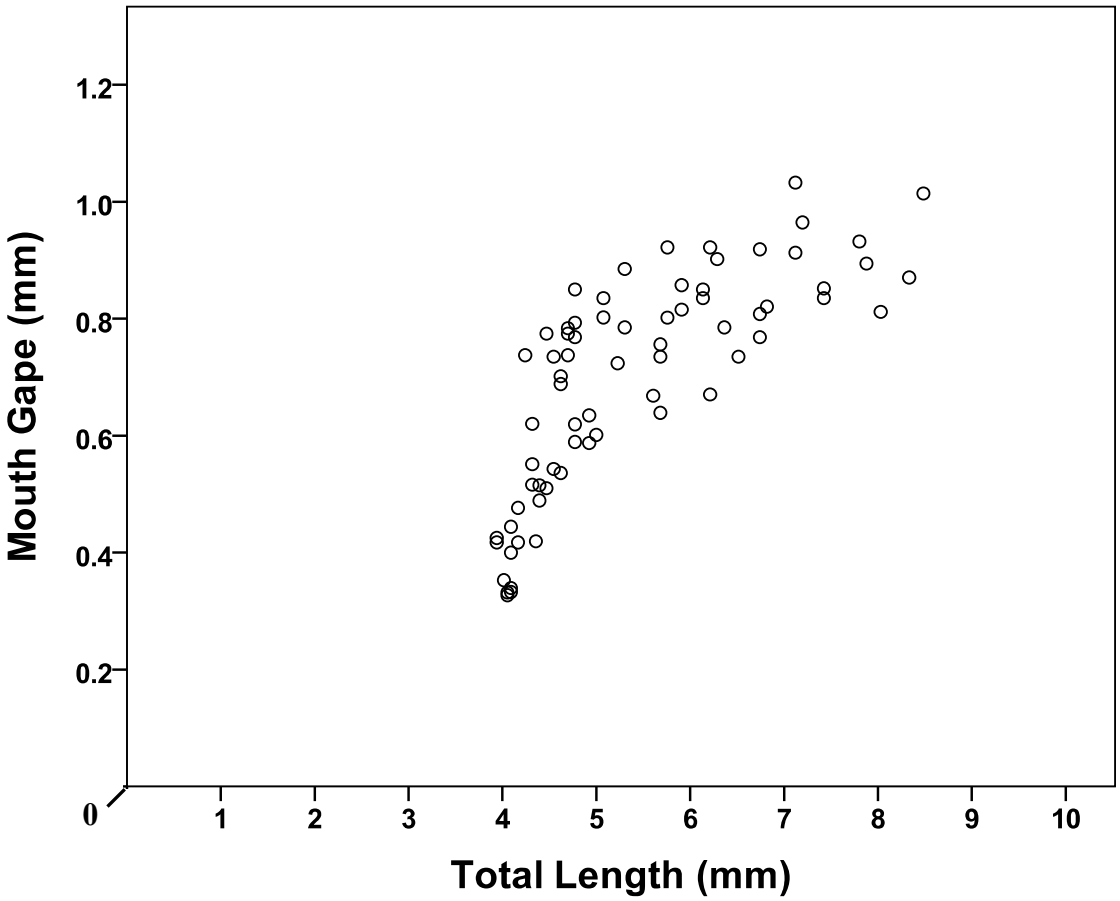


Figure 4. Scatterplot showing *A. ocellaris* mouth gape as a function of total length during the first two weeks of development.

Feeding Experiment I: Single Prey Type Assemblages

A. ocellaris prey capture success resulting from analysis of attacks recorded during experiment I are shown in Figure 5. Three prey types: nauplii, copepodites and

adults were presented separately (i.e. only one prey choice available) during days 1, 3 and 10 post-hatch.

