Determining live prey preferences of larval ornamental marine fish utilizing fluorescent microspheres

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Title: Determining live prey preferences of larval ornamental marine fish utilizing fluorescent microspheres

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Abstract:

As the popularity of marine aquaria grows, the potential exists for increased harvest of marine ornamental fishes and invertebrates from the oceans around the world. Aquaculture may be an alternative source to wild capture fisheries if commercial production protocols can be developed for species of interest, however numerous impediments must be overcome. Experiments were conducted on the reef butterflyfish (*Chaetodon sedentarius*), Pacific blue tang (*Paracanthurus hepatus*), African moony (*Monodactylus seb*ae) and golden trevally (*Gnathanodon speciosus*) to evaluate and define prey preferences at first feeding among the rotifer, *Brachionus plicatiilis*, the copepod, *Parvocalanus crassirostris*, and the ciliate *Euplotes* sp., by marking each prey with a different colored fluorescent microsphere. Prey preferences observed at the larval first feeding stage were species specific. Pacific blue tang larvae preferred rotifers above ciliates, and ciliates above copepod nauplii. African moony larvae preferred ciliates and nauplii equally over rotifers. Reef butterflyfish larvae preferred ciliates over rotifers and rotifers over nauplii. Golden trevally larvae preferred nauplii over ciliates, and ciliates over rotifers. Take together these data provide critical information which may aid in development of feeding protocols for marine ornamental species.

Keywords: Fluorescent microspheres, ciliates, copepods, rotifers, ornamental fish larvae, marine

Highlights:

"Three different colors of fluorescent microspheres were fed to the rotifer (*Brachionus plicatilis*), nauplii of the copepod (*Parvocalanus crassirostris*), and ciliate (*Euplotes* sp.), and were used to mark prey presence in the GI tract of larval Pacific blue tang (*Paracanthurus hepatus*), reef butterflyfish (*Chaetodon sedentarius*), African moony (*Monodactylus sebae*), and golden trevally (*Gnathanodon speciosus*)…. [Pacific blue tang] larvae preferred consuming rotifers over ciliates and ciliates over nauplii…[African moony] larvae preferred nauplii and ciliates over rotifers… [Reef butterflyfish] larvae preferred consuming ciliates over rotifers, and rotifers over nauplii… Golden trevally [larvae] preferred consuming nauplii over ciliates, and ciliates over rotifers.

1. Introduction

Successful first feeding of larval marine fishes represents a major bottleneck in aquaculture that must be investigated. Survival through this stage is affected by mouth gape, nutritional requirements, capture ability and countless other factors. The current industry standard methods used to feed marine fish larvae consists of a diet of enriched rotifers, *Brachionus* spp., followed by *Artemia* nauplii. Commercial marine ornamental hatcheries also utilize this methodology when feasible. When not feasible, those species are fed small marine copepod nauplii or various combinations of live feed organisms (Holt, 2003; Mckinnon et al., 2003; Leu et al., 2013).

1.1 Gut Contents in Wild Fish Larvae

Insights into the gastrointestinal (GI) tract contents of wild larvae may have applications to aquaculture. Provision of natural forage items in aquaculture may not always be possible, especially if the organism can't be easily cultured, like appendicularians (Troedsson et al., 2013). Efforts to discern the gut contents of wild larvae have been made, and the resulting data shows the variety of prey organisms consumed. Investigations into larval gut contents can be readily utilized to identify and quantify organisms that leave behind an exoskeleton, such as copepods, pteropods, or appendicularians (Llopiz and Cowen, 2009). Other studies suggest that protozoans play a much larger role in larval diets. De Figuieredo et al. (2007) reported a variety of ciliates and other protists in the guts of larval fish from the Irish Sea that sustained larval fish growth in the absence of metazoans. Copepods have been found in gut content of many species of larval fish (Sampey et al. 2007; Llopiz and Cowen, 2009). Estimates of digestion rates have been used to determine the number of prey items ingested over time, but the accuracy is unknown (Ohman, 1991; De Figuieredo et al., 2007). Higher rates of digestion of protozoans suggest that these

species may have a significantly larger role in the first feeding of larval fishes, than previously thought.

1.2 Rotifers

Rotifers (*Brachionus* spp.) are the current standard for the first feeding of marine fishes, as they can be easily produced at high densities up to 1000 ind. mL^{-1} and are easily captured by larvae. While these factors make utilizing rotifers attractive, there are many fish species which cannot be successfully cultured with only rotifers. Rotifers must also be enriched in order to provide a significant source of essential fatty acids including eicosapentaenoic acid, decosahexaenoic acid, and arachidonic acid to larval fish (Rainuzzo et al., 1997). *Brachionus plicatilis* and *B. rotundiformis* make up the majority of rotifer species currently used in commercial marine aquaculture. Some larval fish species with small mouth gapes may not be able to physically consume rotifers because they are too large, the movement pattern may not trigger a feeding response, and or the nutritional content or composition may be insufficient. Thus, copepods and ciliates should be further evaluated as alternative first feeds.

1.3 Copepods

Copepods are not common produced at most commercial marine hatcheries, and only a few fish hatcheries worldwide produce copepods on a mass scale. Recent research demonstrated that mass scale production of the copepod *Parvocalanus crassirostris* is possible (Kline and Laidley, 2015). Copepod nauplii can be quite small (<100 μm) and can easily fit into the mouths of larvae whose mouth gape prevents them from consuming rotifers. However, in contrast to rotifers, they can have stronger predator evasion and avoidance responses which can make them more difficult for larvae to detect and prey upon (Titelman and Kioerboe, 2003; Buskey et al., 1993).

1.4 Ciliates

Ciliates are another prey organism for first feeding larvae with aquaculture potential and include *Fabrea salina*, *Euplotes* spp., *Strombidium* spp., and tintinnids (Nagano et al., 2000a & 2000b; Pandey and Yeragi, 2004; Guermazi et al., 2008; Cortes et al., 2012; Baensch, 2014). *Euplotes* spp. are commonly found as contaminants in rotifer, copepod, and algal cultures (Hagiwara et al., 2001; Cheng et al., 2004, Drillet and Dutz, 2014). However, *Euplotes* spp. have also been explored as a potential live feed (Leu et al., 2015; Cortes et al., 2012; Olivotto et al., 2005). *Euplotes* spp. are disc shaped and have sizes ranging from 20 x 30 μm to 135 x 100 μm (Cortes et al., 2012; Leu et al., 2015; Nagano et al., 2000a; Olivotto et al., 2005; Chen et al., 2013). Intensive production is an unknown obstacle that must be investigated with ciliates other than *Euplotes* spp. While many species can be collected in estuaries or bays during blooms, maintaining cultures can prove very challenging (Montagnes, pers. comm.). *Euplotes* spp. can reach densities of 15,000 mL^{-1} (Cortes et al., 2012) while other species such as *Fabrea salina* may only reach 100 mL^{-1} (Rhodes and Phelps, 2008).

Euplotes spp. can be cultured at high densities up to 15,000 mL^{-1} if fed yeast (Cortes et al., 2013). *Euplotes* spp. have been shown to grow best when they consume bacteria. Microalgae such as *Tetraselmis tetrathele* can sustain a population albeit at densities several magnitudes lower (Cheng et al., 2004; Cortes et al., 2012). High tolerance to poor water quality, especially high ammonia and low dissolved oxygen levels, makes *Euplotes* spp. a suitable candidate for high density production (Xu et al., 2014; Freitas da Annunciação, 2015 pers. comm.). *Euplotes* spp. have been shown to be consumed by first feeding larvae such as *P. hepatus* (Nagano et al., 2000a) and the convict grouper, *Epinephelus septemfasciatus* (Nagano et al., 2000b). It has also been used in experiments with cleaner goby, *Gobiosomo evelynae* (Olivotto et al., 2005), barber

goby, *Elacatinus figaro* (Cortes et Tsuzuki, 2012), and bluestriped angelfish, *Chaetodonotoplus septentrionalis* (Leu et al., 2015), with varied results. Unenriched *Euplotes* spp. have been shown to be very low in DHA relative to copepod nauplii and enriched rotifers, but had similar levels of EPA. Enriched *Euplotes* spp. have lower levels of DHA than enriched rotifers on a percent dry weight basis. *Euplotes* spp. have slightly more than half of the relative protein content of copepod nauplii (*Bestiolina* sp.) by percent wet weight (Kraul, 2006). Ciliates such as *Euplotes* spp. are less nutritious by percent dry weight and even more so if calculated on an individual basis.

