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Patrick L Cahill, Javier Atalah, Andrew I Selwood, Jeanne M Kuhajek

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Abstract

In this study, the effects of environmental variables on larval metamorphosis of the solitary ascidian *Ciona savignyi* were investigated in a laboratory setting. The progression of metamorphic changes were tracked under various temperature, photoperiod, substrate, larval density, and vessel size regimes. Metamorphosis was maximised at 18°C, 12:12 h subdued light:dark, smooth polystyrene substrate, and 10 larvae mL^{-1} in a twelve-well tissue culture plate. Eliminating the air-water interface by filling culture vessels to capacity further increased the proportion of metamorphosed larvae; 87 ± 5% of larvae completed metamorphosis within 5 days compared to 45 ± 5% in control wells. The effects of the reference antifouling compounds polygodial, portimine, oroidin, chlorothalonil, and tolylfluanid on *C. savignyi* were subsequently determined, highlighting (1) the sensitivity of *C. savignyi* metamorphosis to chemical exposure and (2) the potential to use *C. savignyi* larvae to screen for bioactivity in an optimised laboratory setting. The compounds were bioactive in the low ng mL^{-1} to high µg mL^{-1} range. Polygodial was chosen for additional investigations, where it was shown that mean reductions in the proportions of larvae reaching stage E were highly repeatable both within (repeatability = 14 ± 9%) and between (intermediate precision = 17 ± 3%) independent experiments. An environmental extract had no effect on the larvae but exposing larvae to both the extract and polygodial reduced potency relative to polygodial alone. This change in potency stresses the need for caution when working with complex samples, as is routinely implemented when isolating natural compounds from their biological source. Overall, the outcomes of this study highlight the sensitivity of *C. savignyi* metamorphosis to environmental variations and chemical exposure.

1 Introduction

The Pacific transparent ascidian *Ciona savignyi* Herdman (Cionidae) is a Japanese native with an expanding invasive range that currently includes Argentina, British Columbia, California, New Zealand, Puget Sound, and Spain (Fofonoff et al. 2003, Lambert and Lambert 1998, Smith et al. 2010). *Ciona savignyi* is recognised as a problematic biofouling organism; it reproduces rapidly in invaded environments and can dominate man-made and natural substrates (e.g., Cohen et al. 1998, Zvyagintsev et al. 2007). This hermaphroditic species can spawn year-round in temperate regions (Nomaguchi et al. 1997; P. Cahill pers obs), with each individual releasing hundreds or thousands of eggs to be fertilised in the water column (Hendrickson et al. 2004, Jiang and Smith 2005).

Any attempt to counteract the invasive tendencies of *C. savignyi* requires an in-depth understanding of this species life-history characteristics. A key step in the biofouling process is the transition from free-swimming larva to sessile adult (Pawlik 1992). Larvae must contact a suitable surface upon which to settle, choose to attach, and then undergo a complex series of morphological changes to form established juveniles. Marine larvae typically respond to a range of environmental and con-specific cues, with a high degree of variability in responses between species (Jackson et al. 2002, Rodriguez et al. 1993). Little information is available on the extrinsic factors that stimulate *C. savignyi* to metamorphose; identifying these cues will facilitate the development of targeted treatment technologies and mitigation techniques.

In particular, the metamorphic process may be sensitive to exposure to chemical compounds. It has been shown that *C. savignyi* metamorphosis is inhibited by the natural antifouling agent polygodial (Cahill and Kuhajek 2014), with other natural (e.g., oridin or portimine; Selwood et al. 2013, Tsukamoto et al. 1996) and synthetic (e.g., chlorothalonil and tolyfluanid; Voulvoulis et al. 1999) compounds also likely to be effective against *C. savignyi* metamorphosis. Screening for effects on *C. savignyi* metamorphosis has potential to identify targeted lead compounds, including both known compounds and novel natural compounds. In the case of novel natural compounds, initial screening and isolation typically involves working with complex biological extracts, with potential for interactions between constituents of the extract (Colegate and Molyneux 2007). Understanding how environmental factors influence metamorphosis will improve our ability to reliably quantify antimetamorphic effects.