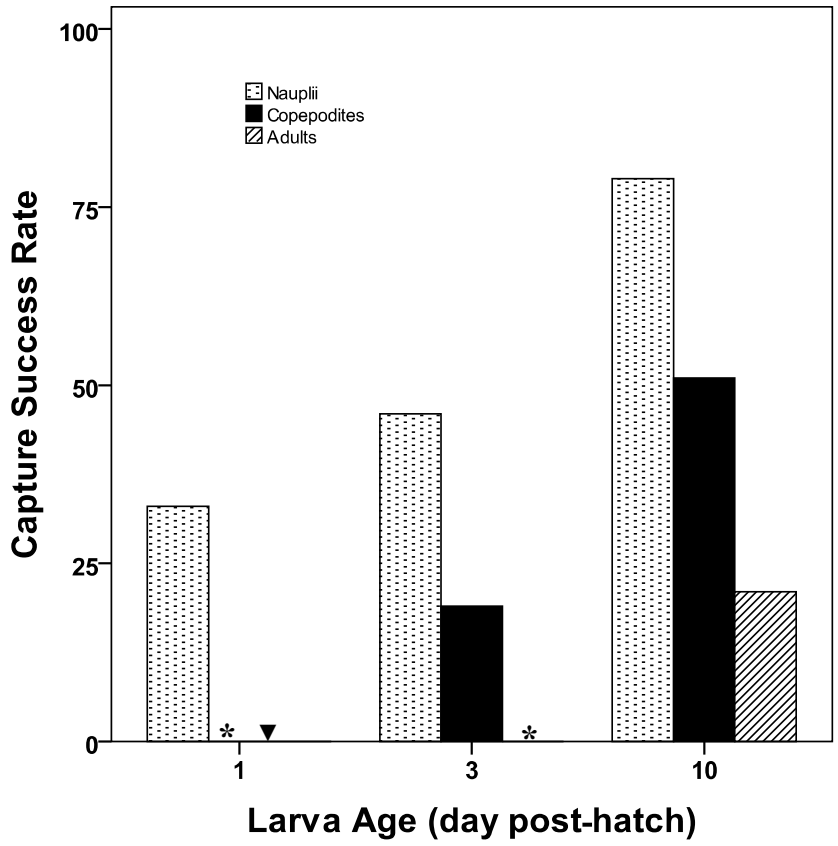


Figure 5. The effect of larval age (day post-hatch) on *A. ocellaris* prey capture success for naupliar, copepodite and adult *P. crassirostris* (presented in single prey-type assemblages) is shown for predation observed on days 1, 3 and 10 post-hatch. Within each age group, all non-zero capture success rates are significantly different between prey types ($p<0.05$).

*= 0% capture success, 100 observed attacks

▼= 0% capture success, although low attack rate resulted in <25 observed attacks

A logistic regression analysis was performed and both prey type and larva age were shown to have a significant effect ($p<0.05$) on attack outcome (see table 4). The effects of each prey type on the outcome of *A. ocellaris* attacks were also tested (see table 5). Attack outcome for naupliar and copepodite prey was significantly affected by the prey type attacked ($p<0.05$), while no significant effect was found for adult prey due to the absence of any successful attacks by larvae on adults for 2/3 of the days studied.

On day 1 post-hatch, *A. ocellaris* larvae were able to successfully capture nauplii with a 31% success rate but unable to capture copepodite or adult prey developmental stages. On day 3 post-hatch, nauplii were captured with a 46% success rate while larvae were able to capture copepodites with a 19% success rate. The encounters with adult copepods resulted in no successful captures on this day. On day 10 post-hatch, the larvae were able to capture all 3 prey types with capture success rates of 75%, 47% and 18% for nauplii, copepodites and adults respectively (see Table 3).

On all three days (1, 3 and 10 post-hatch) studied in this experiment, nauplii were attacked successfully with an increase in capture success across those days. Copepodites were also attacked on all 3 days with no success at day 1 post-hatch followed by successful attacks on days 3 and 10 post-hatch. Capture success for copepodites also increases from day 1 to 10 post-hatch. Due to the low rate of attack for adult prey on day 1 post-hatch it was not feasible to estimate capture success based on 100 attacks, but all attacks that were observed (<25) in these trials were unsuccessful. Adult copepods were attacked at a sufficient rate on days 3 and 10 post-hatch to resume capture success calculation from 100 attacks. This is an important result as it provides evidence that the youngest *A. ocellaris* larvae avoid targeting adult copepods.

Table 3. *A. ocellaris* capture success for three prey types presented in experiment I (in single prey-type assemblages) and experiment II (in mixed prey-type assemblages).