1.5 Fluorescent Microspheres

Polystyrene microspheres, with diameters between 0.01 - 30 μm, are currently manufactured for a variety of marking purposes including biomedical and nanoparticle studies (Jiang et al., 2017a; Jiang et al., 2017b; Mao et al., 2017; Xu et al., 2017; Zhang et al., 2015). Microspheres have also been used for studies investigating the transition of microplastics in planktonic food webs, and to investigate grazing rates of zooplankton on phytoplankton (Agasild et al., 2005; Saba et al., 2011; Cole et al., 2013; Setala et al., 2014) or larvae on zooplankton (Nagano et al., 2000a). Indiscriminate grazers such as some species of rotifers, ciliates and cladocerans will consume microspheres of different sizes at different rates (Nagano et al., 2000a; Agasild et al., 2005; Dolan and Coats, 1991). Incubation rates for microspheres between rotifers, ciliates and copepods differ (Table 1). Copepods have a strong preference for algal exudate coated spheres over non-coated spheres regardless of size, and were also able to distinguish between spheres coated with different species of microalgae (Kerfoot and Kirk, 1991; Agasild et al., 2005). Stage I copepod nauplii (*P. crassirostris*) lack mouth parts and thus are incapable of ingesting any microspheres (Kline and Laidley, 2015).

1.6 Prey Preference

Altricial marine ornamental fish larvae typically have high mortality during the first feeding stage. To improve survival through this stage, it is critical to understand larval prey preference in the presence of more than one prey type. Rotifers, copepod nauplii, and ciliates all have different body sizes, movement profiles, and predator evasion responses (Buskey et al., 1993). How these factors will affect the number and type of prey items consumed by larval fish must be determined. Fluorescent microspheres were utilized for this experiment to mark ciliates, which otherwise would go undetected in the larval GI tract, and would be very difficult to differentiate in the presence of rotifers and copepod nauplii.

To investigate prey preference at the onset feeding, three different colors of fluorescent microspheres were fed to the rotifer (*Brachionus plicatilis*), nauplii of the copepod (*Parvocalanus crassirostris*), and ciliate (*Euplotes* sp.), and were used to mark prey presence in the GI tract of the Pacific blue tang (*Paracanthurus hepatus*), reef butterflyfish (*Chaetodon sedentarius*), African moony (*Monodactylus sebae*), and golden trevally (*Gnathanodon speciosus*).

2. Methods

2.1 Broodstock Acquisition and Management

Adult Pacific blue tangs (*P. hepatus*) were obtained from 2014-2015 from multiple sources including public and private aquaria, and wild collection to establish a population of ten sexually mature individuals. They were stocked into a white 1900 L circular tank equipped with a biofilter, bag filter and a UV sterilizer (Pentair Aquatic Eco-Systems Inc., Cary, NC, USA). Eggs were collected in April 2016. Reef butterflyfish (*C. sedentarius*) were collected as a mated pair in Marathon, FL in May 2015. Reef butterflyfish were stocked into a similar system as the

Pacific blue tangs. Eggs were collected in June 2016. Twenty-five *M. sebae* broodstock have been maintained for ~8 years in a 2500 L (1.8 x 2.4 x 0.5 m^3) rectangular polyethylene tank equipped with a UV sterilizer (Pentair Aquatic Eco-Systems Inc., Cary, NC, USA) and a biofilter. Eggs were collected in June 2016. Golden trevally (*G. speciosus*) eggs were collected from a local private hatchery. Broodstock were maintained in a 6000 L system with flow through natural seawater. Eggs were collected in July 2016.

2.2 Larval Culture System

The larval culture system consisted of twenty-eight 14 L fiberglass tanks (28 cm D x 25 cm H) with black walls and white bottoms. Two air stones placed on each side of the tank provided gentle aeration (<0.1 L min^{-1}). Lighting consisted of two 40 watt fluorescent bulbs (1,250 lux at surface) on a 12 L : 12 D cycle. Sterilized natural seawater (35 g/L salinity) was used for all experiments.

2.3 Live Feeds Culture

Semi-continuous cultures of *P. crassirostris* copepod nauplii were produced using a similar collection method described in detail by Kline and Laidley (2015). Copepods were cultured in 35 g L^{-1} seawater which was diluted with unfiltered tap water to 30 g L^{-1} in 1100 – 2300 L round tanks with (152 cm D x 48 – 74 cm H) on a 14 L:10 D cycle. *P. crassirostris* was fed a 75 : 25 ratio of *T-Isochrysis* (T-iso) and *Chaetoceros gracilis* cultured in 19 L carboys with 30 g L^{-1} sterilized natural seawater, with CO_2 supplementation and 24 L:0 D photoperiod. Nauplii were collected from the tanks via a drain with a 120 μm 316 stainless steel mesh screen (TWP Inc., Berkeley, CA, USA) into a collection tank where they were concentrated in a 38 μm nitex screen. Water from the collection tank was pumped back into the production tank using a 500 gph magnetic drive pump (Danner Manufacturing Inc., Islandia, NY, USA). Copepod

nauplii were sieved through a 75 μm screen prior to the experiment and collected in a 38 μm screen.

Semi-continuous cultures of *B. plicatilis* rotifers were cultured in 200 L cylindrical and conical tanks with heavy aeration (Broach et al., 2015). They were maintained on a 14 L:10 D cycle under ambient room lighting and fed *Nannochloropsis oculata* paste (Nannopaste, Reed Mariculture Inc., Campbell, CA, USA) once a day. Rotifers were sieved through a 100 μm screen and collected with a 40 μm screen. They were then rinsed with sterilized natural seawater prior to incubation in microspheres.

Euplotes sp. were cultured with constant aeration and a 24 L:0 D cycle in ambient lighting in 33 g L^{-1} natural seawater in batch cultures. Stock cultures were maintained in covered six or twelve well plates containing 1.5-3 mL of sterile natural seawater or culture chambers containing 60-300 mL of sterile natural seawater and were not provided any feed or aeration. Production cultures were in 20 L buckets and were fed yeast (Lesaffre Yeast Corp., Milwaukee, WI, USA) at 0.5 g million ind^{-1}. Ciliates were collected with an 11 (Dynamic Aqua-Supply Ltd., Surrey, BC, CAN) or 15 μm nitex screen (Sea-gear Corp., Melbourne, FL, USA). They were rinsed three times with sterile natural seawater to remove uneaten yeast and detritus prior to microsphere incubation.

2.4 Ciliate Dispersion in Water Column

Euplotes spp. are capable of planktonic swimming but will congregate on surfaces to feed on bacteria and detritus (Lawrence and Snyder, 1998). To determine the percentage of ciliates swimming in the water column in clearwater, three density treatments, 10 ciliates mL^{-1}, 20 ciliates mL^{-1}, and 30 ciliates mL^{-1} were added to 14 L tanks, with four replicates per treatment. Water column samples were taken with a 9.5 mm diameter tube from the water surface to the

tank bottom were taken every 15 minutes over one hour and were counted for ciliate density. Ciliate prevalence in the water column remained relatively stable over time for each density (Table 1).

2.5 Microsphere Larval Feeding Experiments and Fluorescent Microscopy

The methods described were utilized for all four fish species unless specifically noted. Eggs were collected 12-16 hpf, rinsed, and stocked into twenty-eight 14 L tanks filled with sterilized seawater filtered through a 1 μm felt filter bag. Water quality was recorded (temperature, salinity, DO, pH) using a YSI 556 Multimeter (YSI Inc., Yellow Springs, OH, USA) and samples were collected the morning of the experiment for total ammonia-nitrogen and nitrite-nitrogen determination using standard methods (HACH, Loveland, CO, USA). Tanks with high mortalities were excluded from the study. All tanks were maintained in static conditions. Aeration was moderate to vigorous prior to the day of first feeding.

Yellow-green (505 nm excitation/515 nmemission) (ciliates), blue (365/415) (rotifers), and red (580/605) (copepod nauplii) fluorescent microspheres (1 μm) were used (FluoSpheres® Carboxylate-Modified Microspheres, Life Technologies Corporation, Carlsbad, CA, USA). Micropheres were stored in a refrigerator at 4°C prior to use. Ciliates and rotifers were incubated at sphere densities described in Table 2. For copepod nauplii, microspheres were mixed for 24 h with T-iso microalgae prior to feeding. All live feeds were then incubated for 1 h prior to the start of the larval feeding experiment.

Tanks were fed on staggered five minute intervals to allow time for larval collection at precisely the same duration of feeding. Live feed samples (200 μL) were collected every 20 min from the concentrated live feeds containers pitchers during the feeding of experiments, and fixed in 10% phosphate buffered formalin to examine incubation success. Rotifers were rinsed three

times with fresh seawater prior to the initiation of the experiment because excessive contact with air resulted in fluctuating numbers of floating rotifers. Ciliates and nauplii did not experience this issue and were left in with microspheres and rinsed three times with fresh seawater immediately prior to feeding each tank every five min. Four treatments were compared: nauplii and ciliates, rotifers and ciliates, rotifers and nauplii, and all three prey types together.

