Experimental Methods

To investigate if different species of macroalgae, and potentially their phenolic content, affect invertebrate larval survival, *C. robusta* larvae were placed in petri dishes with various species of algae and larval survival was recorded. The total phenolic content (TPC) of the algae was analyzed and correlated to larval survival.

Study Species

Ciona robusta (previously known as *Ciona intestinalis* type A (Brunetti et al. 2015)) is a solitary ascidian that occupies the shallow subtidal with an invasive range in all oceans, but is most common in the Mediterranean Sea, the Pacific Ocean, and the Atlantic coast of South Africa (Brunetti et al. 2015). Its native range is, however, currently unknown because the distinction between *C. intestinalis* Type A (now *C. robusta*) and *C. intestinalis* Type B was only recently made (Brunetti et al. 2015). In the Santa Barbara Channel (SBC), as in many other parts of the world, *C. robusta* fouls artificial structures (Lambert and Lambert 2003; Shenkar et al. 2018), allowing individuals to be easily collected from harbors. Larvae are then easily reared in a laboratory (Chiba et al. 2004; Kourakis et al. 2021), and the various life stages manipulated (Hotta et al. 2020).

Ciona species are simultaneous hermaphrodites, releasing both sperm and eggs at the same time but are self-sterile (Jiang and Smith 2005). When the sperm from one individual fertilizes the egg from another, they form embryos that develop into free-swimming tadpole larvae which then metamorphose into sessile adults (Chiba et al., 2004). Because *Ciona* species have become model organisms, the various life stages and their timings have been well studied and, because progression through the life cycle is closely regulated by temperature, this has also

been well documented (Chiba et al. 2004; Hotta et al. 2007; Hotta et al. 2020). At 18°C, healthy tadpoles swim by beating their tails, and this stage occurs between 17.5 and 24 hours post fertilization at 18°C (Hotta et al. 2020). Then, at 27 to 30 hours post fertilization (again at 18°C), tadpoles adhere to a surface by swimming directly into it, followed by absorbing its tail and becoming sessile (Hotta et al. 2020). We used these temperatures, timings, and life stages to inform our experiments that follow.

Larvae Preparation

To generate free-swimming tadpole larvae, adult *C. robusta* individuals were collected from Santa Barbara Harbor and placed in a running seawater tank with full light for several days to stimulate continual gametogenesis. To extract gametes, tunics were removed and, under a dissecting scope, incisions were made into sperm and egg ducts using fine-tipped tweezers. Eggs and sperm were then transferred onto a filter placed in filtered seawater using a glass pipette. After gametes from at least two adults were collected, gametes were washed into a petri dish coated with 1% agarose, with 10 uL of antibiotics also added to keep the larvae healthy, then kept at 18°C for 24 hours. The agarose prevented the larvae from metamorphosing and settling, thus enabling them to be used in subsequent experiments. The 24 hours allowed time for sperm to fertilize eggs, and for zygotes (Fig. 1a) to hatch into free-swimming tadpole larvae (Hotta et al. 2020) (Fig. 1b).

Experimental design

We selected eight species of algae, representative of common species of brown, red, and green algae in the SBC, to test the hypothesis that larval survival is affected by algae species

identity. Five individual samples of each algae species were collected via SCUBA between March and September 2024 and were put in a cooler until delivered to the lab. For each species of algae, we set up 10 petri dishes (60 mm x 15 mm), 5 for the treatments and 5 for the controls, and assigned each treatment dish a specimen of algae (i.e., each dish had the same species of alga but a different individual). We transferred larvae into petri dishes using a P100 micropipette set to 15.0 uL, a volume able to precisely target actively swimming, healthy individuals (larvae were considered healthy if they were free-swimming and without a kink in their tails for up to one hour after transfer) (see Hotta et al. 2020). Once larvae were verified as healthy, we then took two cores (32 mm diameter) from each alga individual and placed them in their respective dish. The petri dish was then filled with filtered seawater to make up a total volume of approximately 30 mL. To provide an upper settlement surface for larvae, a second petri dish was placed on top of the dish containing larvae such that the bottom of this upper dish was in contact with the surface of the water. This was done primarily to prevent larval mortality due to getting 'stuck' in the surface tension at the water-air interface, but also because *Ciona* larvae preferentially settle on the underside of a surface (Rius et al. 2010). Control dishes each had 10 larvae as described above but no algae. Since *Ciona* prefers to settle in darkness (Rius et al. 2010), the dishes were placed in a refrigerator, without lights, set to 18°C and left for 24 hours to facilitate larval settlement (Hotta et al. 2020) (Fig. 1c and 1d). After these 24 hours, survival was quantified. We defined survival as healthy individuals that were either 1) settled on a surface with their tail partially or fully absorbed (Fig. 1c and 1d), 2) tadpoles newly adhered to a surface, or 3) free-swimming tadpoles (Fig. 1b). Unhealthy individuals were defined as dead larvae at the bottom of the dish that were unresponsive to light stimulation, or malformed settlers. Missing data was also