Larva Age (day post-hatch)	Nauplii		Copepodites		Adults	
	Exp I	Exp II	Exp I	Exp II	Exp I	Exp II
1	31%	33%	0%	0%	0%	0%
3	46%	44%	19%	22%	0%	0%
10	75%	79%	47%	51%	18%	21%

Feeding Experiment II: Mixed Prey Type Assemblages

Prey Selectivity

Figure 6 shows Manly-Chesson index values plotted for *A. ocellaris* presented with mixed assemblages of naupliar, copepodite and adult prey. Because there are 3 prey types present in equal proportion, a value close to 1/3 indicates that the predator is exhibiting no selective behavior. A value closer to 0 indicates that the predator is actively bypassing opportunities to attack that prey type while a value closer to 1 indicates that the predator is preferentially seeking out and acting on opportunities to attack that prey type. Thus, the index values showed prey selectivity as the degree that observed feeding behavior deviated from what one would have expected to see, based on the predator's probability of encountering each prey type, if the predator exercised no prey preference. As illustrated by Figure 6, larvae begin feeding preferentially on nauplii while adults are avoided. The strength of the selectivity for nauplii and against adults lessens continuously

until no preference is discernable by the end of the study. Copepodites are neither preferred nor avoided to a significant degree during the first two weeks of development.

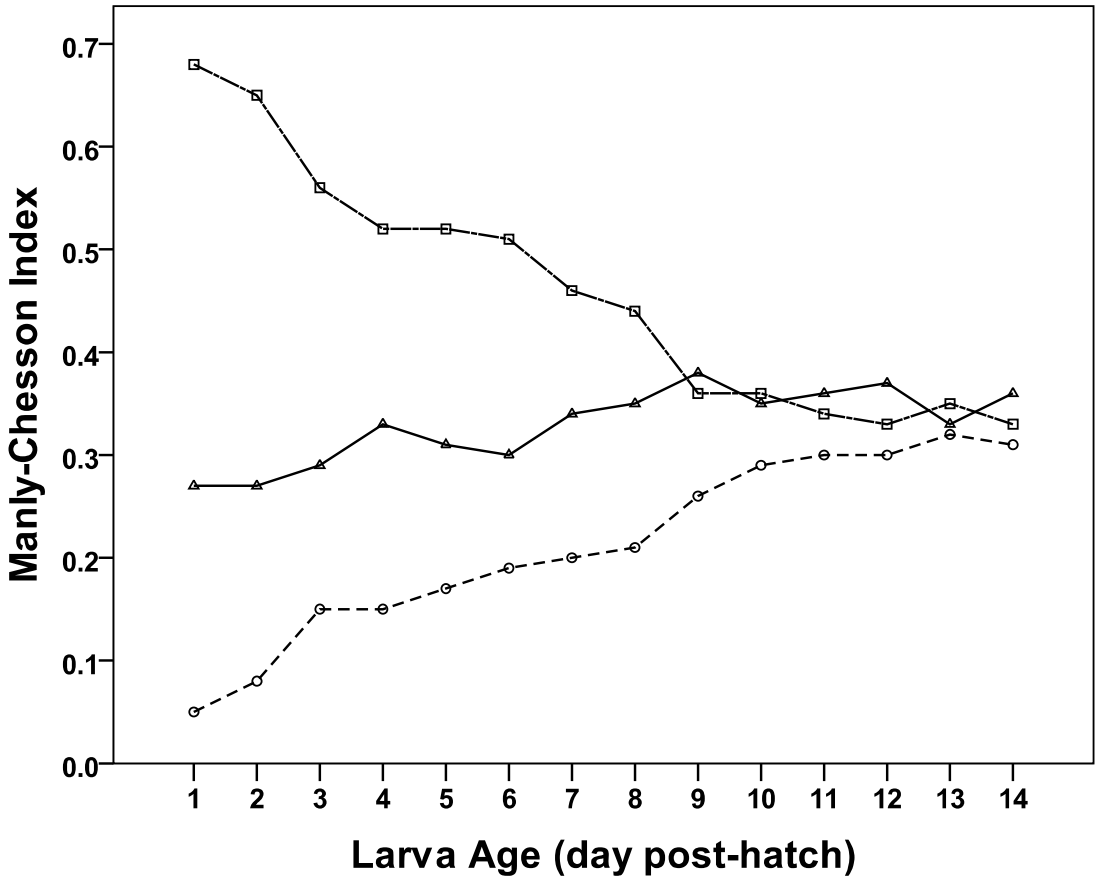


Figure 6. Manly-Chesson prey selectivity index calculated for fish larvae presented with mixed prey assemblages as a function of larval age. Prey types (at cumulative densities of 1 indiv. ml^{-1}): □ = Nauplii, Δ = Copepodites, ○ = Adults.

