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# Selective Feeding and Microalgal Consumption Rates by Crown-of-Thorns Seastar (*Acanthaster cf. solaris*) Larvae

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## ABSTRACT

Outbreaks of the crown-of-thorns seastar (CoTS) represent a major cause of coral loss on the Great Barrier Reef. Outbreaks might be explained by enhanced larval survival supported by higher phytoplankton availability after flood events,

yet little is known about CoTS larvae feeding behaviour, in particular their potential for selective feeding. Here, single- and mixed-species feeding experiments were conducted on CoTS bipinnaria larvae using five algae (*Phaeodactylum tricornutum*, *Pavlova lutheri*, *Tisochrysis lutea*, *Dunaliella* sp. and *Chaetoceros* sp.) and two algal concentrations (1000 and 2500 algae mL<sup>-1</sup>). Cell counts using flow-cytometry at the beginning and end of each incubation experiment allowed us to calculate the filtration and ingestion rates of each species by CoTS larvae. In line with previous studies, CoTS larvae ingested more algae when initial algal concentration was higher. We found evidence for the selective ingestion of some species (*Chaetoceros* sp., *Dunaliella* sp.) over others (*P. lutheri*, *P. tricornutum*). The preferred algal species had the highest energy content, suggesting that CoTS selectively ingested the most energetic algae. Ultimately, combining these results with spatio-temporal patterns in phytoplankton communities will help elucidate the role of larval feeding behaviour in determining the frequency and magnitude of CoTS outbreaks.

**KEYWORDS:** electivity; feeding behavior; filtration rate; Great Barrier Reef; phytoplankton

## INTRODUCTION

Outbreaks of the crown-of-thorns seastar (*Acanthaster* cf. *solaris*, hereafter referred to as CoTS) represent a major threat for coral reefs, and in particular, for the Australian Great Barrier Reef (GBR). CoTS outbreaks are among the main causes of coral loss on the GBR since 1985 [1], and unlike other causes such as

bleaching or cyclones, may be amenable to local or regional management. One of the most widely discussed hypotheses explaining the increased frequency of CoTS outbreaks relates to the availability of phytoplankton, which is intimately linked to elevated terrestrial nutrient runoff [2-6]. As most planktotrophic echinoderm larvae, CoTS larvae feed on nano-phytoplankton and larval survivorship increases with food availability [7-9]; although the latter reference also suggests that too high food concentrations may be detrimental to development and survival.

While documented descriptions of feeding mechanisms of CoTS in the bipinnaria stage are scarce, it can be assumed that similarities to the feeding of other asteroid taxa exist (*Luidia* spp., *Patiria* spp., *Evasterias* spp., *Pisaster* spp., *Pycnopodia* spp.) [10]. These asteroid larvae might facilitate food ingestion via the beating of epidermal cilia that generate a feeding current for the concentration and ingestion of particles (Strathmann, 1971). Particles accumulate in the mouth and are subsequently swallowed via the action of muscles surrounding the oesophagus. The sphincter between the oesophagus and stomach then opens, allowing the particles to enter the stomach where they can be sorted, digested or rejected by dorsal flexion [10,11]. Rejection of particles that have not reached the stomach is accomplished by a reverse beating of the oesophagus and mouth cilia, and the reverse sequence of muscle contractions. This might be a mechanism for food selection [11].

Studies of larval feeding in CoTS conducted to date have focused on size selectivity and the influence of food concentration on feeding behavior [7,12,13]. In particular, these investigations have demonstrated the ability of CoTS larvae to discriminate particle ingestion based on size (feeding in the range of 5 to

20µm in diameter) and a modulatory effect of algal species on feeding and development. It has also been demonstrated that CoTS larvae can feed on other organic matter such as mucus derived from corals [14]. As suggested by Ayukai [12], algae size and concentration are likely not the only relevant factors controlling ingestion, with other factors such as food quality potentially influencing CoTS feeding behavior. While each of the aforementioned studies contributed knowledge to our understanding of the larval phase of this species, the corresponding experiments were short in duration (approximately 30 minutes) [12,13], or conducted using high food concentrations of little relevance to natural conditions [7]. Thus, there is currently limited knowledge on the feeding ecology (e.g., preferred food items, consumption) of the larvae of this important coral predator, in particular in terms of potential preference for some algal species and corresponding consumption rates.

In the present study, early bipinnaria larvae of *A. planci* were fed with five different algae species (in isolation or in 1:1 choice experiments) and tested over 18 hours of consumption. Initial and final algae concentrations were measured by flow cytometry. This technique uses the fluorescence properties of each algal species for discrimination [15,16]. It simultaneously computes cell concentrations and, by comparing concentrations before and after consumption by CoTS, allows calculation of filtration and ingestion rates. Flow cytometry has been used to study feeding behavior of several zooplankton species including copepods [17], oyster larvae [18] zebra mussel larvae [19] and decapod larvae [20] but never for studying CoTS feeding behavior (nor, to our knowledge, that of any other echinoderm). Therefore, flow cytometry represents an alternative to the more traditional methods implemented previously in CoTS feeding studies,

and potentially an efficient way to increase accuracy in algal counts [12]. Specifically, we used this technique to determine the filtration and ingestion rates of the different algal species under two different ecologically relevant algal cell concentrations. In addition, we investigated the ability of CoTS larvae to selectively ingest or reject individual algal species in food mixes. Finally, we related these patterns of food selection to the energetic content of the algae species tested.

## MATERIAL AND METHODS

### *Spawning and maintenance of CoTS larvae*

Adult CoTS were collected on Rudder Reef in the Northern section of the GBR (16.21°S, 145.47°E) in mid-September 2014, transported to the Australian Institute of Marine Science and maintained under natural flow-through seawater (FSW) conditions. Adult CoTS were spawned and oocytes fertilized as described previously [9]. Briefly, a small ( $\approx 1$  cm) incision was made near the proximal end of one of the arms and 3–4 gonadal lobes were removed from each specimen. After macroscopic sex determination, testes of six males and ovaries of six females were collected. Testes were placed in covered 6-well plates to prevent desiccation. Ovary lobes were rinsed with filtered seawater (FSW) and subsequently submerged in a  $10^{-5}$  M 1-methyladenine/seawater solution to induce maturation. After 60–70 min, mature oocytes were washed through a 500- $\mu$ M mesh, and oocytes from all females combined. Oocytes were diluted in seawater to achieve a stock solution of  $\sim 400$  eggs  $\text{mL}^{-1}$ . Two mL of dry sperm from each male were combined and added to 2500 mL of the egg solution, resulting in a concentration of  $10^6$ – $10^7$  sperm  $\text{mL}^{-1}$ . This yielded a fertilization

success of >99%. After 20 min, eggs were washed repeatedly using a 50 mm mesh to remove excess sperm. Fertilized eggs were then divided evenly across three holding tanks (70L each) and kept at 27°C in FSW (0.2µm) under a 12h:12h light dark cycle. CoTS larvae were maintained at a density of 0.6 to 1.5 larvae mL<sup>-1</sup> and fed with *Dunaliella* sp. (3000 cells mL<sup>-1</sup>) once per day starting two days post-hatching. Because feeding behavior of CoTS larvae changes over the course of larval development [7], all experiments were conducted using larvae less than 13 days old.