After 4-6 h, 20 larvae from each tank were euthanized with 100 mg L^{-1} MS-222, rinsed with fresh seawater and then fixed in 10% phosphate buffered formalin. For the reef butterflyfish experiment, between 5 and 20 larvae were collected from each tank depending on survival. Larval samples were examined microscopically using an inverted fluorescent scope with DAPI (350/460 nm), MB-1 (470/515 nm), and TRITC (540/605 nm) filters (VWR International Inc., Radnor, PA, USA). Incidence was determined by detection of any microsphere present, regardless of density. Quantification of prey items per larvae was conducted by utilizing microsphere patterns inside live feed organisms found in preliminary investigations. Rotifers had a circular pattern (Fig. 4 A, B) or a few smaller clusters (Fig. 3 A; Fig. 4 D). Ciliates had small clusters of spheres (Fig. 3 C). Nauplii had a large cluster in the head and or in the digestive tract (Fig. 3 B) and sometimes this would appear as a solid line (Fig. 4 D). Photos were taken with an EVOS FL imaging system using DAPI (357/447 nm), GFP (470/510 nm), and RFP (531/593 nm) filters (Thermo Fisher Scientific Inc., Waltham, MA, USA). For each experiment, the proportions of larvae with each prey type and the numbers of each prey items ingested per larvae were recorded.

2.5.1 Pacific blue tang prey preference of first feeding larvae 3 dph after 6 h of feeding

Eggs were stocked at 10 L^{-1}. Treatments (n=6-7) were: 1) Rotifers 5 mL^{-1}, Nauplii 5 mL^{-1}; 2) Rotifers 5 mL^{-1}, Ciliates 15 mL^{-1}; 3) Nauplii 5 mL^{-1}, Ciliates 15 mL^{-1} 4) Rotifers 5 mL^{-1}, Nauplii 5 mL^{-1}, Ciliates 15 mL^{-1}.

2.5.2 African moony prey preference of first feeding larvae 3 dph after 6 h of feeding

Eggs were stocked at 10 L^{-1}. Treatments (n=7) were 1) Rotifers 5 mL^{-1}, Nauplii 5 mL^{-1}; 2) Rotifers 5 mL^{-1}, Ciliates 15 mL^{-1}; 3) Nauplii 5 mL^{-1}, Ciliates 15 mL^{-1} 4) Rotifers 5 mL^{-1}, Nauplii 5 mL^{-1}, Ciliates 15 mL^{-1}. Larvae were squashed with glass coverslips prior to fluorescent microscopy as they could not be examined in welled plates due to black pigmentation surrounding the digestive tract.

2.5.3 Reef butterflyfish prey preference of first feeding larvae 4 dph after 6 h of feeding

Eggs were stocked at 5 L-1. Treatments (n=3-4) were 1) Rotifers 5 mL^{-1}, Nauplii 5 mL^{-1}; 2) Rotifers 5 mL^{-1}, Ciliates 15 mL^{-1}; 3) Nauplii 5 mL^{-1}, Ciliates 15 mL^{-1} 4) Rotifers 5 mL^{-1}, Nauplii 5 mL^{-1}, Ciliates 15 mL^{-1}.

2.5.4 Golden trevally prey preference of larvae 3 dph after 4 h of feeding

Eggs were stocked at 10 L^{-1}. Treatments (n=5-6) were 1) Rotifers 3 mL^{-1}, Nauplii 3 mL^{-1}; 2) Rotifers 3 mL^{-1}, Ciliates 9 mL^{-1}; 3) Nauplii 3 mL^{-1}, Ciliates 9 mL^{-1} 4) Rotifers 3 mL^{-1}, Nauplii 3 mL^{-1}, Ciliates 9 mL^{-1}.

2.6 Statistical analysis

The proportion of larvae that ingested each prey type and the number of prey items ingested by each larvae were standardized by the proportion of each prey item that consumed fluorescent microspheres (Table 3).

Prey items ingested were normalized using the following equation:

$$\frac{\#Prey\ Items\ Ingested}{\%Fluorscence}$$

The proportion of larvae that ingested each prey type was calculated by adding normalized values obtained from prey combinations.

$$Proportion\ Larvae\ that\ ingested\ R = \frac{\#R + \#(R+N) + \#(R+Ci) + \#(R+N+Ci)}{Total\ larvae\ collected\ per\ tank}$$

The statistical program SAS was used to perform logistic regressions on all normalized proportion data. Larvae with a prey item in the gut were coded as 1 (Full) where as larvae lacking the presence of a prey item were coded as 0. Binary output of the variable Full was run against categorical variables Treatment and Organism type for prey proportion. If Treatment and Organism interactions were not significant, a logistic regression with the binary output Full was run against organism types, for each treatment. Preference across all treatments for prey organism was determined using post-hoc Tukey's test.

Binary output Full was run against continuous variables Quantity of Ciliates, Quantity of Rotifers, and or Quantity of Nauplii for quantity of prey proportion ingested. If Treatment and Organism interactions were not significant, a logistic regression utilizing the poisson distribution of output Full was run against quantity of prey organisms ingested for each treatment. Significance was determined by likelihood ratio P-values, and 95% confidence interval of odds ratio estimates were used for pair-wise comparisons if significance was found. Odds ratio estimates that did not contain a value of 1.00 within a 95% confidence interval were considered significant. Likelihood ratio statistics were utilized to determine significance between and among treatments, organisms, and interactions between treatments and organisms.

3. Results

3.1.1 Pacific blue tang prey preference of first feeding larvae 3 dph after 6 h exposure

Larvae were significantly more likely to have ingested a prey item when given rotifers and ciliates compared to rotifers and nauplii but no significant differences were observed among other treatments ($F_{3,50}$=3.64, P=0.0187) (Table 4). Larvae preferred consuming rotifers over ciliates and ciliates over nauplii ($F_{2,50}$=114.77, P<0.0001). When fed only rotifers and ciliates, the proportion of larvae feeding were 4.184 (95% CL: 2.481-7.042) times more likely to ingest a rotifer than a ciliate ($F_{1,10}$=37.32, P<0.0001) (Fig. 4 A). When fed rotifers and nauplii, the proportion of larvae feeding were 20 (95% CL: 10.10-40.00) times more likely to ingest a rotifer over a nauplii ($F_{1,12}$=91.57, P<0.0001). When fed all three prey types, larvae were 3.57 (95% CL: 2.247-5.682) times more likely to consume a rotifer than a ciliate and 17 (95% CL: 7.634-38.462) times more likely to a consume a rotifer than a nauplii ($F_{2,15}$=38.63, P<0.0001). There were no significant differences when larvae were fed nauplii and ciliates ($F_{1,10}$=2.97, P=0.1155).

When fed with nauplii and ciliates, larvae ingested significantly more ciliates ($F_{1,238}$=31.47, P<0.0001). When fed rotifers and ciliates, larvae ingested significantly more rotifers than ciliates ($F_{1,238}$=35.68, P<0.0001) (Fig. 4 B). When fed rotifers and nauplii, larvae ingested significantly more rotifers ($F_{1,278}$=146.46, P<0.0001). When fed all three feed types, larvae ingested significantly more rotifers than nauplii, and more ciliates than nauplii ($F_{2,357}$=33.62, P<0.0001).

3.1.2 African moony prey preference of first feeding larvae 3 dph after 6 h exposure

African moony larvae were significantly more likely to have ingested food items when fed nauplii and ciliates when compared to the treatment containing all food types, but no other significant differences were observed among other treatments ($F_{3,57}$=4.31, P=0.0083) (Table 4). Larvae preferred nauplii and ciliates over rotifers ($F_{2,57}$=73.72, P<0.0001). When larvae were fed ciliates and rotifers, larvae preferred to ingest a ciliate 1.899 (95% CL: 1.241-2.904) times more

than a rotifer ($F_{1,12}$=10.81, P<0.0065) (Fig. 5 A). When fed nauplii and rotifers, larvae preferred to ingest a nauplii 9.297 (95% CL: 5.050-17.116) times more than a rotifer ($F_{1,12}$=63.36, P<0.0001). When fed all three prey types, larvae ingested a ciliate 21.982 (95% CL: 8.939-54.056) times more than a rotifer and a nauplii 20.181 (95% CL: 8.195-49.698) times more than a rotifer ($F_{2,18}$=26.44, P<0.0001). There were no significant differences when fed with ciliates and nauplii ($F_{1,12}$=1.62, P=0.2275).

When larvae were fed nauplii and ciliates, they ingested significantly more nauplii than ciliates ($F_{1,270}$=296.55, P<0.0001) (Fig. 5 B). When larvae were fed rotifers and ciliates, they ingested significantly more ciliates than rotifers ($F_{1,278}$=51.53, P<0.0001). When fed rotifers and nauplii, larvae ingested significantly more nauplii than rotifers ($F_{1,278}$=261.26, P<0.0001). When fed all three prey types, larvae ingested significantly more nauplii than ciliates and ciliates than rotifers ($F_{2,411}$=69.36, P<0.0001).