recorded, i.e., any larvae/settlers of the original ten free-swimming larvae that could not be found. All observations were made using a dissecting microscope set to 160x magnification.

The experiment was repeated for the 7 remaining species and, to ensure adequate replication, the entire experiment was repeated for two more rounds, thus larvae for survival for each species of algae was quantified a total of 15 times (n = 5 x 3 rounds).

Phenolics analysis

To assess TPC of algae individuals, we used the UV method, adapted from Day (1993), Levizou and Manetas (2002), and Jarchow and Cook (2009). This method uses a UV spectrophotometer to measure the total concentration of phenolic compounds, based on their property of absorbing UV-B (Day 1992). Overall, this method is faster, easier and less costly than the more commonly employed Folin-Ciocalteu (FC) method (Levizou and Manetas 2002).

Firstly, when cores were taken from individual algae specimens for the experimental treatment dishes (see above), an additional 0.1 g of algae tissue from each sample was taken. This was rinsed in DI water, blotted dry, and then placed in a previously weighed 15 mL tube. The tube with the sample was weighed again and the weight recorded. Tubes were then placed in the drying oven at 55°C with the lids off for one week and then reweighed afterwards to get the dry weight of the algae sample. 12.5 mL of acid methanol (methanol, HCl and distilled water, 90:1:1 volume) was then added to each tube, and tubes were then shaken and boiled in a water bath set to 67°C for ten minutes. After ten minutes, the absorbance of the sample was read at 300 nm on a UV spectrophotometer in a Quartz cuvette, after first blanking the UV spectrophotometer with Nanopure water and taking a methanol only reading. Absorbance readings were then compared to a standard curve created using gallic acid with concentrations,

and phenolic content was calculated as gallic acid equivalents (GAE). We chose gallic acid as our standard instead of phloroglucinol (phloroglucinol is more commonly used as a standard for calculating phenolic content in brown algae, whereas gallic acid is more commonly used with red and green) as it is crucial to select one reference standard when comparing across groups (Torres et al. 2024), and we found gallic acid easier to work with in the lab (specifically, gallic acid resulted in reproducible linear standard curves).

Statistical Analyses

Survival, S, was calculated as the proportion of healthy individuals that were still alive after 24 hours and, to meet assumptions of normality and homogeneity of variance, was logit transformed (Equation 1), where p is the proportion of healthy larvae + 0.025 (this small value, 0.025, was added to avoid values of 0 or 1).

$$S = ln\left(\frac{p}{1-p}\right) \qquad (1)$$

Because the experimental design did not fit any conventional ANOVA model (each treatment had a paired control; controls paired to treatments because every time we ran the experiment, we had to generate a new batch of larvae), a one-way ANOVA was first carried out to test that there was no difference between the control treatments. When no difference was found, controls were eliminated from subsequent analyses.

To test that there was no difference in invertebrate larval survival between species of algae, a two-way ANOVA, with species as a fixed factor and round as a random factor, was carried out. Here, we used delta, δ, as our variable. To calculate delta for each algae treatment

dish, survival was first averaged for the five control replicates, and then the survival of each algae treatment replicate was subtracted from this (Equation 2).

$$\delta = \underline{S}_{control\ replicates} - S_{algae\ treatment\ replicate} \quad (2)$$

To test for differences in TPC between algae species, a two-way ANOVA was again employed with species as a fixed factor and round as a random factor. To meet assumptions of all ANOVAs, normality was visualized with Q-Q plots and verified using the Shapiro-Wilk test and homogeneity of variance was visualized with plots of residuals versus fitted values and verified using Levene's median test. Raw TPC data did not meet assumptions, so the data was log-transformed. When ANOVA results revealed significant differences among treatments, Tukey's post-hoc pairwise comparison tests were carried out.