### ***Phytoplankton cultures and analysis***

CoTS larvae were fed cultured phytoplankton over the course of the feeding experiments. Five pure algae species were sourced from the Australian Algal Culture Collection (Hobart, Australia), consisting of two diatom species: *Phaeodactylum tricornutum* (Species No. CS-29) and *Chaetoceros* sp. (CS-256), two haptophytes: *Pavlova lutheri* (CS-182) and *T. lutea* (T-ISO, CS-177) and the green algae *Dunaliella* sp. (CS-353). All algae were grown at 26°C under a 12h:12h light dark cycle, in F/2 medium. Experimental algal species were selected because they have been frequently used in CoTS larval culture (*Dunaliella* sp., *T. lutea*, *P. tricornutum*, *P. lutheri*) [7,9,13] or are naturally occurring on the GBR (*Chaetoceros* sp.) [21]. Length and width of the microalgae were determined for 30 cells of each species under a 400x magnification.

To quantify algal nutritional content, algae were analyzed for total lipid content following standardized procedures described previously [22]. Briefly, lipids were extracted with dichloromethane: methanol (2:1). Protein content was determined via the application of a nitrogen-to-protein conversion factor of

5.0, taking into account the significant sources of chlorophyll, nucleic acids, free amino acids and inorganic nitrogen [23], while the energetic content of each algal species was determined on a pg/cell basis from the enthalpies of combustion values published by Bureau et al. [24]: lipid (39.5 kJ g<sup>-1</sup>) and protein (23.9 kJ g<sup>-1</sup>).

Organic Carbon (OC), Nitrogen (N) and Chlorophyll content of each algal species were measured in duplicate using standard water quality methods [e.g., 25]. In brief, 100 ml (for chlorophyll) or 50 ml (for OC, N) of stock solution was filtered on pre-combusted 25mm filters (Whatman GF/F) and stored frozen (-20°C) until analysis. Nitrogen was analysed on an ANTEK 9000 NS analyser and OC on a Shimadzu TOC-V carbon analyser, equipped with a solid sample module (SSM-5000A) after removing inorganic carbon using 2 M hydrochloric acid. Chlorophyll-a concentrations were measured fluorometrically (Turner Designs 10 AU fluorometer) after grinding filters in cold (4°C) acetone (90%).

### ***Larval feeding experiments***

Prior to incubations, larvae were starved for approximately 20h, concentrated and placed in six glass jars containing 200mL of FSW. Larvae were stocked at an approximate concentration of 1 larvae mL<sup>-1</sup> and counted both at the beginning and the end of each experiment. Initial counts in each experiment showed that the actual numbers varied slightly (mean ± standard deviation: 1.00 ± 0.17 larvae mL<sup>-1</sup>; range: 0.57 - 1.50 larvae mL<sup>-1</sup>). We accounted for this variation by adding the actual initial larval density as a factor in the statistical model (see below). To document possible intrinsic changes in algal concentrations without larvae, six jars were used as no larvae controls and filled with 200mL of FSW.

Jars were then placed on a shaker plate and larvae starved a further five hours. Thus, larvae had sufficient time to acclimate to their new environmental conditions. Prior to each experiment, algae stock concentrations were obtained using an Accuri C6 flow cytometer. Feeding concentrations were then calculated and the respective number of algae added to each jar (experimental and control). Each jar was adjusted to a final concentration of 1000 cells mL<sup>-1</sup> for the low-level treatment and 2500 cells mL<sup>-1</sup> for the high-level treatment. These concentrations were in the range reported by [26] in flood plumes on the Great Barrier Reef lagoon.

In addition to the aforementioned experimental and control jars, three experimental (with larvae) and three control jars (without larvae) were run with *Dunaliella* sp. at a density of 2500 cells mL<sup>-1</sup> during each incubation. These served as an inter-experiment reference to control for possible variation in CoTS algal consumption between experiments. In other words, we obtained a feeding rate for *Dunaliella* sp. at a standard concentration for each experiment.

Following the addition of algae, each jar was gently agitated with a customized plunger and a 2mL subsample of water taken through a 100µm mesh (to avoid removing larvae). The jars were placed back on the shaker plate and left for 18h. All subsamples were immediately analyzed with the flow cytometer, and the initial algal concentration calculated. This step ensured that the actual initial algal concentrations (as measured by flow cytometry) that slightly varied around the expected value both in the single and mixed-species experiments could be taken into account in the statistical model. However, in each case low- and high-level treatments were spanning non-overlapping ranges (Table S1). After 18h, each jar was agitated again, and a final sample was taken and cell



numbers analyzed. Larvae were then counted in a 25mL subsample from each replicate to determine larval densities for each replicate.

We used the initial experiments to investigate the feeding rates on individual algal species. Subsequently we conducted choice experiments with mixtures of two algal species. The four mixtures each consisted of 50% of *Dunaliella* sp. and 50% of either of the remaining species. Two cell concentrations were used for single food and choice experiments: 1000 cells mL<sup>-1</sup> for the low treatment and 2500 cells mL<sup>-1</sup> for the high treatment.

Filtration rate (*Fr*) for each algal species was calculated according to Coughlan [27]. For each treatment, a growth constant *k* was calculated from *C'*<sub>0</sub> and *C'*<sub>*t*</sub>, i.e. the initial and final algae concentrations in the control jars respectively.

$$C'_t = C'_0 e^{kt} \Leftrightarrow k = \frac{1}{t} \ln\left(\frac{C'_0}{C'_t}\right)$$

Where *t* is the duration of the experiment (18h). A grazing coefficient *g* was calculated in the same way from *C*<sub>0</sub> and *C*<sub>*t*</sub>, i.e. initial and final algae concentrations in each replicate, respectively:

$$g = \frac{1}{t} \ln\left(\frac{C_0}{C_t}\right) + k$$

Filtration (or clearance) rate (μL larva<sup>-1</sup> h<sup>-1</sup>) was then calculated as

$$Fr = \frac{V}{N_{larv}} g$$

Where *N*<sub>larv</sub> is the number of larvae in the jar, and *V* the volume of water in the jar (200mL).