3.1.3 Reef butterflyfish prey preference of first feeding larvae 4 dph after 6 h exposure

Larvae were significantly more likely to ingest a food item when fed ciliates and nauplii than any other treatment ($F_{3,23}$=12.66, P<0.0001) (Table 4). Larvae preferred consuming ciliates over rotifers, and rotifers over nauplii ($F_{2,26}$=104.23, P<0.0001). When fed ciliates and nauplii, larvae were 25.375 (95% CL: 10.397-61.928) times more likely to ingest a ciliate than a nauplii ($F_{1,4}$=101.27, P=0.0005). Larvae fed with ciliates and rotifers were 1.954 (95% CL: 1.070-3.566) times more likely to ingest a ciliate than a rotifer ($F_{1,26}$=7.42, P=0.0345). When fed all three prey types, larvae were 27.760 (95% CL: 10.328-74.613) times more likely to ingest a ciliate over a rotifer ($F_{2,9}$=52.74, P<0.0001). There were no significant differences when larvae were fed nauplii and rotifers ($F_{1,4}$=2.52, P=0.1878) (Fig. 6 A).

When fed nauplii and ciliates, reef butterflyfish larvae consumed significantly more ciliates than nauplii ($F_{1,108}$=105.02, P<0.0001). When fed rotifers and ciliates, larvae ingested significantly more ciliates than rotifers ($F_{1,80}$=91.48, P<0.0001). When fed all three prey types, larvae ingested significantly more ciliates than nauplii or ($F_{1,122}$=73.46, P<0.0001). There were no significant differences in ingestion rate when fed rotifers and nauplii ($F_{1,91}$=1.92, P=0.1689) (Fig. 6 B).

3.1.4 Golden trevally prey preference of first feeding larvae 3 dph after 4 h exposure

Based on the feeding regimes tested, larvae were significantly less likely to have ingested a prey item when given rotifers and ciliates compared to other treatments ($F_{3,44}$=7.18, P=0.0005) (Table 4). Golden trevally preferred consuming nauplii over ciliates, and ciliates over rotifers ($F_{2,44}$=28.98, P<0.0001). When fed copepod nauplii and ciliates, golden trevally larvae were 3.15 (95% CL: 1.46-6.80) times more likely to ingest a copepod nauplii than a ciliate ($F_{1,10}$=11.3, P=0.0075) (Fig. 7 A). When fed nauplii and rotifers, larvae were 8.196 (95% CL: 2.67-25.14) times more likely to ingest a copepod nauplii than a rotifer ($F_{1,8}$=18.74, P=0.0025). When fed the choice of all three food types, larvae were 4.205 (95% CL: 1.428-12.382) times more likely to ingest a ciliate over a rotifer and 8.771 (95% CL: 3.138-24.517) times more likely to ingest a copepod nauplii than a rotifer ($F_{2,15}$=11.49, P=0.0009). No significant differences were detected when larvae were fed rotifers and ciliates ($F_{1,8}$=0.85, P=0.3824).

Larvae consumed significantly more nauplii when fed nauplii and ciliates ($F_{1,238}$=95.06, P<0.0001). When fed rotifers and ciliates, larvae ingested significantly more ciliates than rotifers ($F_{1,196}$=7.13, P=0.0082). When fed rotifers and nauplii, larvae ingested significantly more nauplii ($F_{1,198}$=99.15, P<0.0001). When fed all three prey types, larvae ingested significantly more nauplii than ciliates, and significantly more ciliates than rotifers ($F_{2,357}$=71.87, P<0.0001).

4. Discussion

4.1 Microsphere Larval Feeding Experiments and Fluorescent Microscopy

This study presents a new understanding of prey preference of ornamental marine fish larvae by utilizing fluorescent microspheres. Fluorescent microspheres have been employed in marine ecology research to examine the transfer of microplastics throughout planktonic food webs (Setala et al., 2014; Cole et al., 2013; Agasild et al., 2005). Investigation into the feeding of larvae with microsphere-labeled naked ciliates in fish larvae has been previously performed (Nagano et al., 2000a; Nagano et al., 2000b). However, fluorescent microspheres have not been utilized to examine consumption of other live feeds such as rotifers and copepods by fish larvae. To the authors' knowledge this study represents the first use of microspheres in conjunction with live food organisms [the rotifer (*B. plicatilis*) and copepod (*P. crassirostris*)] for aquaculture research.

Ecological studies reported difficulties when examining ingestion rates of microspheres by copepods as they show high taste discrimination. However, mixing microspheres with microalgae has been documented to eliminate this issue (Kerfoot and Kirk, 1991; Agasild et al., 2005). Rotifers and ciliates exhibit less prey discrimination making these organisms an excellent choice for us in prey preference studies using microspheres (Saba et al., 2011).

Larval prey preference at first feeding may be influenced by factors such as size, prey visibility, contrast, encounter rate, predator evasion response, and swimming pattern (Buskey et al., 1993). Utilizing Gerritsen and Strickler's (1977) encounter model, larvae have lower encounter rates for copepod nauplii than rotifers or ciliates. Since larvae require movement to differentiate zooplankton from inanimate particles, live feed organisms that have more constant swimming patterns and shorter motionless phases have been reported to be preferred (Buskey et

al., 1993; Peterson and Ausubel, 1984; Titelman and Kiørboe, 2003). Larval turbot (*Scophthalmus maximus* L.) ingested mainly rotifers for the first six days of feeding before including copepod nauplii into the diet (Van der Meeren, 1991). Grouper larvae (*Epinephelus coioides*) have much lower ingestion rates of rotifers compared to copepod nauplii at first feeding (Toledo et al., 1999). Larval gulf menhaden (*Brevoortia patronus*) have higher preference for dinoflagellates and tintinnids over copepod nauplii (*Acartia tonsa*) (Stoecker and Govoni, 1984). Atlantic cod larvae (*Gadus morhua*) have increased preference for protozoa over nauplii (*Pseudodiaptomus* sp.) at first feeding (Herbing and Gallager, 2000).

Ciliates represent an understudied prey organism in larval fish diets which may be due in part to an implicit bias resulting in poor detection and identification of these soft-bodied prey (Fukami et al., 1999; Nagano et al., 2000a; 2000b; De Figuieredo et al., 2007). Soft bodied ciliates are estimated to be digested within two hours compared to rotifers and copepods which may take upwards of five hours (Ohman et al., 1991; Conway et al., 1993; Lough and Mountain, 1996; De Figuierdo et al., 2007). Healthy larvae with "empty guts" discovered in the wild have been hypothesized to contain protozoan prey where metazoan populations are not high enough to support larval development (De Figuieredo et al., 2007). Evaluation of consumption rates of soft bodied ciliates such as *Euplotes* spp. is difficult as the feed organism does not remain intact inside the larval gut for a long time, but previous studies have endeavored to do so (Nagano et al., 2000; Tew et al., 2015). Nagano et al. (2000b) showed that 57.8% of convict grouper (*E. septemfasciatus*) ingested fluorescent labelled *Euplotes* spp. on 4 dph after one day of acclimation to eating the ciliate. Larval survival has also been shown to increase with the use of ciliates as an alternative live feed in the larval culture protocols of marine larvae such as *Gobiosoma evelynae* and *Lutjanus campechanus* (Olivotto et al., 2005; Rhodes and Phelps,

2008). Aside from increased rate of digestion, the mechanism behind observed increases in survival for larvae fed with ciliates is not clear (Ohman, 1991). Further investigation into these properties is warranted.

In the current study, microspheres had to be consumed by nauplii in order to be marked, and the first naupliar stage reported to feed is N3 (Lawson and Grice 1984; Kline and Laidley 2015). The nauplii used in the current experiments hatched between 6-30 hours before being used in the feeding experiments because they were harvested from a large population with timers used to control daily collection times. According to Alajmi and Zeng (2015), N2 nauplii development occurred from 0.5-0.8 days post-embryonic and N3 development occurred 0.8-1.0 days post-embryonic. In the current study, it is possible that a small proportion of nauplii had not reached the N3 stage. If all nauplii were not N3 and they were not marked, the reported percentages marked with microspheres may have been affected.

Copepod nauplii increase their predator avoidance response as they grow (Titelman and Kiorboe 2003). The reactive distance of *Parvocalanus crassirostris* N2 and N3 nauplii was measured to be slightly greater than N1 nauplii (Bradley et al. 2013); and N2 and N3 nauplii were reported to have longer total lengths than N1 nauplii (N1 80±0.6 µm, N2 87.4±0.4 µm, N3 112±0.4 µm) (Alajmi et al. 2015). However, even though nauplii are more difficult to capture than other food organisms, they have been shown to be more energetically efficient and are frequently observed in the GI tracts of many larval fish species (Llopiz and Cowen, 2009; Sampey et al., 2007; von Herbing et al., 2001). If all nauplii were not N3 and they were not marked, the resultant nauplii consumed may have been greater than these reported results, and could have varied by larval fish species.

4.1.1 Pacific blue tang prey preference of first feeding larvae 3 dph after 6 h exposure

Previous studies have shown wild Acanthurid larvae in the 2-3 mm size range ingested mainly (>50%) *Limacina* spp. pteropods and nauplii (~30%) (Llopiz et al., 2009). *P. hepatus* larvae have been shown to ingest ciliates because of research with fluorescent microspheres (Nagano et al., 2000a; 2000b). Nagano et al. (2000a) concluded that *P. hepatus* larvae will ingest both *Euplotes* sp. and the tintinnid *Amphorellopsis acuta*. Feeding exclusively ciliates, they were able to grow the larvae to 7 dph (*Euplotes* sp.) and 9 dph (*A. acuta*).