A Pearson chi-square analysis was also performed on these data to test the null hypothesis that attacks on each prey type occurred in proportion to the availability of each prey type in the environment. This showed that attacks on nauplii were observed at a

significantly different ($p<0.5$) proportion than expected for no selection from day 1 until day 8 post-hatch. On days 9-14 post-hatch, attacks on nauplii no longer showed selection. The observed proportion of attacks on adult copepods was significantly different ($p<0.05$) than that which would have been expected for no selection from day 1 until day 9 post-hatch. On days 10-14 post-hatch, adults were attacked with no selective preference.

Feeding Success

The effect of larval age on prey capture success rate is shown for each prey type in Figure 7. The larval ages at which *A. ocellaris* begin to successfully consume each of the three copepod life-stage groupings (i.e. prey types) are shown. *A. ocellaris* larvae successfully capture *P. crassirostris* nauplii at day 1 post-hatch, larvae begin capturing copepodites successfully at day 3 post-hatch and larvae begin capturing adults successfully at day 8 post-hatch. A trend of increasing attack success is clear for all three prey categories. The abrupt change in the trend of larval growth reported above coincides with the time point at which larvae begin to feed on adult copepods: day 8 post-hatch (see Figure 7). Also noteworthy is the abrupt change in capture success for copepodites between days 2 and 3 post-hatch (0 to 22%) compared to the gradual increase in capture success rates for adult copepods from days 7 to 10 post-hatch (0%, 4%, 9% and 19% respectively).

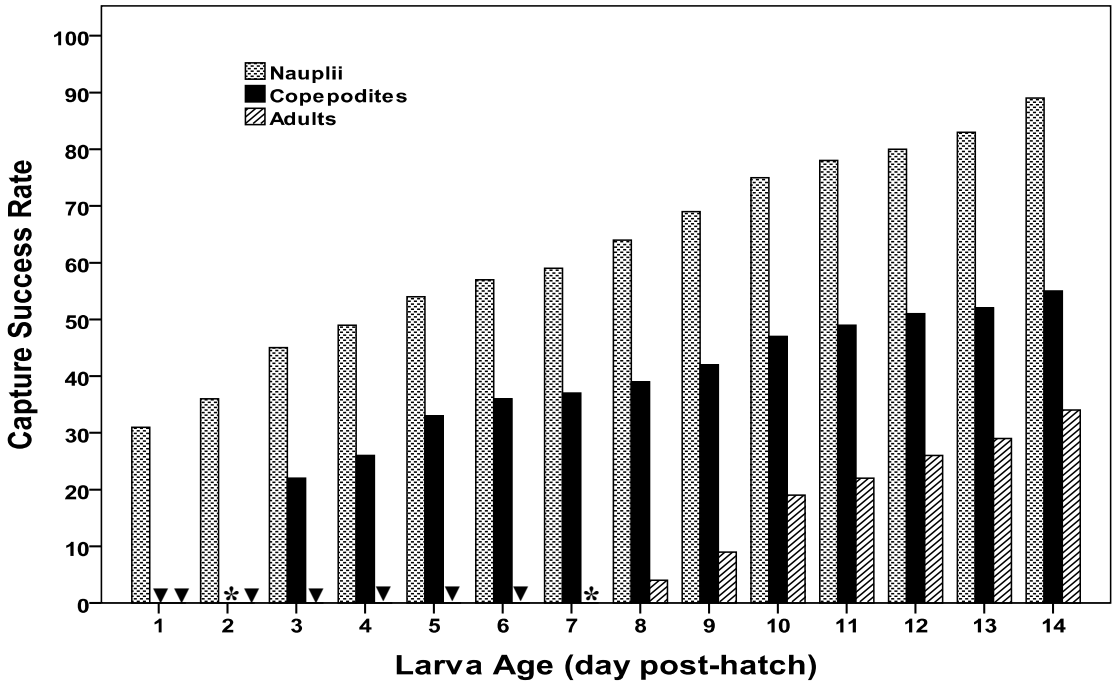


Figure 7. The effect of larval age (day post-hatch) on *A. ocellaris* prey capture success rate when presented with *P. crassirostris* naupliar, copepodite and adult life-stages in mixed assemblages is shown by the proportion of successful captures for each prey type presented during the first 14 days of larval development. N=100 attacks per prey type per day post-hatch.