Mean ingestion rate (cells larva<sup>-1</sup> h<sup>-1</sup>) was calculated from

$$I_r = F_r C_{mean}$$

Where  $C_{mean}$ , the mean concentration (cells μL<sup>-1</sup>), is calculated as

$$C_{mean} = \frac{C_0(e^{(k-g)t} - 1)}{(k - g)t}$$

Selective feeding by CoTS larvae in the choice experiments was then evaluated as described by Baldwin [18] and Fileman et al. [20], and through an electivity index proposed by Vanderploeg and Scavia [28]:

$$E_i = \frac{Fr_i}{\sum_j Fr_j}$$

Where  $Fr_i$  is the filtration rate for algae  $i$  in the mixture and  $\sum_j Fr_j$  the sum of filtrations rates of all species present in the mixture. For each species  $i$ ,  $E_i$  ranges between 0 and 1, with 0.5 meaning no selectivity, 0 to 0.5 representing avoidance and 0.5 to 1 representing selection. The utilization of this index is particularly relevant here, because it is unaffected by the initial proportions of each algal species in the mixture [18].

### **Modelling**

We predicted filtration and ingestion rates of CoTS larvae as a function of algal species and initial concentration using a Poisson error-distributed generalized linear mixed-effect model (GLMM) [29]. Initial larval concentration and age, and

initial algal concentrations were included in the models as fixed effects. This allowed us to test for the effect of initial algal concentration on ingestion and filtration rates as suggested by Marin et al. [30]. All combinations of fixed effects including their interactions were considered in three model sets for each response variable (i.e. filtration and ingestion rates): (i) in single-species experiments, (ii) for *Dunaliella* sp. in mixed-species experiment and (iii) for the second species in the mixed-species experiments. We included the date of the experiment as a random effect to account for the non-independence of replicates tested on a same day and from the same batch of larvae.

We assessed GLMM performance using the marginal  $R^2$  ( $R_m$ , variance explained by the fixed effects), and the conditional  $R^2$  ( $R_c$ , variance explained by both the fixed and random effects) to provide an index of the model's goodness-of-fit [31]. We also used the Akaike's information criterion corrected for small sample sizes ( $AIC_c$ ) that provides an index of Kullback-Leibler information loss and corresponding weights ( $wAIC_c$ ) to assign relative strengths of evidence to the different competing models [32]. This information-theoretic approach offers a more robust method than standard regression techniques for testing alternative hypotheses, because it uses a multi-model inference framework without discarding any models or predictors based on arbitrary thresholds such as  $p$  values [33]. We fitted the GLMM using the function `lmer` (library `lme4`) in R 3.0.1 [34].

We then used the GLMM to compare the effect of algal species and initial concentration (high vs. low treatment) on the filtration and ingestion rates both in single- and mixed-species experiments. To do this, we predicted the partial effects of the different algae species and their initial concentrations on the

filtration and ingestion rates of CoTS larvae. We used a model-averaging approach whereby predictions from each model in the model set were weighted by their relative strength of evidence ( $wAIC_c$ ) then averaged. This procedure allowed the estimation of mean filtration and ingestion rates in each treatment while correcting for the known variation in initial algae concentration and the possible measurement errors due to non-independence in the original dataset. We then compared the predicted filtration and ingestion rates of each algal species in single vs. mixed species and high vs. low treatments using  $t$ -test pairwise comparisons.

## RESULTS

### *Single species experiment*

In the single-species experiments, the model predicting filtration rate from larval age, initial algal concentration and their interaction with algal species received the strongest support based on  $AIC_c$ , and explained over 80% of variation in filtration rates (with 66% attributed to fixed effects) (Table 1). The same model without the interaction between algal species and initial concentration received the strongest  $AIC_c$  support in explaining ingestion rates, accounting for up to 90% of their variation (with 74% attributed to fixed effects).

Filtration rates of individual algal species were either not significantly affected by initial food concentration (Fig.1a. *P. tricornutum*, *P. lutheri* and *Dunaliella* sp., *T. lutea*;  $t$ -test;  $p > 0.05$ ), or decreased with increasing algae concentration (*Chaetoceros* sp.;  $p < 0.001$ ). When initial algae concentrations increased from 1000 to 2500 algae  $mL^{-1}$ , the filtration rate for *Dunaliella* sp. remained around 60  $\mu L$  larvae $^{-1}$  h $^{-1}$  for both concentrations (Fig. 1a) while the

ingestion rates for the same species increased from 36.3 cells larva<sup>-1</sup> h<sup>-1</sup> on average (95% confidence interval: 31.8–40.8) to 87.0 (84.2–89.8) cells larva<sup>-1</sup> h<sup>-1</sup> (Fig. 1b). Similarly, ingestion rates significantly increased at higher algal concentrations for all algal species tested (Figure 1;  $P < 0.001$ ). The lowest mean ingestion rate of 10.3 cells larvae<sup>-1</sup> h<sup>-1</sup> (5.9–14.8) was observed for *P. lutheri* in the 1000 cells mL<sup>-1</sup> concentration, while the highest 104.3 cells larva<sup>-1</sup> h<sup>-1</sup> on average (96.4–112.1) was found for *T. lutea* under the 2500 cells mL<sup>-1</sup> concentration.

CoTS larvae filtered and ingested *T. lutea*, *Dunaliella sp.* and *Chaetoceros sp.* at higher rates (filtration > 63.1  $\mu$ L larva<sup>-1</sup> h<sup>-1</sup> and ingestion > 77.2 cells larva<sup>-1</sup> h<sup>-1</sup> for the high treatment), with lower rates of filtration and ingestion for *P. tricornutum* and *P. lutheri* (28.5 and 24.6  $\mu$ L larva<sup>-1</sup> h<sup>-1</sup> for filtration and 63.0 and 46.4 cells larva<sup>-1</sup> h<sup>-1</sup> for ingestion, respectively) (Figure 1).