Pacific blue tang have recently been cultured to metamorphosis utilizing a diet of *P. crassirostris* nauplii, *B. plicatilis*, and live *Artemia* nauplii (DiMaggio et al., 2017). Copepod nauplii were used exclusively during the period of first feeding for Pacific blue tangs, but significant mortalities were still observed during this period. It is also important to note that preference may not indicate suitability of the prey type for survival of larvae. In the current study, first feeding Pacific blue tang larvae showed a preference for *B. plicatilis* and *Euplotes* sp., however, successful larval culture trials have only been completed when *P. crassirostris* nauplii were available at the first feeding (DiMaggio et al. 2017). Preference again may be influenced by ease of capture.

4.1.2 African moony prey preference of first feeding larvae 3 dph after 6 h exposure

Africany moony fish larvae have exhibited similarly survival when fed (*P. crassirostris* & *Pseudodiaptomus pelagicus*) and rotifers (*B. plicatilis*) (Elefante, 2014). While copepod nauplii and ciliates may not be necessary for commercial culture of this species, it may be of interest to investigate the effects of a wider diversity of food types on survival.

4.1.3 Reef butterflyfish prey preference of first feeding larvae 4 dph after 6 h exposure

Degidio (2014) found that *Chaetodon miliaris* larvae did not ingest rotifers after 6 h in clearwater and had very low ingestion rates (<10%) in their guts in T-Isochrysis (400,000-

600,000 cells mL^{-1}), which is inconsistent with the results from the current study. Ingestion of nauplii in greenwater was significantly higher than rotifer ingestion, while ingestion rates in clearwater was not significantly higher. Also the addition of green water was shown to increase feeding prevalence on copepod nauplii by *C. miliaris* larvae (Degidio, 2014). These results suggest that conducting this trial under greenwater conditions may yield different results.

4.1.4 Golden trevally prey preference of first feeding larvae 3 dph after 4 h exposure

Golden trevally larvae have been successfully cultured using rotifers (*B. plicatilis*) and copepods nauplii (*Parvocalanus* sp.) (Broach et al., 2015). Larvae are currently commercially produced using rotifers followed by *Artemia* nauplii. Increased ingestion of copepod nauplii over rotifers and ciliates indicates that first feeding larvae of this species are more developed, which corresponds to higher survival in a commercial setting.

5. Conclusion

The prey preferences of the four marine ornamental fish studied varied among species. Pacific blue tang larvae ingested more rotifers above ciliates, and ciliates above copepod nauplii. African moony larvae ingested more ciliates and nauplii equally over rotifers. Reef butterflyfish larvae ingested more ciliates over rotifers and rotifers over nauplii. Golden trevally larvae ingested more nauplii over ciliates, and ciliates over rotifers. Further research is required in order gain a deeper understanding in the differences reported in this study.

Microspheres have a variety of inherent advantages and disadvantages when utilized for feed preference experiments. The primary disadvantage is that after microspheres are ingested, they will pass through the digestive system over a period of time. To control for variation due to this factor, larvae were all given the same amount of time to feed. The retention duration of rotifers, ciliates, and copepods needs to be more thoroughly investigated as other factors such as

temperature or stress may also influence metabolic rates and thus the transit time of the microspheres through the gastrointestinal tract. The presence of microalgae or yeast may also affect retention time of microspheres in the digestive tract or food vacuole and further studies evaluating these variables could be valuable. Experiments performed using greenwater may give different results because the spheres inside the GI tract of feed organisms could be replaced with newly ingested microalgae and this may vary with larval species. One possible solution that needs to be evaluated is the use of a dye or another compound to color the water that live feed organisms will not ingest. Another aspect that has not been well researched is the residence time of microspheres in the guts of larval fish if the prey item is fully digested, and microspheres potentially may be distributed throughout the digestive tract. During this study, it was observed that larval fish had different dispersal and quantities of microspheres within the gut. Thus, the possibility exists that a prey organism had already been digested and or passed through the gut but residual spheres remained. Blue tang and reef butterflyfish larvae did not have dispersal of spheres ingested by copepod nauplii, whereas golden trevally and African moony larvae did. Microspheres from ciliates were spread throughout the gut cavity of all larval species. Instances of microspheres from rotifers spread throughout the digestive tract were observed in blue tang larvae only.

Fluorescent microspheres appear advantageous for us in prey preference studies with marine finfish larvae. Elucidation of preferred food organisms, prey size preferences, and diet phase shifts and preferences at different stages of larval developmentcan all be accomplished using techniques developed for working with microspheres in this manuscript.

Prey preference experiments with microspheres revealed a need for further research to quantify retention time of microspheres within different prey species. Research with fluorescent

microspheres and other types of food organisms such as large dinoflagellates, oyster trochophores, other ciliates, rotifers, and copepods is justified.

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Table 1. Percent ciliates remaining in water column at three ciliate densities. Ciliate densities were determined using three treatments: 10 ciliates mL^{-1}, 20 ciliates mL^{-1}, and 30 ciliates mL^{-1} added to 14 L tanks. Ciliates were sampled using a 9.5 mm diameter tube sampler.

Table 2. Fluorescent microsphere incubation rates for prey preference experiments. Stock densities of microspheres were $2.0E_{+}11$ microspheres mL^{-1}.

Table 3. Fluorescence of live feeds prior to experiment start.

Table 4. Empty gastrointestinal tracts of larval preference experiments utilizing fluorescent microspheres.

Table 5. Water quality (temperature, salinity, dissolved oxygen, pH, total ammonia nitrogen, and total ammonia nitrite) of first feeding prey preference experiments.

Figure 1. Live feeds labeled with fluorescent microspheres: rotifers (A), copepod nauplii (B) and ciliates (C). Photos courtesy of author.

Figure 2. Photos of larvae exhibiting the presence of fluorescent microspheres. A) Pacific blue tang larvae that had ingested rotifers, nauplii and ciliates. B) Reef butterflyfish larvae that had ingested ciliates and nauplii. C) Pacific blue tang larvae that had ingested both rotifers and nauplii. D) Golden trevally larvae that had ingested nauplii, rotifers, and ciliates. E) African moony larvae with rotifers in GI tract. Photos courtesy of author.

Figure 3. Proportion of larvae with different prey combinations in the GI tract. A) Pacific blue tang, B) African moony, C) reef butterflyfish and D) golden trevally with rotifers (R), copepod nauplii (N), ciliates (Ci), rotifers and nauplii (R+N), rotifers and ciliates (R+Ci), nauplii and ciliates (N+Ci), all three food types (All) and larvae with empty GI tracts (X).

Figure 4. Prey preference of Pacific blue tangs utilizing fluorescent microspheres in clearwater after 6 h. A) Proportion of prey items rotifers (R), copepod nauplii (N) and ciliates (Ci) ingested by larvae. B) Quantity of prey items consumed by larvae. Different lowercase letters indicate statistically significant differences among or between prey organisms within treatments.

Figure 5. Prey preference of African moony utilizing fluorescent microspheres in clearwater after 6 h. A) Proportion of prey items rotifers (R), copepod nauplii (N) and ciliates (Ci) ingested by larvae. B) Quantity of prey items consumed by larvae. Capital letters indicate statistically significant differences among organisms throughout all treatments. Lowercase letters indicate statistically significant (differences among or between prey organisms within treatments.

Figure 6. Prey preference of reef butterflyfish utilizing fluorescent microspheres in clearwater after 6 h. A) Proportion of prey items rotifers (R), copepod nauplii (N) and ciliates (Ci) ingested by larvae. B) Quantity of prey items consumed by larvae. Capital letters indicate statistically significant differences among organisms throughout all treatments. Lowercase letters indicate statistically significant differences among or between prey organisms within treatments.

Figure 7. Prey preference of golden trevally utilizing fluorescent microspheres in clearwater after 4 h. A) Proportion of prey items rotifers (R), copepod nauplii (N) and ciliates (Ci) ingested by larvae. B) Quantity of prey items consumed by larvae. Lowercase letters indicate statistically significant differences among or between prey organisms within treatments.