*= 0% capture success for 100 observed attacks

▼= 0% capture success, although low attack rate resulted in <25 observed attacks

A logistic regression analysis of attack outcome in response to prey type and larval age was performed. This analysis tested the null hypothesis that that the type of prey presented and/or the age of the larvae would have no effect on the outcome of attacks on the prey by the larvae. The effects of both prey type and larval age on attack outcome were statistically significant ($p<0.05$) such that the null hypothesis can be

rejected (see Table 4). A logistic regression analysis of attack outcome in response to each prey type was also performed (see Table 5). The presentation of naupliar and copepodite prey types had a significant effect ($p<0.05$) on the outcome of attacks, while no significant effect was found for adult prey due to the absence of any successful attacks by larvae on adults for 1/2 of the days studied.

Table 6 shows the results of an additional logistic regression analysis that was performed for capture success data collected only from days 7-14 post-hatch. This was done to test the significance of differences in capture success rates once *A. ocellaris* began preying on adult *P. crassirostris* with sufficient attack rates to allow for feeding based on 100 attacks. This analysis showed significant effects ($p<0.05$) on attack outcome for all three prey types.

Table 4. Results of logistic regression performed for Experiment I and II to test the effect of larva age and prey type on attack outcome.

Experiment I	*df*	*p-value*
Attack Outcome by Larva Age	2	<0.005
Attack Outcome by Prey Type	2	<0.005
Experiment II	*df*	*p-value*
Attack Outcome by Larva Age	13	<0.005
Attack Outcome by Prey Type	2	<0.005

Table 5. The effect of each prey type on attack outcome during Experiments I and II. *denotes non-significant result due to zero capture success for multiple larval ages e.g. In experiment I: *A. ocellaris* presented with adult copepods demonstrated no successful captures on 2 of the 3 days examined.

Experiment I: days 1, 3 & 10 post-hatch

Attack Outcome by Prey Type	*df*	*p-value*
Nauplii	1	<0.005
Copepodites	1	<0.005
Adults	1	0.988*

Experiment II: days 1-14 post-hatch

Attack Outcome by Prey Type	*df*	*p-value*
Nauplii	1	<0.005
Copepodites	1	<0.005
Adults	1	0.997*

Table 6. The effect of prey types presented in experiment II on attack outcome for days 7-14 post-hatch. This shows that when larval ages with insufficient attack rates for adult copepods are excluded from the analysis, the effect of the adult prey type on attack outcome becomes significant.

Experiment II: days 7-14 post-hatch only

Attack Outcome by Prey Type	*df*	*p-value*
Nauplii	1	<0.005
Copepodites	1	<0.005
Adults	1	<0.005

Discussion

Prey selectivity, capture success and growth were monitored daily for the planktonic larval phase of *A. ocellaris* spanning 14 days post-hatch. During this time *A. ocellaris* larvae approximately doubled in size and demonstrated changes in predation ability and selectivity for three distinct groups of copepod life-stages. Larval mouth gape size was not a valid predictor for these changes. Larvae shifted from only being able to successfully consume nauplii to feeding on nauplii and copepodites and towards the end of their planktonic larval phase with the ability to consume all copepod life-stages.

Growth

Mean total length at day 1 post-hatch (4.1 mm) is in agreement with previous studies that report lengths in the 3-4mm range for day 1 post-hatch *A. ocellaris* larvae. Mean total length at day 14 post-hatch (7.9mm) is 2mm less than that of previously reported total length data for *A. ocellaris* of the same age (Frakes and Hoff 1982, Avella et al. 2007). The larvae in this study grew slower than those fed rotifers and *Artemia* in previous studies. The growth reported in this study is potentially more reflective of larval growth in the natural environment. However, a feeding experiment using rotifer & *Artemia* prey performed on the same *A. ocellaris* cohort measured would be necessary to isolate prey type as the only difference. The shape of the growth curve for the planktonic larval phase examined is also different from previous studies in which logarithmic growth was reported when clownfish were fed rotifers and *Artemia*. Considering prey densities

were high, this may reflect the difficulty of capturing copepod prey. In contrast, other studies that investigated clownfish growth fed the larvae non-evasive prey (Green and McCormick 2001, Olivotto et al. 2008a, Olivotto et al 2008b). In my data, a distinct inflection point between days 7 and 8 post-hatch is evident where growth shifts to a more rapid rate (see figure 4). This coincides with an important increase in predation competency; at this time the larvae gain the ability to capture and consume adult copepods. Taking *A. ocellaris* as a model for reef fish development, these results generally suggest a relationship between the size of a larval fish predator and its feeding success. These results also suggest that the type of prey on which a larval reef fish feeds may affect its growth rate during development.