In addition to the interest in *C. savignyi* as a marine invader, this species has been increasingly studied as a model organism for developmental biology (Corbo et al. 2001, Sasakura et al. 2012, Satoh 2003). Ascidians occupy an intriguing evolutionary position as sister clade to the vertebrates (Lemaire 2011, Satoh and Levine 2005, Schubert et al. 2006), meaning they a can afford insights into developmental biology in general. Embryos and larvae of *C. savignyi* can be produced in large numbers in the lab, the latter undergoing a defined progression of metamorphic changes to form established juveniles within 7 days (Cirino et al. 2002, Hendrickson et al. 2004, Kourakis et al. 2010). Many studies have examined the intrinsic determinants driving the progression from egg, to free-swimming larvae, to sessile adult in *C. savignyi* (e.g., Imai et al. 2002a, Imai et al. 2002b, Kimura et al. 2003). Identifying extrinsic conditions that

stimulate ascidian larvae to settle and metamorphose will also provide a context for these molecular and biochemical investigations (Morse 1990).

In this study, the effects of extrinsic environmental factors on *C. savignyi* metamorphosis were examined in a laboratory setting. Variables investigated included temperature, photoperiod, substrate, larval density, and vessel size. The most susceptible stages of the larval settlement and metamorphosis processes were identified, providing a relevant experimental end-point for investigating the effects of reference antifouling compounds on the larvae.

2 Material and Methods

2.1 Culture and Spawning

Adult *C. savignyi*, collected from the underside of pontoons at Nelson Marina, Nelson, New Zealand, were housed in water lily baskets suspended in 10-L glass aquaria for up to three weeks. Aquaria were supplied with 10 L seawater h^{-1} as part of a 1000-L recirculating system held at 18 ± 1°C, 34 ± 1 psu, and 300 ± 50 mV ORP; constant full-spectrum florescent light prevented premature spawning. Daily, the flow of water to the aquaria was cut off for 3 h while *C. savignyi* were fed 250 mL of an 8 – 9 × 10^6 cells mL^{-1} *Isochrysis galbana* Parke culture. Three gravid individuals with densely packed egg and sperm ducts were spawned according to Cirino et al. (2002). Ventral incisions were made to expose the egg and sperm ducts. The egg duct of each individual was pierced with a Pasteur pipette and the eggs transferred to a glass Petri dish (90 mm dia., 68 mL vol.) filled with 20 mL of 0.3-µm filtered and UV-sterilized seawater (FSW). Sperm were then harvested and transferred to a glass Petri dish (90 mm dia., 68 mL vol.) containing 50 mL FSW. Each dish of eggs received eight drops of sperm suspension from each of the two other individuals. After incubating at 18°C for 1 h, fertilized eggs were strained through a 20-µm sieve, rinsed three times with 25 mL reconstituted seawater (RSW; 33 ± 0.5 psu; Red Sea Salt, Red Sea Aquatics, Cheddar, UK), transferred to a glass Petri dish along with 25 mL RSW, and held at 18°C for 18 h to hatch. Hatched larvae were transferred to conical flasks and diluted with RSW as necessary to yield desired larval densities.

2.2 Temperature, photoperiod, and substrate

115 The temperature, photoperiod, and substrate preferences of larvae were assessed in
116 three separate experiments. Firstly, the effects of exposing larvae to 15, 18, 21, and
117 25°C were evaluated. For the photoperiod experiment, larvae were exposed to the
118 following lighting regimes: 24:0, 12:12, 0:24 h intense (100 ± 10 µmol m^{-2} s^{-1}) or
119 subdued (10 ± 5 µmol m^{-2} s^{-1}) light:dark. Substrate type (polystyrene, acrylic, or glass)
120 and texture (smooth or rough) were assessed using unlined wells (polystyrene) or wells
121 lined with acrylic discs (35-mm diameter) or glass cover slips (35-mm diameter, Gerhard
122 Menz GmbH, Saarbrücken, GE). Untreated unlined wells, acrylic discs, and glass cover
123 slips comprised smooth treatments. Sandblasted acrylic and sanded (800-grit
124 sandpaper) glass and polystyrene comprised rough treatments; it should be noted that
125 these surface treatments likely resulted in different feature sizes for each of the three
126 substrate types and that the walls of the wells remained untreated in all cases.