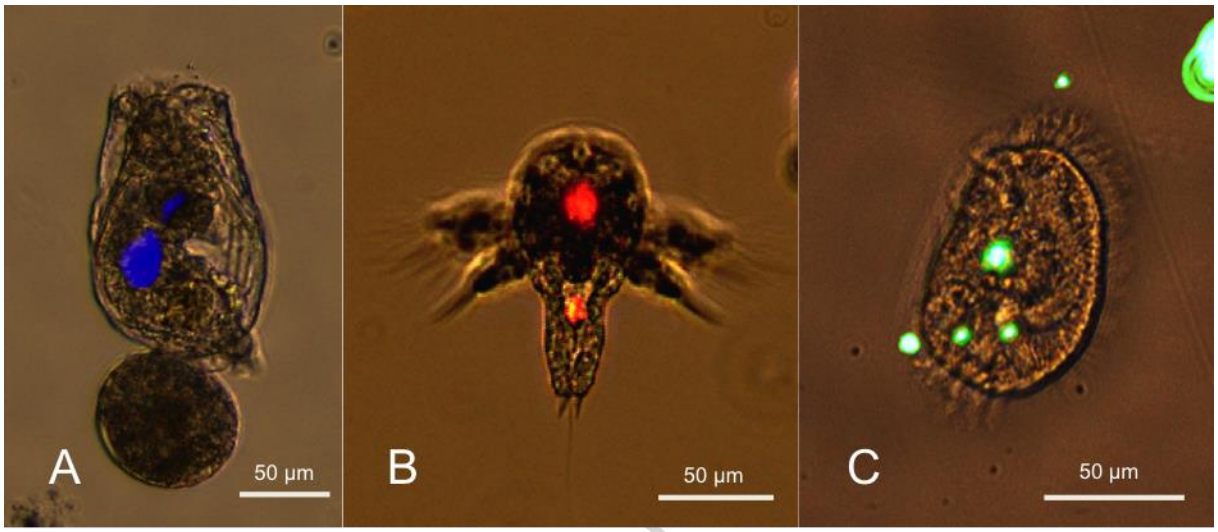
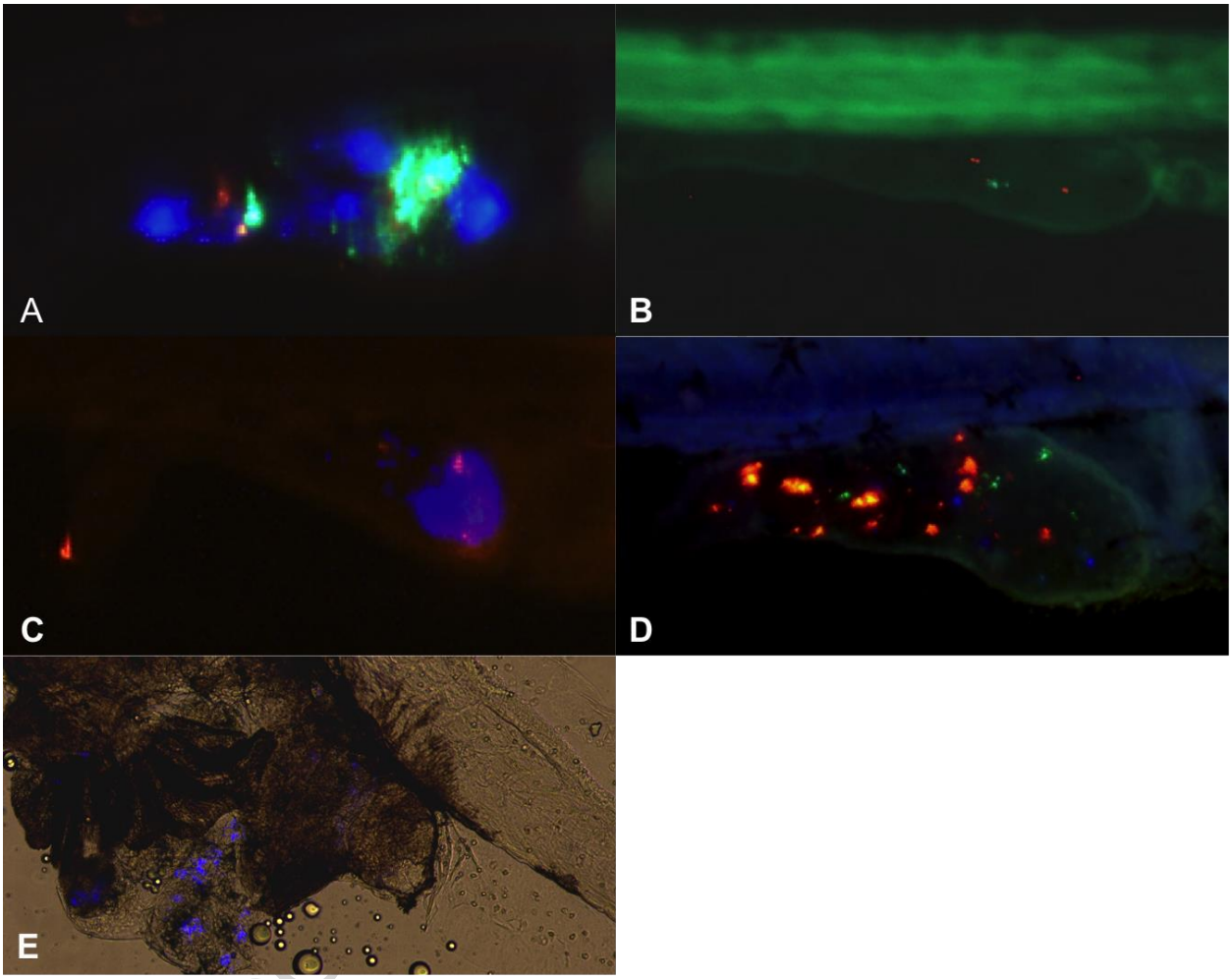


Figure 1.



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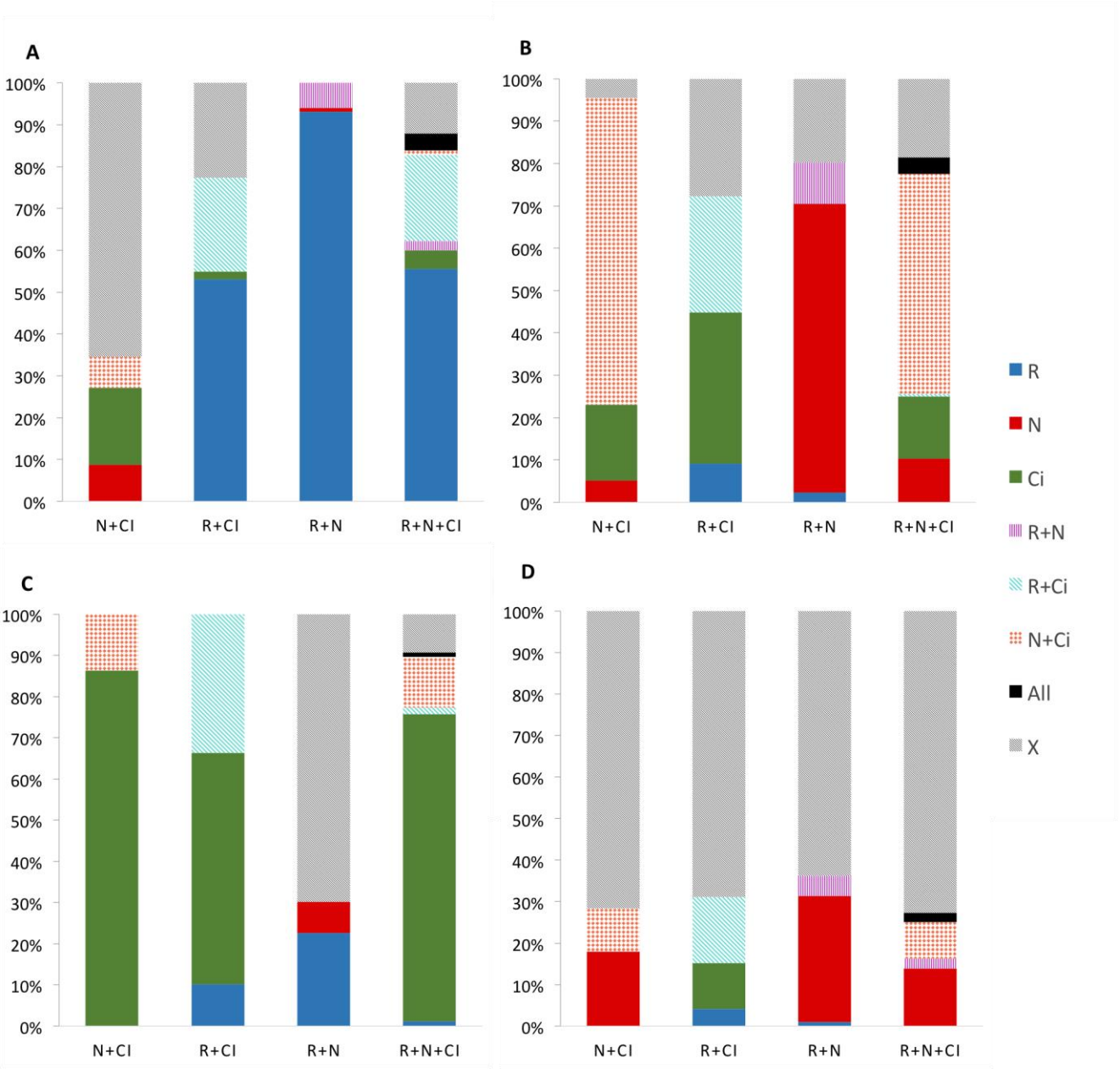