Mouth Gape

When compared with the mouth gape measurements, the total length data showed much less variability. It is possible that the measurements obtained reflect true variability given that the morphology of the head and mouth changes greatly during the first two weeks of development and mouth gapes vary to a greater extent than total lengths within a cohort during this time. During the first week of development mouth gape also increases in size more rapidly than total length. This growth trend may reflect the importance of the larval fish's ability to consume larger and more calorically valuable prey over the importance of increasing body size during the first week of development.

The size of a fish's mouth gape is commonly used as a predictor for the maximum size of prey that may be consumed by that fish (Schmitt and Holbrook 1984, Bremigan

and Stein 1994, DeVries et al. 1998). Based on mouth gape measurements, *A. ocellaris* would be able to feed on adult *P. crassirostris* by day 2 post-hatch. However, the results of the mouth gape measurements and feeding experiments presented above demonstrate that mouth gape is not a useful predictor for feeding success in this situation. Because the fish larvae in this study were provided only highly evasive copepod prey, a given prey item's size relative to the larval predator's mouth gape was not the limiting factor for feeding success, as is often the case for non-evasive prey types (e.g. rotifers and *Artemia*). Instead, the sensory and evasive ability of the prey relative to the predator's ability to successfully capture prey appears to be the deciding factor for feeding success. As a model for larval reef fish development, these results suggest that the mouth gape of a larval reef fish may not be a useful predictor for feeding success when that larval fish is feeding on highly evasive prey such as copepods.

Feeding Selectivity

Predator feeding selectivity, as shown by the Manly-Chesson Index results, provides an overview of what prey type our model fish larvae prefer and which they avoid at each day during their planktonic larval phase. During the final 4 days of the study period, leading up to and including the time of metamorphosis, the larval fish exhibit no feeding selectivity on any copepod prey type. Metamorphosis marks the end of the planktonic larval phase, the time of settlement and the beginning of the juvenile stage. The lack of prey preference shown in the last days of the planktonic larval phase may not remain so. However, the current study did not address the question of prey selectivity

beyond the conclusion of the planktonic larval phase. Further research on *A. ocellaris* examined after metamorphosis will be required to determine prey preference beyond what was examined here.

An interesting qualitative observation was made during the final days of the study. When some of the most mature larvae performed an unsuccessful attack on naupliar prey, they then swam to the new location of the nauplius and performed a second attack. As described previously (on page 30), all unsuccessful attacks prior to these observations were not followed by a second attack on the same prey. This behavior was observed too infrequently to assess in a quantitative way. However, it is a noteworthy observation as the capacity to relocate escaped nauplii marks an improvement in predation ability.

The size, and presumably value, of individual copepod prey items should vary greatly between the three prey types provided in this study. Using methods presented in Mauchline (1998), *P. crassirostris* average dry weight was calculated from average body length measurements. Mean dry weights of 0.024 µg for nauplii (stages NI-NIII) 0.42 µg for copepodites (stages CI-CIII) and 1.13 µg for adults (stage CVI) reflect an approximately 1:17:47 nauplii:copepodite:adult weight ratio. This indicates that that copepodite and adult stages have far greater mass and thus likely provide far greater caloric value than naupliar stages. As discussed previously, *A. ocellaris* mouth gape should accommodate copepodites as early as day 1 post-hatch. Together this information would lead one to predict strong feeding selectivity for the copepodite stages over naupliar stages from hatching onward. However, this was not the observed behavior, which provides further evidence that the evasive abilities of the prey play a much more

important role than the mouth gape size of larval *A. ocellaris* or the caloric value of *P. crassirostris* life-stages. These results suggest that prey selection expressed by a larval reef fish may not be readily determined from estimates of abundance and caloric value. Larval reef fish prey selection may be greatly influenced by other factors, such as prey evasive ability.