### ***Mixed species experiment***

In the mixed-species experiment, the model including larvae age and the interaction between algal species and initial algal concentrations (both species) received the strongest AIC<sub>c</sub> support in explaining both the filtration and ingestion rates for *Dunaliella sp* (Table 2). This model explained 63 and 87% of variation in *Dunaliella sp* filtration and ingestion rates respectively (with 27 and 71% attributed to fixed effects). The same model also received the strongest support in explaining filtration and ingestion rates for the second species (with 79 and 92% of variation explained in filtration and ingestion rates, respectively) (Table 3).

In a similar manner to the trends observed in single algal experiments, all larval ingestion rates increased with initial algal concentration ( $P < 0.05$ ; Table 4) (Figure 2). Conversely, the filtration rates remained constant for *P. tricornutum*, *P. lutheri* and *Dunaliella* sp. (mixed with either the former or the latter) ( $P > 0.05$ ) and significantly decreased for *T. lutea* and *Chaetoceros* sp. ( $P < 0.001$ ) (Table 4).

When offered in a 1:1 mixture, the ingestion rate for *Chaetoceros* sp. was higher than for *Dunaliella* sp. regardless of the initial concentration (56.6 and 44.2 cells larvae<sup>-1</sup> h<sup>-1</sup>, respectively in the high treatment) ( $P < 0.001$ ; Fig. 2b). In contrast, ingestion rates of *P. lutheri* (17.3 cells larvae<sup>-1</sup> h<sup>-1</sup>) and *P. tricornutum* (29.7 cells larvae<sup>-1</sup> h<sup>-1</sup>) in the high treatment were lower than those observed for *Dunaliella* sp. (25.8 and 40.2 cells larvae<sup>-1</sup> h<sup>-1</sup> for each mixture, respectively), although the difference was only significant for *P. tricornutum*. *T. lutea* was ingested at a similar rate to *Dunaliella* sp ( $P > 0.05$ ) (Fig. 2b). These results are further substantiated by the electivity indexes (Fig. 3): *Dunaliella* sp. was preferably consumed over both *P. tricornutum* and *P. lutheri*, but not over *Chaetoceros* sp. There was no significant difference in electivity between *Dunaliella* sp. and *T. lutea*. These preferences for individual algae were independent of algal concentration at the two different concentrations measured.

The importance of the predictor 'species' in all models of filtration and ingestion rates for *Dunaliella* sp. (Table 2) and for the second species (Table 3) showed that both rates were significantly influenced by the presence of the other species. This effect was mainly caused by *P. lutheri*: when *Dunaliella* sp. was offered combined with *P. lutheri*, its ingestion rate (25.8 algae larvae<sup>-1</sup> h<sup>-1</sup>) was

reduced compared to when *Dunaliella sp.* was offered in isolation (44.2 algae larvae<sup>-1</sup> h<sup>-1</sup>) (dashed line on Fig 2b, top panel). The presence of other species did not have a significant effect on *Dunaliella sp.* consumption, with the exception of *T. lutea* at the low concentration. In that case, the consumption of *Dunaliella sp.* appeared slightly enhanced in the presence of *T. lutea*: indeed, we measured an ingestion rate of 28.5 algae larvae<sup>-1</sup> h<sup>-1</sup> for 500 algae mL<sup>-1</sup> of *Dunaliella sp.* offered in a 1:1 mixture with *T. lutea* against 20.3 algae larvae<sup>-1</sup> h<sup>-1</sup> for twice the same amount of *Dunaliella sp.* offered as a single species (Fig. 2).

### ***Algae quality***

The five algal species presented a variety of morphologies and crude nutritional compositions (Table 4). *P. tricornutum* was long and thin in shape, whereas the other species were roughly spherical. All species have different size, *Chaetoceros sp.* being the largest with a length of 9.2 µm. The content of each species varied with respect to the concentration (pgcell<sup>-1</sup>) of total lipid, total protein, carbon, nitrogen and chlorophyll. In accordance with its larger size, *Chaetoceros sp.* contained the highest cell specific content of all constituents with the exception of chlorophyll, which was found in highest abundance in *Dunaliella sp.* Conversely, *T. lutea* (the smallest species) contained the lowest concentrations of these constituents followed by *P. lutheri* and *P. tricornutum*. Differences in crude nutritional composition subsequently manifested in clear trends relating to the energetic content of the algal cells. *Chaetoceros sp.* and *Dunaliella sp.* contained the highest energy levels with 1.6 and 1.4 10<sup>-9</sup> kJ cell<sup>-1</sup>, respectively, while in comparison, *T. lutea* contained less than half the amount of total energy (0.6 10<sup>-9</sup> kJ cell<sup>-1</sup>).

Based on algal energetic contents combined with effective ingestion rates, the greatest energy gain in the single species experiment resulted from the ingestion of *Dunaliella* sp. in the high concentration treatment ( $12.2 \cdot 10^{-8}$  kJ larvae<sup>-1</sup> h<sup>-1</sup>). In the same experiment, the greater ingestion of *T. lutea* only resulted in  $5.7 \cdot 10^{-8}$  kJ larvae<sup>-1</sup> h<sup>-1</sup> due to its lower energy content. In the mixed-species experiment, the greatest energy gain was obtained from the ingestion of *Dunaliella* sp. and *Chaetoceros* sp. in combination ( $15.32 \cdot 10^{-8}$  kJ larvae<sup>-1</sup> h<sup>-1</sup>) followed by that of *Dunaliella* sp. mixed with *T. lutea* ( $9.15 \cdot 10^{-8}$  kJ larvae<sup>-1</sup> h<sup>-1</sup>).

## DISCUSSION

*Acanthaster cf. solaris* (CoTS) larvae on the Great Barrier Reef are exposed to a diverse array of food choices to fuel their development through to settlement and metamorphosis [35]. The rate at which they select and consume these food items ultimately drives their rate of development and probability of survival, which is likely a major driver of CoTS population dynamics and probability of outbreak [36,37]. In the current experiments, algal consumption by CoTS larvae increased with algal concentration. As generally observed for other echinoderm larvae [10] and in CoTS subject to short-term experiments [7,12,13], this result is directly linked to an increased opportunity for larvae-algae interactions when algae are provided in abundance. However, clear differences appeared in larval consumption based on the algal species being offered, whether or not in mixes.