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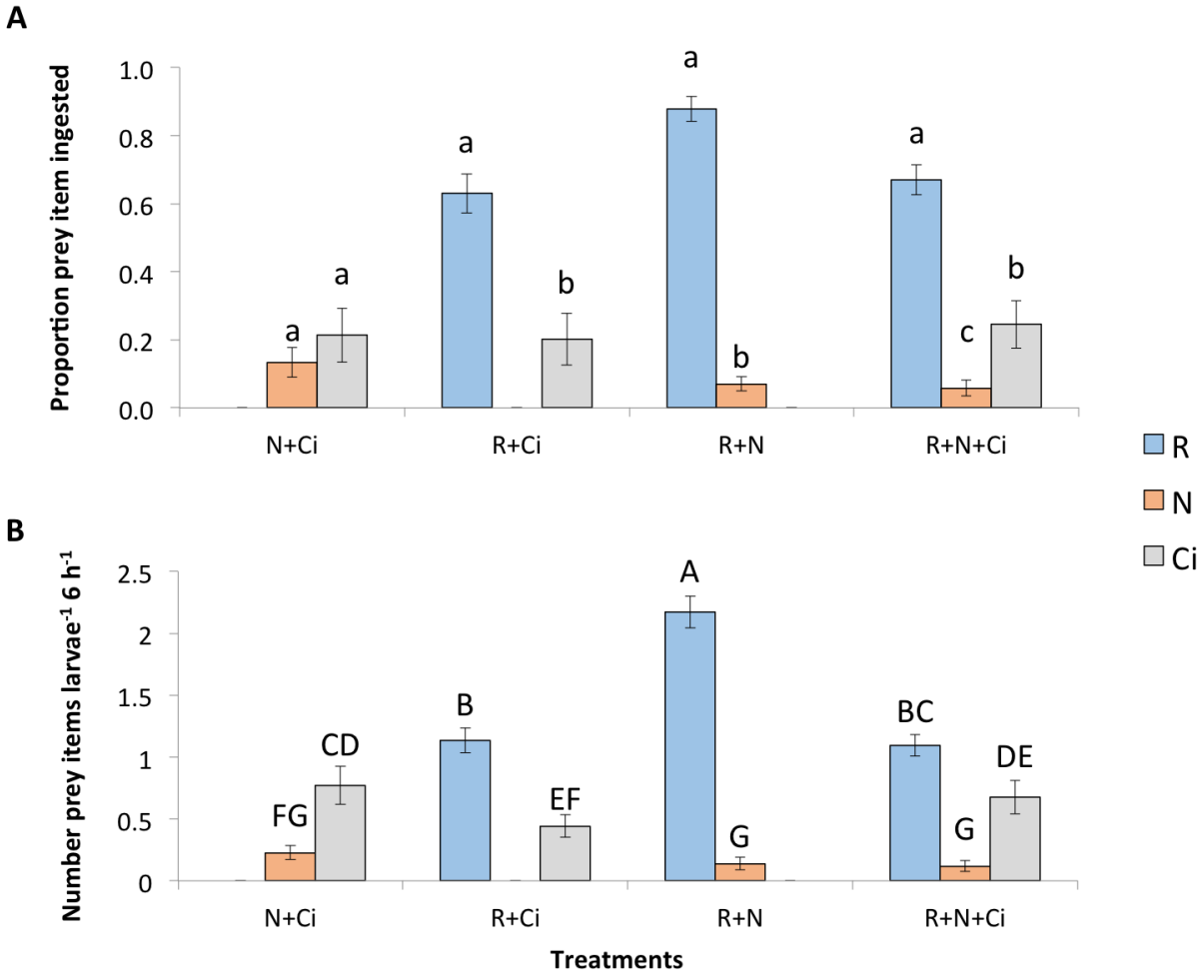

Figure 4.

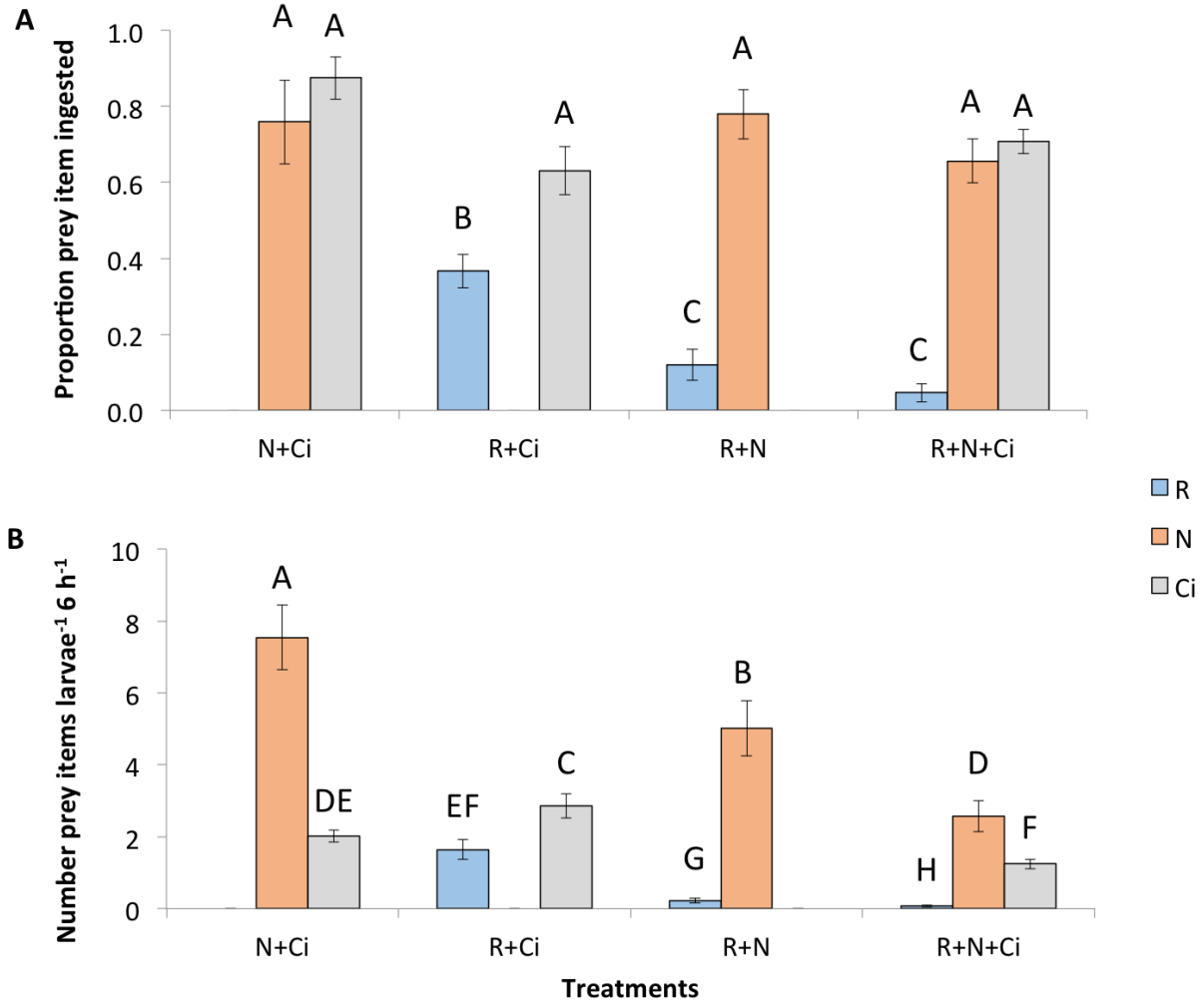

Figure 5.