To test for a correlation between larval survival and TPC, we used the Spearman Rank-Order Correlation. All analyses were carried out in R version 4.2.1 (R Core Team 2022) using packages 'car' (leveneTest function) (Fox et al. 2001) and 'agricolae' (HSD.test function) (de Mendiburu 2006). All proportions of healthy larvae did not include missing individuals, since they could not be verified as healthy or deceased.

Figures

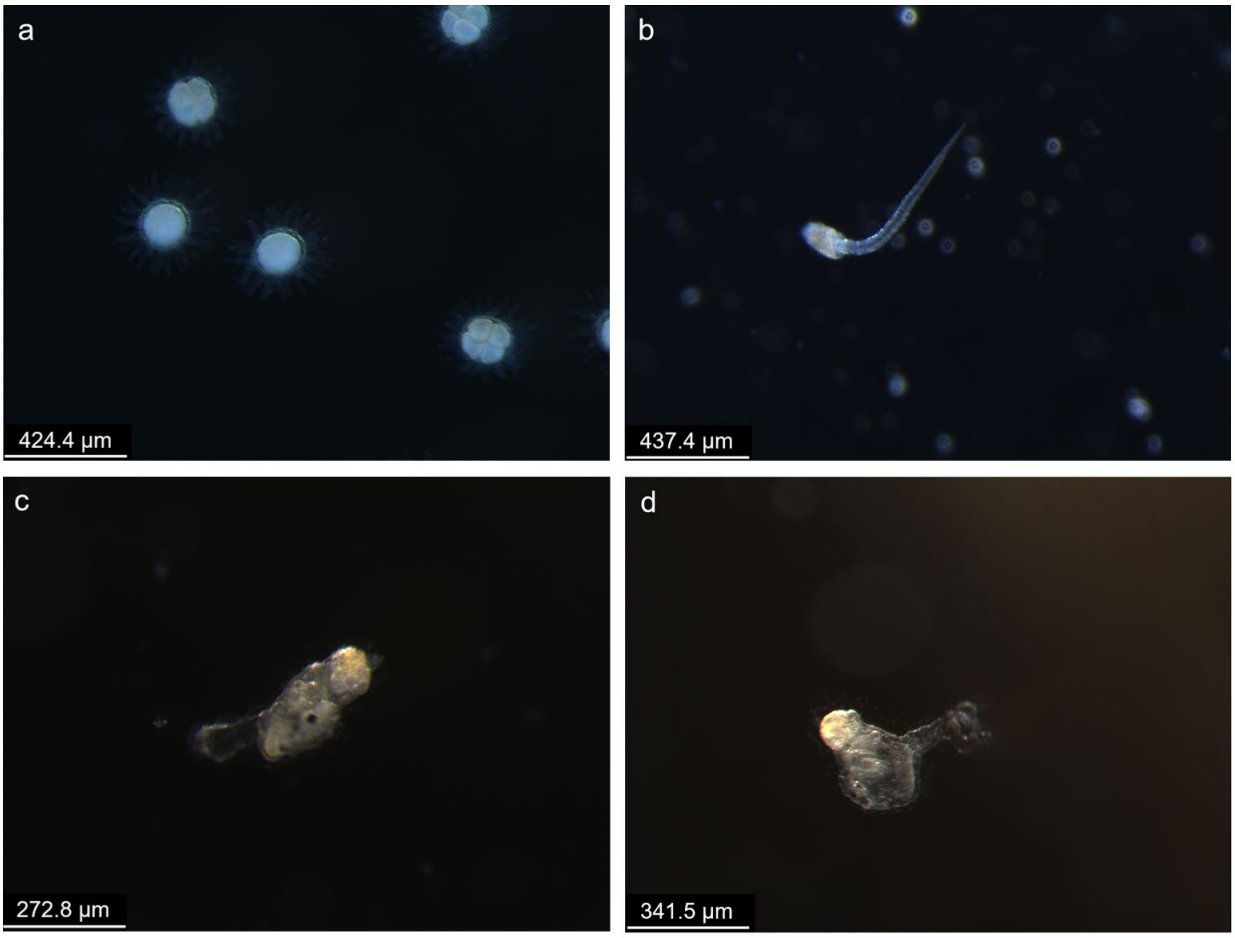

Figure 1. *Ciona robusta* larvae in dishes. (**a**) Eggs on day 1, 1-2 hours after fertilization. (**b**) *C. robusta* on day 2, 24 hours after fertilization. (**c**) *C. robusta* on day 3, 48 hours after fertilization (less advanced life stage). (**d**) *C. robusta* on day 3, 48 hours after fertilization (more advanced life stage)

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