The ability of CoTS larvae to discriminate and select the food they ingest was evident in the present study. For example, a clear preference appeared in the mixed-species experiment for *Chaetoceros* sp., which was selectively ingested over *Dunaliella* sp. In contrast, *Dunaliella* sp. was preferred over both *P. lutheri*



and *P. tricornutum*, as indicated by the lower ingestion rates and electivity indices observed for these two species. Interestingly, these results follow the trends observed in food quality and energy content, with *Chaetoceros* sp. having the highest content of energy, lipid, protein, carbon and nitrogen, whereas the lowest energy content was found for *P. lutheri* followed by *P. tricornutum*. This result indicates that, when offered a mix of different algal species, CoTS larvae selectively ingested the most energetic ones when cells are present an abundant concentration. However, the mechanisms behind this discriminatory ability currently remain unknown and ultimately require further investigation.

Algal species that were selectively avoided by CoTS larvae (*P. lutheri*, *P. tricornutum*) were both characterized by lower energy content, which might lead to lower development and survival rates. Indeed, Lucas [7] found differences in development and survival for larvae fed with different algal species, with larvae fed with *P. lutheri* rarely making it through their development cycle. In addition to energy content, the size and/or shape of a particular algal species could also influence its selective ingestion by CoTS larvae, with the second least preferred species (*P. tricornutum*) being the only long and thin one. Okaji et al. [13] showed an effect of phytoplankton size on feeding rates, as well as a preference for eukaryotes over cyanobacteria (probably also resulting from their difference in size). Okaji et al. [13] also demonstrated that CoTS typically consume particles of 5 to 20µm in equivalent spherical diameter. The length of *P. tricornutum* (19µm), being close to the end of this range, might thus hinder its ingestion by CoTS larvae.

The case of *T. lutea* was somewhat particular: while no preference or avoidance was detected in the mixed-species experiment, *T. lutea* was ingested at

a much higher rate when offered as a single species. Its very low energy content meant that, despite more algal cells being ingested, the consumption of this species led to the lowest energy gain for CoTS larvae. Such an impact was demonstrated for other zooplankton species: for example, Baldwin [18] showed that oyster larvae ingestion rates were three times higher for algae with a low C/N ratio than for algae with a high ratio. Here, *T. lutea* was characterized by the lowest nitrogen content and second lowest C/N ratio, indicating that CoTS larvae might need to consume more of this species than the others to satisfy energy requirements.

The interaction between algal species appeared to affect their filtration and ingestion rates by CoTS larvae, with both rates for *Dunaliella* sp. being significantly influenced by the other species present in the mixture. Notably, the presence of *P. lutheri* reduced the ingestion of *Dunaliella* sp. by CoTS larvae, without increased uptake of the former species. A similar phenomenon was previously reported for larvae of the brittle star *Ophiopholis aculeata* and the sea urchin *Strongylocentrotus droebachiensis* [10]. In these species, the presence of the diatom *Ditylum brightwellii* reduced the feeding rate on smaller cells. Similarly, the inadvertent ingestion of *P. lutheri* by CoTS larvae may have stopped the larvae from feeding all together, but the exact mechanisms underpinning food selection (and how the ingestion of one species influences that of another) remain unclear. In fact, the filtration theory for suspension feeders is still controversial, as it does not explain the variable ingestion rates observed for different particles [38,39]. A study on sand dollar *Dendraster excentricus* larvae found that they selectively fed on specific particles and refused to capture or ingest others [40], while a more recent study showed that those

larvae concentrated food particles without filtration [41]. Instead they use a different mechanism, actively capturing the particles by reversing the beat of a small portion of their cilia. In doing so, they reversed the movement of a small and targeted amount of water and were thus able to select which particle to consume. The ability of CoTS larvae to select their food could be explained by such a mechanism, but this hypothesis needs to be further investigated.

The ingestion and filtration rates found in this study were consistent with those reported by Lucas [7] based on incubation times of 24h, but differed from the higher rates reported by Ayukai [12] and Okaji et al. [13] in short-term studies (5 to 30 minutes). In a copepod feeding experiment, Frost [42] found that copepods had abnormally high ingestion rates during the first few hours of feeding, which could be an artifact of the imposed period of starvation prior to the beginning of the experiment. As such, the high feeding rates reported by Ayukai [12] and Okaji et al. [13] may correspond to the elevated cell intake that manifested only over a short experimental duration.

Preference for a particular algal species is likely to be taxon-specific. Together with *T. lutea*, *P. lutheri* represented the best diet for the larvae of *Saccostrea commercialis*, a commercially important oyster [43]. *Pecten maximus* larvae (scallop) have been shown to perform favorably when subjected to *P. lutheri* as a food source, but poorly when *Dunaliella tertiolecta* was offered [44]. In contrast, in the present study, CoTS larvae demonstrated a significant preference for *Dunaliella* sp. over *P. lutheri*, a trend previously observed for *Crassostrea gigas* larvae (oyster). Indeed, oyster larvae fed with *P. lutheri* exhibited significantly lower growth and survival rates than the same larvae fed with *Chaetoceros calcitrans* [45]. Like for CoTS, this trend might reflect the

positive effect of a more energetic food source on ingestion, and subsequently growth and survival. However, nutritional requirements seem to vary widely between species, meaning that the presence/absence of a single essential nutrient (specific to each species) will likely have a major impact on larval performance.

Phytoplankton concentration in the natural environment is often measured using chlorophyll *a* as a proxy. On the Great Barrier Reef, long term monitoring programs on the inner shelf region estimated a chlorophyll *a* concentration of 0.5  $\mu\text{g L}^{-1}$  on average [4], ranging between 0.2 and 4.6  $\mu\text{g L}^{-1}$  in flood plumes [26]. In this experiment, chlorophyll *a* concentrations ranged from 0.3 to 1.0  $\mu\text{g L}^{-1}$  in the low treatment (1000 cells  $\text{mL}^{-1}$ ) and from 0.8 to 2.6  $\mu\text{g L}^{-1}$  in the high treatment (2500 cells  $\text{mL}^{-1}$ ). These ranges are consistent with those reported on the Great Barrier Reef, and coupled with the nutritional metrics collected, suggests that chlorophyll *a* might be a poor proxy for algae nutritional content, with no clear correlation between nutritional content and chlorophyll *a* concentration.