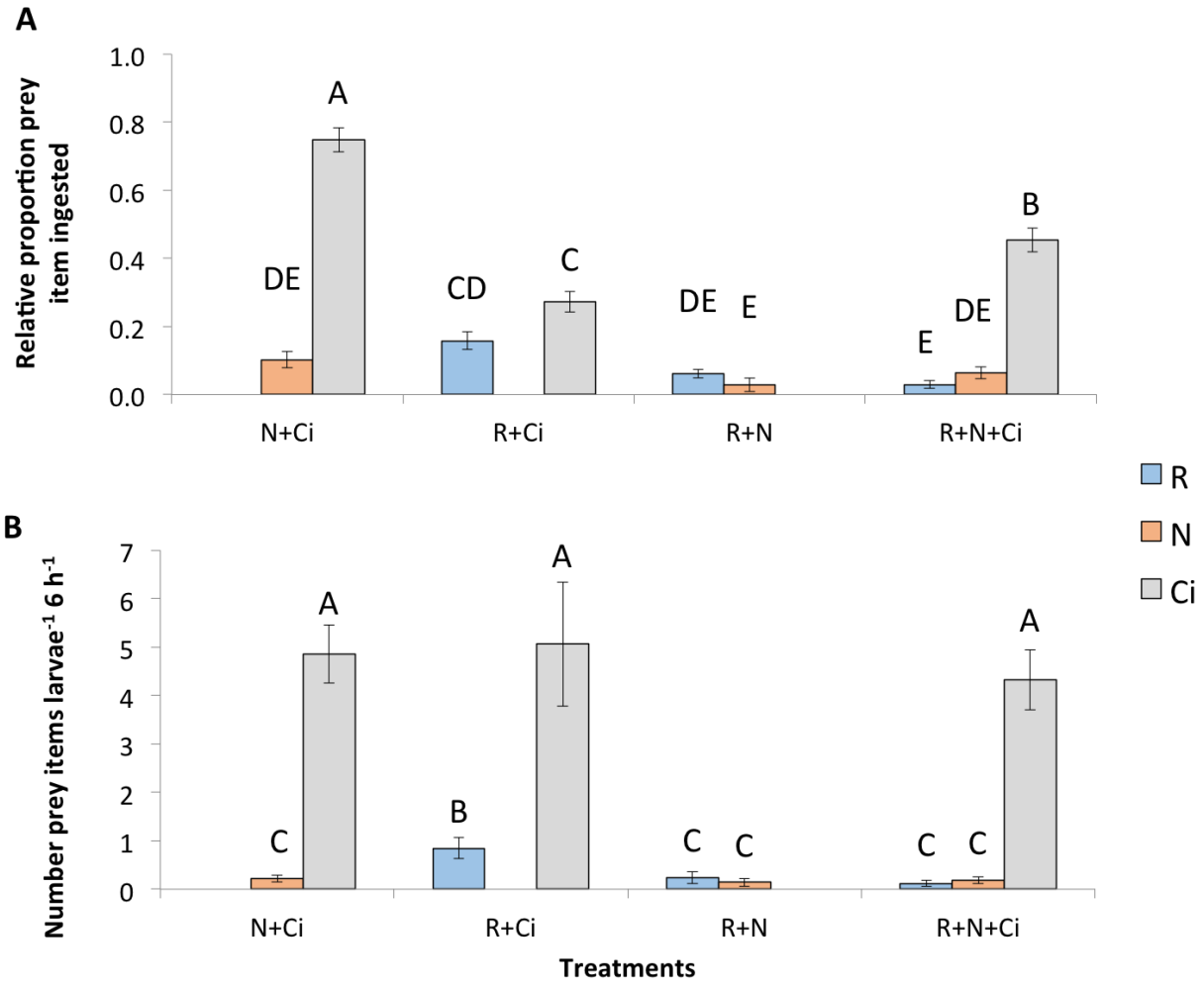

Figure 6.

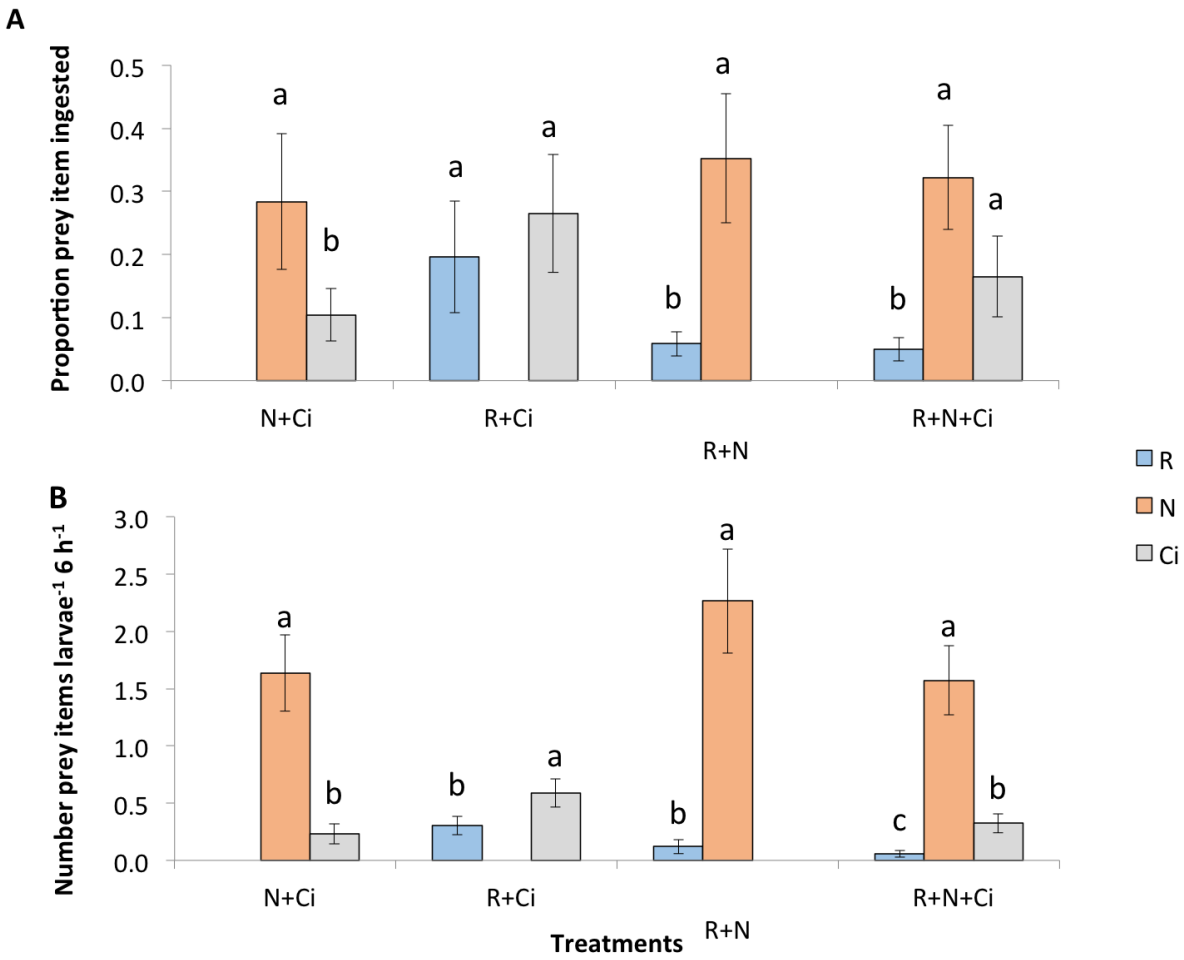

Figure 7.

Table 1.

Ciliates mL^{-1}	% in water column	±SE
10	35.30%	2.69%
20	31.24%	4.55%
30	32.48%	7.53%

Table 2.

	Volume microspheres (μL)			Microspheres mL^{-1}		
	Ciliates	Nauplii	Rotifers	Ciliates	Nauplii	Rotifers
C. sedentarius	400	400	400	4.57E+07	4.71E+07	4.85E+07
P. hepatus	400	400	400	5.52E+07	4.32E+07	4.21E+07
G. speciosus	400	400	400	4.71E+07	5.00E+07	4.21E+07
M. sebae	400	400	400	4.00E+07	3.20E+07	3.20E+07

Table 3.

	Ciliates	±SE	Nauplii	±SE	Rotifers	±SE
C. sedentarius	31.8%		89.0%	1.9%	51.8%	6.7%
P. hepatus	96.3%	3.7%	77.1%	4.9%	80.0%	6.8%
G. speciosus	82.5%	5.7%	79.0%	2.2%	100.0%	0.0%
M. sebae	98.3%	1.7%	83.8%	1.8%	93.3%	1.0%

Table 4.

	N+Ci	±SE	R+Ci	±SE	R+N	±SE	R+N+Ci	±SE	*P*
C. sedentarius	18.39% (a)	±9.24%	39.50% (a)	±5.57%	81.67% (b)	±1.67%	38.75% (a)	±13.9%	0.010
P. hepatus	67.50% (a)	±9.38%	35.83% (b)	±5.69%	15.00% (b)	±3.62%	26.67% (b)	±4.22%	<0.001
G. speciosus	77.50% (b)	±8.54%	72.00% (a)	±11.02%	71.00% (b)	±7.31%	74.17% (b)	±6.64%	<0.001
M. sebae	13.57% (a)	±4.97%	30.00% (ab)	±4.63%	32.14% (b)	±4.86%	25.00% (ab)	±3.27%	0.033

Table 5.

	Temp (°C)	±SE	Salinity (g L^{-1})	±SE	DO	±SE	pH	±SE	NH3-N	±SE	NO2-N	±SE
C. sedentarius	29.07	0.012	33.05	0.030	5.32	0.032	8.052	0.002	0.006	0.003	0.004	0.001
P. hepatus	26.59	0.012	37.64	0.350	6.02	0.042	7.951	0.003	0.423	0.005	0.003	0.000
G. speciosus	26.59	0.014	30.00	0.077	5.97	0.038	8.029	0.002	0.002	0.002	0.004	0.000
M. sebae	28.68	0.013	18.86	0.075	6.32	0.037	7.831	0.002	0.070	0.002	0.006	0.000

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