127 All three experiments (temperature, photoperiod, and substrate) were performed
128 in six-well tissue culture plates (Corning® Costar®, Corning Inc., Corning, NY; 36 mm
129 dia.; 17.5 mL vol.) filled with 10-mL aliquots of 2.5 larvae mL^{-1} RSW. Default parameters
130 were 18°C, 12:12 h subdued light:dark, and unlined smooth wells; three replicates were
131 performed in all cases (n = 3). Culture waters were renewed with RSW every other day
132 and settled larvae were scored according to metamorphic stage (Figure 1) after 1, 3, 5,
133 and 7 days. Unattached larvae or larvae adhered to the meniscus were not counted. A
134 proxy for daily metamorphic progress was calculated based on the number of larvae
135 counted at each developmental stage as follows:

136
$$\begin{aligned} &Metamorphic\ progress \\ &= (Stage\ A\ larvae \times 1) + (Stage\ B\ larvae \times 2) + (Stage\ C\ larvae \times 3) \\ &+ (Stage\ D\ larvae \times 4) + (Stage\ E\ larvae \times 5) + (Stage\ F\ larvae \times 6) \end{aligned}$$

2.3. Vessel size, larval density, and fill volume

Vessel size was assessed in conjunction with larval density. Polystyrene Petri dishes (LabServ, Thermo Fisher Scientific, AU; 90 mm dia.; 68 mL vol.), six-well plates, and twelve-well plates (Corning® Costar®; 23 mm dia.; 6.5 mL vol.) were filled with 25-, 10-, or 5-mL aliquots of larval suspension, respectively. Four larval densities (2.5, 5, 10, and 15 larvae mL^{-1}) were tested in each of the three vessel sizes, yielding twelve vessel size × larval density treatments in total (n = 3). Vessels were held at 18°C, 12:12h subdued light to dark and culture waters were renewed every other day. After 5 days, the number of larvae that had completed stage E of metamorphosis were counted (Figure 1).

Fill volume was assessed using twelve-well plates and a larval density of 10 larvae mL^{-1}. Treatment wells were filled to capacity with 7.1 mL of larval suspension so that the seawater contacted the underside of the lid, eliminating the air-water interface. Controls wells were filled with 5 mL of larval suspension (n = 3). The temperature, photoperiod, and experimental end-point matched those used for the vessel size and larval density experiments but culture waters were not renewed for the duration of this experiment.

2.4 Carrier solvent and reference compounds

The sensitivity of larvae to two common carrier solvents was assessed in twelve-well plates with 7.1 mL aliquots of 10 larvae mL^{-1} RSW; held at 18°C, 12:12 h subdued light to dark. Concentrations of ethanol and dimethyl sulfoxide (DMSO) evaluated were 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 40 µL mL^{-1}; controls contained no solvent (n = 3). Larvae that had completed stage E of metamorphosis were counted after 5 days.

159 Five reference compounds, polygodial (ENZO Life Sciences, Farmingdale, NY),
160 portimine (Cawthron Natural Compounds, Nelson, NZ), oroidin (ENZO Life Sciences),
161 chlorothalonil (Sigma-Aldrich, St Louis, MO), and tolylfluanid (Sigma-Aldrich), were
162 screened using the protocol outlined for carrier solvent. With the exception of polygodial
163 (1, 2.5, 5, 10, 15, 20, 40, 80 ng mL^{-1}) and portimine (0.05, 0.1, 0.5, 1, 5, 10, 20 ng mL^{-1}),
164 concentrations tested against the larvae were 0.001, 0.005, 0.01, 1, 5, and 10 µg mL^{-1}.
165 Stock solutions prepared in 20% (v/v) ethanol (polygodial), RSW (portimine), or 20%
166 (v/v) DMSO (oroidin, chlorothalonil, tolylfluanid) were added to wells to yield the desired
167 test concentrations; control wells contained solvent only (n = 3).

168 Polygodial treatments (5, 10, and 50 ng mL^{-1}; n = 3) were subsequently included
169 in eight independent experiments performed over a 1-year period, allowing repeatability
170 (i.e., intra-run variability) and intermediate precision (i.e., inter-run variability) to be
171 estimated. In a separate experiment assessing the potential for interactions among
172 complex mixtures of natural compounds, the potency of pure polygodial was compared
173 to that of polygodial enriched with an environmental extract. The extract was produced
174 by eluting 10 L seawater that ~ 400 g of green-lipped mussels, *Perna canaliculus*
175 Gmelin, had been cultured in for 1 day through a 20-cm^3 column of Diaion HP20 resin
176 (Mitsubishi Chemical, Tokyo, JP). The column was flushed with 50 mL ethanol, and the
177 resulting extract dried and re-suspended in 1 mL 20% (v/v) ethanol. Polygodial stock
178 solutions prepared in 1 mL of 20% (v/v) ethanol or 1 mL of extract were added to wells
179 to yield final polygodial concentrations of 2.5, 5, 10, and 50 ng mL^{-1} (n = 3).