Phytoplankton composition is highly variable through different regions and flood conditions [26], yet little is known on the exact species being present, their seasonal succession and spatial distribution. The highest phytoplankton concentrations in flood plumes of the Great Barrier Reef were dominated by *Skeletonema* sp. (a coastal diatom of 2 to 21  $\mu\text{m}$  diameter) that reached up to 4422 cells  $\text{mL}^{-1}$ , followed by *Chaetoceros* sp. that reached concentrations of 150 cells  $\text{mL}^{-1}$  [26]. Although the other algal species considered in this study are commonly used in aquaculture and in the size range consumed by CoTS, they are not naturally present on the Great Barrier Reef and future studies should focus

on natural assemblages, pending sufficient knowledge on their composition. High-throughput genetic techniques such as Next Generation Sequencing now allow further insight into GBR phytoplankton dynamics and composition. Ultimately, improved knowledge of phytoplankton composition and its effect on food uptake by CoTS larvae will help elucidate the CoTS outbreak dynamics and triggers.

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## AUTHOR CONTRIBUTIONS

SU, DF and KO conceived and designed the experiments; CL performed the experiments; CM and CL analyzed the data; SU, DF and KO contributed materials and analysis tools; CM and CL wrote the paper, with contributions from all other authors.

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## Tables

**Table 1 Generalized linear mixed-effects model (GLMM) results for filtration and ingestion rates of larvae fed with a single algal species.** Filtration and ingestion rates are predicted as a function of algal species, larvae concentration (LC), larvae age (LA), initial algae concentration (IC) and their interaction. All models include experimental date as the random effect. Shown are the number of model parameters ( $k$ ), maximum log-likelihood ( $LL$ ), the information-theoretic Akaike's information criterion corrected for small samples ( $AIC_c$ ),  $AIC_c$  weight ( $wAIC_c$ ) marginal and conditional R-squared ( $R_m$  and  $R_c$ ) as measures of the model's goodness-of-fit. Models are ordered by decreasing  $wAIC_c$ . Only models with  $wAIC_c > 0.001$  are shown, in addition to the null model.

Model	$k$	$LL$	$AIC_c$	$wAIC_c$	$R_m$	$R_c$
<i>Filtration rate</i>						
Species*IC + Species*LA + (1 date)	17	-324.8	744.7	0.920	65.70	81.80
Species*IC + Species*LA + LA + (1 date)	18	-322.4	749.5	0.080	64.50	82.10
1 + (1 date)	3	-426.1	858.4	0.000	0.00	39.80
<i>Ingestion rate</i>						
Species*LA + IC + (1 date)	13	-339	740.3	0.996	73.70	90.90
Species + IC + LA + (1 date)	9	-357.4	751.2	0.004	73.70	91.40
1 + (1 date)	3	-447.3	900.9	0.000	0.00	41.20

**Table 2. Generalized linear mixed-effects model (GLMM) results for filtration and ingestion rates of *Dunaliella sp.* for larvae fed with 1:1 mixture.** Filtration and ingestion rates of *Dunaliella sp.* are predicted as a function of algal species, larvae concentration (LC), larvae age (LA), initial algae concentration of *Dunaliella sp.* (IC dun), initial algal concentration for the second species (IC), and their interaction. All models include experimental date as the random effect. Shown are the number of model parameters ( $k$ ), maximum log-likelihood ( $LogL$ ), the information-theoretic Akaike's information criterion corrected for small samples ( $AIC_c$ ),  $AIC_c$  weight ( $wAIC_c$ ) marginal and conditional R-squared ( $R_m$  and  $R_c$ ) as measures of the model's goodness-of-fit. Models are ordered by decreasing  $wAIC_c$ . Only models with  $wAIC_c > 0.001$  are shown, in addition to the null model.

Model	k	LL	$AIC_c$	$wAIC_c$	$R_m$	$R_c$
<i>Filtration rate</i>						
Species * IC * IC dun + LA + (1   date)	22	-317.1	779.9	0.980	27.0	63.5
Species * IC * IC dun + LA + LA + (1   date)	23	-315	788.7	0.012	29.0	68.6
Species * IC + Species * IC dun + Species * LA + (1   date)	21	-328.3	790.2	0.006	27.5	67.5
Species * IC + Species * IC dun + LA + (1   date)	17	-349.7	792.3	0.002	26.8	64.3
1 + (1   date)	3	-422.4	851.1	0.000	0.0	38.0
<i>Ingestion rate</i>						
Species * IC * IC dun + LA + (1   date)	22	-243.8	633.3	0.980	71.4	87.2
Species * IC + Species * IC dun + LA + (1   date)	17	-274.6	642.3	0.011	69.2	86.7
Species * IC * IC dun + LA + LA + (1   date)	23	-242.4	643.6	0.006	71.3	88.0
1 + (1   date)	3	-399.4	805	0.000	0.0	27.8

**Table 3 Generalized linear mixed-effects model (GLMM) results for filtration rates and ingestion rates of the second species for larvae fed with 1:1 mixture.**

Filtration and ingestion rates of the second species offered along *Dunaliella sp.* in a 1:1 mixture are predicted as a function of algal species, larvae concentration (LC), larvae age (LA), initial algae concentration of *Dunaliella sp.* (IC dun), initial algal concentration for the second species (IC) and their interaction. All models include experimental date as the random effect. Shown are the number of model parameters (k), maximum log-likelihood (LL), the information-theoretic Akaike's information criterion corrected for small samples (AICc), AICc weight (wAICc), and the marginal and conditional R-squared (R<sub>m</sub> and R<sub>c</sub>) as measures of the model's goodness-of-fit. Models are ordered by decreasing wAICc. Only models with wAICc > 0.001 are shown, in addition to the null model.