Feeding Success

Results of experiment I (where larvae were presented with each prey type in single-type assemblages at three larval ages) showed that *A. ocellaris* exhibited significantly different capture success rates for the three prey types presented separately during days 1, 3 and 10 post-hatch. One of the key differences between the design of this experiment and experiment II (where larvae were presented with all three prey types in mixed assemblages daily) is that here the larval fish had access to each prey type separately, thus ruling out the influence of choice. On day 3 post-hatch larvae were able to capture copepodites in addition to nauplii and by day 10 they were able to capture all prey types, including adult copepods. Capture success rates between single prey-type assemblages presented in experiment I (Figure 5) and mixed prey type assemblages presented in experiment II (Figure 7) are similar and all zero values are consistent between the two experiments (see Table 3). One would not expect such a result if capture success rates for copepodite and adult stages were strongly influenced by predator preference. This comparison also demonstrates that the changes in capture success for

each prey type shown by experiment II are a result of the fish larvae's inability to capture those copepod life-stages and not a result of predator selection.

Results of experiment II showed capture success as it was examined for mixed prey-type assemblages daily during the planktonic larval phase. *A. ocellaris* larvae hatched with the ability to successfully capture *P. crassirostris* nauplii. Day 3 post-hatch is the point at which larvae begin consuming copepodites in addition to nauplii. This change in copepodite capture success (from 0% at day 2 post-hatch to 22% at day 3 post-hatch) appears abrupt relative to the more gradual increase in adult copepod capture success (4% at day 8 post-hatch, 9% at day 9 post-hatch and 19% at day 10 post-hatch). This suggests that the change in predatory ability that allowed for the capture of copepodite stages is of a more sudden onset, on day 3 post-hatch, than the more gradual improvement in predatory ability, which occurred during days 8, 9 and 10 post-hatch.

Interestingly day 3 post-hatch is the same age at which Coughlin (1994) reported that larvae of the closely related *Amphiprion perideraion* adopted the more efficient suction method of prey capture over the simpler method of ram feeding. It also coincides with the age at which Gordon and Hecht (2002) reported that the closely related *Amphiprion percula* yolk sacs were fully absorbed and stomachs were fully formed.

Taking the *A. ocellaris* larvae and their copepod prey as a model system, the results of the feeding experiments suggest that larval reef fish feeding success is likely to change rapidly and significantly during the planktonic larval phase, allowing for the capture of more evasive prey as the larval fish develop.

Prey Behavior

Maximum velocities of copepod escape responses increase as a power function of fish length (Lenz et al. 2004) and escape speed is certainly an important facet of copepod evasive ability. However, as discussed previously, sensory ability is crucial to the escape response. Bradley (2003) examined attributes of sensory ability for the escape response of *P. crassirostris* NI-NIII, NVI, CI and CVI life-stages stimulated by the electronically controlled vertical motion of a submerged sphere. As later stages of *P. crassirostris* were examined, an increase in reactive distances and a decrease in response latencies were recorded. The use of actual larval fish predators as opposed to an artificial stimulus, although far more difficult to control, provides a more realistic model. The qualitative observations of attacks made in the current study, where successful outcomes are always preceded by an absence of evasive action by the copepod, provide further evidence that sensory detection is the key to successful escape in *P. crassirostris*.

Although the earliest copepod life-stages (NI-NII) do not possess the same sensory and escape capabilities of later stages, they may have a slight advantage with respect to their movement pattern. As mentioned previously, zooplankton movement has been shown to be a key factor in prey identification by mature fish (Buskey et al. 1993). *P. crassirostris* NI-NII stages are non-feeding and very immobile (Bradley 2009). It is possible that such inactivity would help guard against predation. When nauplii begin feeding (NIII) they become significantly more active (Bradley 2009) and thus may be easier for larval fish predators to locate. This would become a more important

consideration when prey densities are lower than those densities used in this study (e.g. in the field). However, the current study was not able to investigate this.

Ecological Considerations

The planktonic larval phase is commonly broken into three sub-stages: pre-flexion, flexion and post-flexion. This study spanned all three sub-stages for *A. ocellaris*: pre-flexion (hatching - day 5 post-hatch), flexion (day 6 post-hatch) and post-flexion (days 7-14 post-hatch). In contrast, the critical time at which larval fish shift from consuming only nauplii to consuming nauplii and copepodite prey (day 3 post-hatch) is missed by field studies that examine larvae primarily in the post-flexion stage (e.g. Pepin et al. 2003, Sampey et al. 2007).