180 ***2.5 Data Analyses***

Temperature, photoperiod, and substrate data were analyzed using one-way linear mixed-effects modelling (Bolker et al. 2009), with metamorphic progress (Section 2.2) as the response variable, treatment as a fixed factor, and time as a continuous covariate. Replicate was included as a random effect to account for the repeated-measures experimental design. Reported p-values are based on the *t* distribution of the ratios between the estimates and their standard errors (Pinheiro and Bates 2006). Principal response curves (PRC), a redundancy analysis for multivariate responses in repeated-measures design (Van den Brink and Ter Braak 1998, Van den Brink and Ter Braak 1999), were used with 999 permutations to identify the metamorphic stages that were driving treatment effects (> 0.5 signifies strong treatment effect). Mean absolute PRC coefficients for the number of larvae in each metamorphic stage (A – F) at each time point were calculated by averaging the values from the temperature, photoperiod, and substrate datasets. The effects of larval density and vessel size were assessed using a two-way factorial ANOVA with the number of Stage E larvae on day 5 as the response variable. The effect of fill-volume on the number of larvae reaching Stage E within 5 days was tested using a Student's t-test, and carrier solvent using one-way ANOVA followed by Dunnett's post-hoc test. Dose-response curves were plotted for the reference compounds using four parameter logistic curve fitting, and the corresponding concentrations that reduced the number of larvae reaching Stage E by 50% relative to blank controls (EC_{50}) calculated (Kuo et al. 1993). Repeatability (relative bias) and intermediate precision for the replicate polygodial experiments were calculated in accordance with USP <1033> (2012). Analyses were performed using R 2.13.1 (R Development Core Team 2015) and SigmaPlot 11.0 (Systat Software Inc. 2015).

3 Results

Overall, larvae reached stages A and B of metamorphosis within 1 day, while stage C took 1.2 ± 0.06 days, stage D 3.1 ± 0.02 days, stage E 4.3 ± 0.03 days, and stage F 6.6 ± 0.04 days.

3.1 Temperature, photoperiod, and substrate

Larvae responded to variations in temperature, photoperiod, and substrate. Daily metamorphic progress was comparable for larvae reared at 15, 18, or 21°C but hindered at 25°C ($t = -4.6$, $p < 0.001$; Figure 2A). Metamorphic progress was enhanced relative to the other photoperiod regimes evaluated when exposed to 12:12 h subdued light:dark ($t = 2.1$, $p = 0.04$), and slowed by exposure to constant intense light ($t = 3.0$, $p = 0.004$; Figure 2B). Metamorphic progress was reduced when the substrate was smooth acrylic ($t = -3.5$, $p = 0.04$; Figure 2C) but there were no discernable differences between the other substrate types tested. The statistical differences observed for the temperature (PRC, $F = 10.1$, $p = 0.005$), photoperiod (PRC, $F = 8.4$, $p = 0.005$), and substrate (PRC, $F = 1.9$, $p = 0.005$) datasets were driven by the latter stages of metamorphosis. Mean absolute PRC coefficients revealed a strong treatment response for stages E (4.1 ± 0.9) and F (5.1 ± 1.5), compared to weak effects for stages A (0.0002 ± 0.0001), B (0.0005 ± 0.0003), C (0.017 ± 0.011), and D (0.27 ± 0.14).

3.2 Larval density, vessel size, and fill volume

Both density (ANOVA, $F = 21.4$, $p < 0.005$) and vessel size (ANOVA, $F = 13.3$, $p < 0.005$) significantly influenced metamorphosis. The proportion of larvae reaching Stage E within 5 days was enhanced above 10 larvae mL^{-1} for Petri dishes or 5 larvae mL^{-1} for six- and twelve-well plates. Overall, the greatest proportion of Stage E larvae were

observed in twelve-well plates (Figure 3). Larval metamorphosis was enhanced when wells were filled to capacity ($t = 5.1$, $p = 0.007$). In wells filled to capacity, 87 ± 5% of larvae completed stage E of metamorphosis within 5 days compared to 45 ± 5% in control wells.