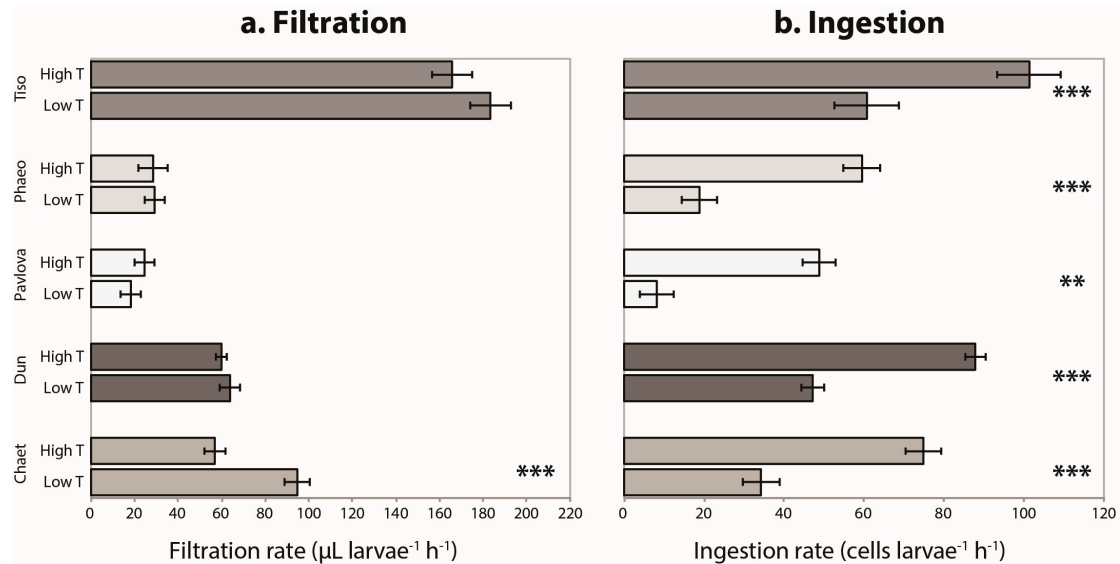
Model	k	LL	AIC <sub>c</sub>	wAIC <sub>c</sub>	R <sub>m</sub>	R <sub>c</sub>
<i>Filtration rate</i>						
Species * IC * IC dun + LA + (1   date)	22	-317.3	780.4	0.985	75.0	79.4
Species * IC * IC dun + LA + LA + (1   date)	23	-316	790.7	0.006	74.3	79.6
Species * IC + Species * IC dun + (1   date)	16	-353.3	790.9	0.005	75.5	80.1
Species * IC + Species * IC dun + LA + (1   date)	17	-349.7	792.4	0.002	75.1	80.0
Species * IC + Species * IC dun + Species * LA + (1   date)	21	-329.6	793.0	0.002	74.7	79.6
1 + (1   date)	3	-461.6	929.4	0.000	0.0	39.9
<i>Ingestion rate</i>						
Species * IC * IC dun + LA + (1   date)	22	-243.2	632.2	0.980	83.4	91.9
Species * IC + Species * IC dun + LA + (1   date)	17	-274	640.9	0.012	81.2	91.2
Species * IC * IC dun + LA + LA + (1   date)	23	-242.2	643.1	0.004	83.1	92.1
Species * IC + Species * IC dun + (1   date)	16	-279.6	643.5	0.003	78.9	91.4
1 + (1   date)	3	-410.1	826.5	0.000	0.0	45.0

**Table 4. Morphometry, chemical composition and energetic value of the five algal species on a per cell basis (mean and 95% confidence interval, in brackets).**

Values pertaining to lipid, carbon, nitrogen and chlorophyll concentration (pg/cell) were obtained via direct analytical measurement, whilst protein concentration and energetic content were calculated according to published conversion factors. For each species, a sample size of N = 30 was used to determine length and width, and N = 2 for the other parameters.

	<i>Dunaliella</i> sp.	<i>Isochrysis</i> sp	<i>Chaetoceros</i> sp.	<i>P. lutheri</i>	<i>P. tricornutum</i>
Length (µm)	7.92 (7.37-8.47)	5.01 (4.77-5.25)	9.18 (8.65-9.72)	5.91 (5.51-6.3)	18.92 (17.94-19.9)
Width (µm)	5.43 (5.15-5.71)	5.7 (5.46-5.94)	6.5 (6.1-6.9)	5.17 (4.94-5.4)	3.48 (3.31-3.64)
Carbon (pg/cell-1)	33.29 (33.01-33.58)	15.39 (15.09-15.68)	49.87 (48.56-51.18)	22.97 (22.14-23.79)	24.2 (23.71-24.69)
Nitrogen (pg/cell)	6.58 (6.54-6.61)	2.53 (2.44-2.62)	7.9 (7.44-8.36)	3.57 (3.52-3.62)	3.85 (3.73-3.97)
C/N	5.06 (4.99-5.13)	6.07 (5.97-6.17)	6.31 (6.11-6.51)	6.42 (6.28-6.57)	6.27 (6.2-6.34)
Chlorophyll (pg/cell)	1.05 (0.99-1.12)	0.35 (0.32-0.38)	0.75 (0.7-0.8)	0.58 (0.55-0.6)	0.31 (0.3-0.33)
Lipid (pg/cell)	15.58 (15.49-15.66)	6.79 (5.48-8.09)	17.33 (16.53-18.13)	10.54 (8.96-12.12)	9.93 (9.5-10.36)
Protein (pg/cell)	32.9 (32.7-33.05)	12.65 (12.2-13.1)	39.5 (37.2-41.8)	17.85 (17.6-18.1)	19.25 (18.65-19.85)
Energy (10 <sup>-9</sup> kJ/cell)	1.39 (1.38-1.40)	0.57 (0.50-0.63)	1.62 (1.53-1.70)	0.84 (0.77-0.91)	0.85 (0.81-0.88)

## 1. Figures



**Figure1. Filtration and ingestion rates for larvae fed with one algal species.**

Predicted filtration (a) and ingestion (b) rates of larvae fed with 5 different species.

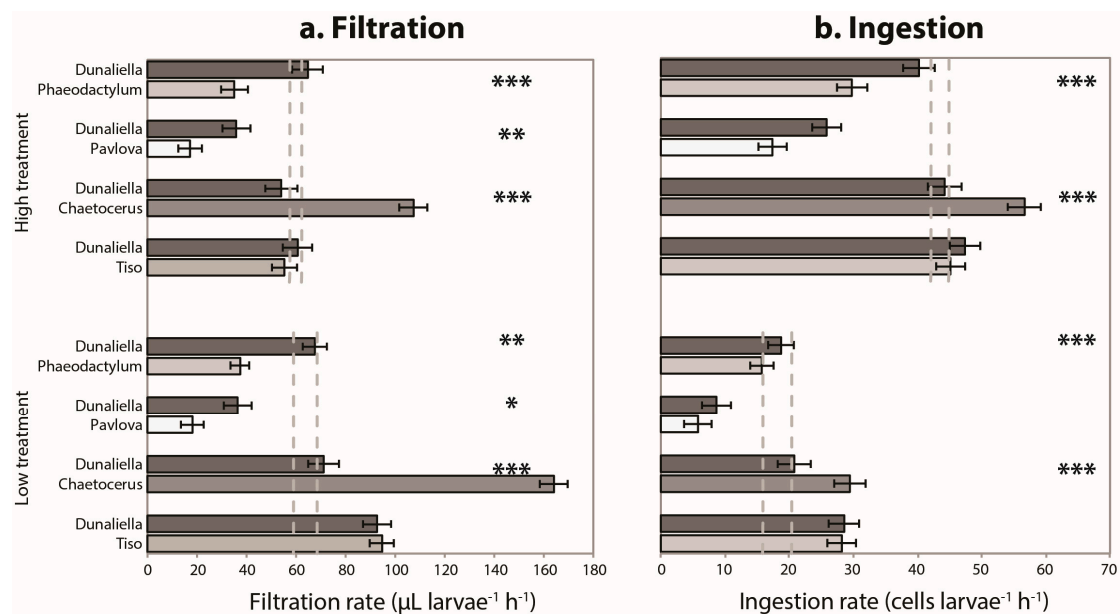
Predictions are made using GLMM models. High treatment represents an initial concentration of 2500 cells  $\text{mL}^{-1}$ , low treatment an initial concentration of 1000 cells  $\text{mL}^{-1}$ . The error bars represents the 95% confidence intervals. Asterisks represent significantly different rates between low- and high-concentration treatments (Student's