Clownfish brood their eggs, which are relatively large, and such parental investment allows the larvae a "head start" with respect to size and motor capabilities compared to other fish, especially temperate and pelagic species, which broadcast spawn relatively small eggs that produce smaller and less developed larvae (see Table 7.) Although larval *A. ocellaris* differ in significant ways from these other species, their size and morphological differentiation is similar to those data reported for the planktonic larval phases of other reef fishes (Liew 1983, McCormick and Molony 1992, Morinière et al. 2003). However, this statement is not intended to downplay the diversity in larval reef fish growth and development (Hamner et al. 1988, Carassou et al. 2009, Shima and Swearer 2010). Although *A. ocellaris* has proven very valuable as a model larval reef fish

predator, future studies of other captive-reared reef fish species would further expand the limited scope of knowledge for larval reef fish feeding behavior.

Table 7. Reproductive characteristics of captive-reared marine fish species. * denotes non-spherical eggs

Species	**Egg Diameter (mm)**	**Parental Investment**	**Mean Total Length at Hatching (mm)**	**Source**
Pacific Threadfin *Polydactylus sexfilis*	0.8	Broadcast Spawning	2.75	Santerre and May 1977
Atlantic Cod *Gadus morhua*	1.4	Broadcast Spawning	4.6	Marteinsdottir and Steinarsson 1998
European Seabass *Dicentrarchus labrax*	1.3	Broadcast Spawning	4.45	Kennedy and Fitzmaurice 1968
Atlantic Salmon *Salmo salar*	6.2	Brooding	16	Christiansen and Torrissen 1997
Nile Tilapia *Oreochromis niloticus*	2.4	Brooding	4.5	Campos-Mendoza et al. 2004
False Percula Clownfish *Amphiprion ocellaris*	2.0 x 1.0*	Brooding	3.8	personal measurements
Flame Angelfish *Centropyge loriculus*	6.9	Brooding	15.6	Callan 2007

Future Directions

The change in size and morphology during larval *A. ocellaris* development likely has significant effects on the hydrodynamic signal produced. This cannot be addressed by my data, but future studies could address this with the use of Holographic Particle Image Velocimetry (HPIV) techniques (Malkiel et al. 2003, Sheng et al. 2003).

The data suggest that learning may also be playing a significant role in the larval fish behavioral changes observed. Learning in the context of animal behavior has been defined by Hinde (1970) as a change in an animal's capacity for behavior as a result of experience excluding the effects of fatigue, sensory adaptation or maturation of the nervous system. However the data presented in this study are not sufficient to answer this question. To address the issue of learning, further experiments would need to be specifically designed to manipulate the experience of different groups of larval fish (e.g. groups naïve to evasive prey vs. groups experienced with evasive prey) and then to compare feeding behaviors (e.g. test capture success rates of the two groups in the presence of evasive prey).

Conclusions

This study provides novel additions to the body of knowledge of larval reef fish feeding behavior. The results demonstrate the complexity of larval reef fish predatory ability and prey preference during the critical planktonic larval phase. Any model of the relationships between larval fish and their zooplankton prey that underestimates the

importance of behavior (e.g. by assuming feeding rates are proportional to encounter rates) will be missing a key factor that will likely greatly limit predictive value.

In the past, the use of model larval reef fish predators and natural prey has been hindered by the difficulties associated with the culturing natural prey species along with the captive rearing of reef fish. The success I was able to achieve in maintaining healthy and highly reproductive cultures of *P. crassirostris* while rearing healthy *A. ocellaris* allowed for the study of their interactions under controlled conditions. For the consistent observation of normal feeding and evasion behavior, larval fish and copepods must be healthy and unstressed.

Future experiments employing a larval reef fish predator and zooplankton prey model system are needed to further advance our understanding of larval fish life history strategies and food webs in tropical environments. Such studies should be designed to further examine the progress of predatory abilities, and the causal factors behind that progress, for reef fish larvae presented with different zooplankton prey types. Rearing of healthy animals was critical for the success of this study. For the planning of further research efforts, I recommend that this challenge be addressed first.

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