3.3 Carrier solvent and reference compounds

No detectable effects on larval metamorphosis were observed when carrier solvent was added to the vessels at or below 20 µL mL^{-1} for ethanol or 10 µL mL^{-1} for DMSO. When dosed at 40 µL mL^{-1}, the number of larvae that reached stage E was reduced to zero in both cases (ethanol ANOVA: $F = 12.6$, $p < 0.005$; Dunnett's $p = 0.009$; DMSO ANOVA: $F = 16.0$, $p < 0.005$; Dunnett's $p = 0.02$).

Polygodial (EC_{50} = 4.5 ng mL^{-1}), portimine (EC_{50} = 1.0 ng mL^{-1}), oroidin (EC_{50} = 1.1 µg mL^{-1}), chlorothalonil (EC_{50} = 0.1 µg mL^{-1}), and tolylfluanid (EC_{50} = 0.3 µg mL^{-1}) potently inhibited metamorphosis; dose-response curves closely fit the data ($R^2 > 0.9$). The polygodial positive controls from the eight independent experiments had negative dose-dependent effects on metamorphosis, with mean reductions in the proportions of larvae reaching stage E varying by 14 ± 9% within experiments (i.e., repeatability) and 17 ± 3% between experiments (i.e., intermediate precision; Figure 4, Table 1). However, enrichment with an environmental extract decreased the potency of polygodial (Figure 5). The extract alone had no detectable effect on metamorphosis but the observed shift in potency for enriched *vs.* pure polygodial represents an approximately 80% increase in EC_{50} (7.8 vs. 4.3 ng mL^{-1}).

4 Discussion

The larvae of *C. savignyi* were well suited to laboratory culture and, as has been reported previously, they completed metamorphosis to established juveniles inside seven days (Cirino et al. 2002, Hendrickson et al. 2004). The proportion of larvae completing metamorphosis to stage E within five days exceeded 90% in some cases, but the performance of the larvae was highly dependent on environmental factors. Temperature and photoperiod strongly influenced metamorphic progress. Although no observable differences were found between 15 and 21°C, exceeding 21°C effectively halted the progression of metamorphosis. A similar optimal temperature range of 12 – 20°C has previously been reported for *C. savignyi* embryogenesis (Nomaguchi et al. 1997). Likewise, the observed enhancement of metamorphic progress for larvae reared under 12:12 h subdued light:dark fits the general rule that ascidians are initially positively phototactic but switch to being negatively phototactic when competent to settle (McHenry 2005). It follows that free swimming larvae ought to prefer a lighted environment and, when competent to settle, will utilize the dark period to settle and begin metamorphosis.

Compared to temperature and photoperiod, substrate had only limited impact on *C. savignyi* larvae. Ascidians are generally considered to be one of the least discerning marine taxa in relation to surface selection (Aldred and Clare 2014), yet there are anecdotal reports of surface selectivity in some species: *Ascidia nigra* Savigny settlement is negatively correlated with surface wettability (Gerhart et al. 1992), *Ascidia interrupta* Heller prefer sandblasted surfaces (Rae Flores and Faulkes 2008), and *Ascidiella* spp. tend to be most abundant on concrete pilings (Andersson et al. 2009).

Many other marine larvae will only attach to surfaces with defined chemical, biological, texture, or wettability characteristics (Pawlik 1992, Wahl 1989). For example, barnacle cyprids prefer smooth surfaces (Berntsson et al. 2000) and *Ulva* zoospores use quorum sensing signal molecules to seek out suitable biofilm communities (Tait et al. 2005). Granting that only a small number of simple substrate variations were tested and larvae were confined within small vessels, *C. savignyi* did not display obvious substrate preferences in the experiments performed here. The sides and bottom of the wells were of dissimilar composition, yet larvae adhered indiscriminately to all surfaces. The exception was wells lined with smooth acrylic, where the overall progression of metamorphosis was reduced by approximately 12% compared to the other surfaces tested. This apparent insensitivity to surface characteristics may contribute to *C. savignyi's* invasive character, whereby larvae will settle on a wide range of available surfaces.