*t*-test; \*:  $P < 0.05$ , \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ). With Tiso: *Tisochrysis lutea*, Phaeo:

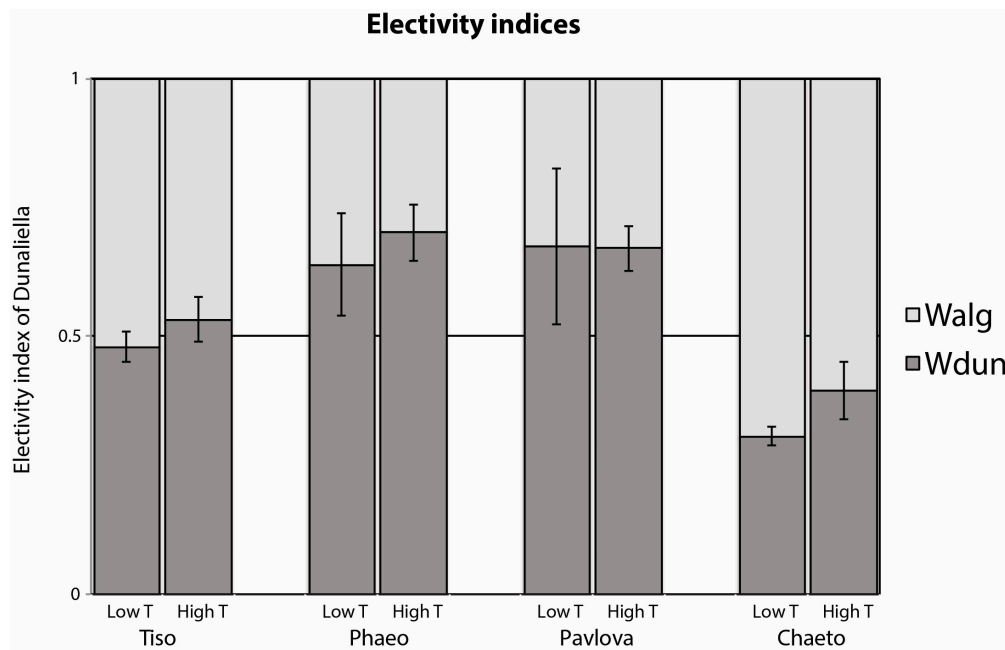
*Phaeodactylum tricornutum*, Pavlova: *Pavlova lutheri*, Dun: *Dunaliella* sp., Chaet:

*Chaetoceros* sp.





**Figure 2. Filtration and ingestion rates for larvae fed with a 1:1 mixture of two algae species.** Predicted filtration (a) and ingestion (b) rates for each of the two algae for the 4 food mixtures. Predictions are made using GLMM models. High treatment represents an initial concentration of 2500 cells mL<sup>-1</sup> (1250 cells mL<sup>-1</sup> of each species), low treatment an initial concentration of 1000 cells mL<sup>-1</sup> (500 of each species). The error bars represent the confidence intervals. The grey dotted lines represent the predicted 95% confidence interval of filtration and ingestion rates for larvae fed with *Dunaliella* sp. alone (filtration rate for 2500 and 1000 cells mL<sup>-1</sup>, half of the ingestion rate for 2500 and 1000 cells mL<sup>-1</sup> of *Dunaliella* sp.). Asterisks represent significantly different rates between *Dunaliella* sp. and the second species (Student's *t*-test; \*:  $P < 0.05$ , \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ). With Tiso: *Tisochrysis lutea*, Phaeo: *Phaeodactylum tricornutum*, Pavlova: *Pavlova lutheri*, Dun: *Dunaliella* sp., Chaet: *Chaetoceros* sp. Differences in filtration and ingestion rates between high and low treatments were significant for all species ( $P < 0.05$ ), except for the filtration rate of *Dunaliella* sp. when mixed with *P. lutheri* ( $P = 0.50$ ) or *P. tricornutum* ( $P = 0.81$ ), or that of *P. lutheri* when mixed with *Dunaliella* sp. ( $P = 0.90$ ).



**Figure 3. Electivity indices for larvae fed with a 1:1 mixture of two algal species.**

Mean electivity index for each of the 4 food mixtures. High treatment represents an initial concentration of 2500 cells mL<sup>-1</sup> (1250 cells mL<sup>-1</sup> of each species), Low treatment an initial concentration of 1000 cells mL<sup>-1</sup> (500 of each species). The lower bars (Wdun, dark grey) represent the electivity for *Dunaliella* sp., and the upper bar (Walg) the electivity for the second algae present in the mixture. Electivity 'Wdun' above 0.5 (grey dotted line) indicates that the larvae selectively ingested *Dunaliella* sp. over the other species. The error bars are 95% confidence interval.

**Table S1** Initial algal concentrations determined by flow cytometry in comparison to expected values.

Experiment/treatment	Expected initial concentration (cells mL <sup>-1</sup> )	Actual measured concentration (cells mL <sup>-1</sup> )	
		Mean $\pm$ standard deviation	Minimum-maximum
<i>Single-species</i>			
Low concentration	1000	848 $\pm$ 180	520 – 1210
High concentration	3000	2563 $\pm$ 500	1530 - 3780
<i>Mixed-species (total concentration)</i>			
Low concentration	1000	947 $\pm$ 131	740 – 1310
High concentration	3000	848 $\pm$ 180	1860 – 3630
<i>Mixed-species (individual concentrations)</i>			
Low concentration	500	461 $\pm$ 110	340 – 760
High concentration	1500	1315 $\pm$ 252	850 - 2070