When the temperature, photoperiod, and substrate datasets were combined, the earliest developmental stage of metamorphosis to show strong treatment response was stage E when the branchial basket, siphons, and stigmata develop. It has previously been noted that tail-resorption is a particularly sensitive stage of ascidian metamorphosis (e.g., Bishop et al. 2001, Eri et al. 1999, Green et al. 2002) but, in the case of *C. savignyi*, the tail resorption process (Stage B) was little affected by variations in temperature, photoperiod, or substrate. It is not clear why the later stages of metamorphosis were more sensitive to environmental variations but the lecithotrophic nature of *C. savignyi* may be partly responsible. Energy reserves within the larvae may become depleted later in metamorphosis (e.g., Jaeckle 1994, Jaeckle and Manahan

294 1989, Moran and Manahan 2003), with energy deficient larvae becoming increasing
295 susceptible to sub-optimal environmental conditions.

296 When larval density and vessel size were subsequently evaluated, the proportion
297 of larvae reaching Stage E of metamorphosis was enhanced above 5 or 10 larvae mL^{-1},
298 with twelve-well plates having the greatest proportion of Stage E larvae overall. Density-
299 dependent behaviours are common among marine larvae (Hadfield and Paul 2001);
300 examples include the gregariousness of larvae of the barnacle *Balanus amphitrite*
301 Darwin (Head et al. 2003), the tubeworm *Hydroides dianthus* Verrill (Toonen and Pawlik
302 1996), and the oyster *Ostrea edulis* L. (Bayne 1969). Enhanced metamorphosis in
303 smaller vessels could be a result of conspecific settlement cues or reduced surface area
304 to volume ratio. Under the later scenario, larvae are more likely to contact, and thus
305 adhere to, the internal surfaces of a smaller vessel.

306 Filling wells to capacity further increased the proportion of Stage E larvae
307 observed after 5 days. When wells were not filled to capacity, some *C. savignyi* larvae
308 became trapped at the air-water interface and where not considered to have
309 successfully attached (P. Cahill pers. obs.). This phenomenon has been reported
310 previously for other ascidians (e.g., Fletcher and Forrest 2011), and is probably due to
311 the larvae swimming upwards against the force of gravity for the first few hours after
312 hatching (Hendrickson et al. 2004). Because culture waters contact the underside of the
313 lid in wells that were filled to capacity, larvae are provided with an inverted surface upon
314 which to settle and are prevented from them becoming trapped at the air-water
315 interface. In line with these findings, it has been anecdotally observed that *C. savignyi*

316 adults tend to be congregated on the underside of man-made structures in the sea (P.
317 Cahill pers. obs.).

318 Evaluating the effects of select reference compounds on *C. savignyi*
319 metamorphosis highlighted the potential to use *C. savignyi* larvae to screen for
320 bioactivity under an optimised laboratory setting. The natural antifouling agent
321 polygodial (Cahill and Kuhajek 2014), the algal biotoxin portimine (Selwood et al. 2013),
322 and the synthetic antifouling biocides chlorothalonil and tolyfluanid (Voulvoulis et al.
323 1999) were bioactive in the low ng mL^{-1} to high µg mL^{-1} ranges. Tolerance of *C. savignyi*
324 larvae for the carrier solvents ethanol (20 µL mL^{-1} max.) and DMSO (10 µL mL^{-1} max.)
325 facilitated screening these compounds, which, with the exception of portimine, have
326 only limited water solubility. The results reported here meet or exceed potency
327 estimates previously determined for these compounds against other organisms. For
328 example, polygodial is effective against fungal pathogens at approximately 1 µg mL^{-1}
329 (Kubo and Himejima 1991), chlorothalonil kills water fleas and fathead minnows at 0.03
330 − 0.2 µg mL^{-1} (Sherrard et al. 2002), and tolylfluanid controls seaweed zoospores at
331 0.03 µg mL^{-1} (Wendt et al. 2013). However, systems that test the bioactivity of
332 compounds in solution must be carefully interpreted. In nature, marine larvae are
333 typically only exposed to allelopathic metabolites at surfaces, such as when they
334 contact another benthic organisms (Pawlik 1993). Perfusing larval tissues with a
335 compound, as was done here, provides a largely pharmacological rather than ecological
336 context. This pharmacological data is often used as a first step to identify promising
337 bioactive compounds but should always be followed by additional investigations, either

laboratory or field based, where the compound of interest is bound to/released from a surface (Bressy et al. 2014).

When polygodial treatments were included in eight independent experiments performed over a one year period, mean reductions in the proportion of larvae reaching stage E varied by 14 ± 9% and 17 ± 3% within and between experiments, respectively. These values represent a relatively high degree of consistency for the effects of polygodial exposure, with comparable larval systems returning repeatability estimates in the order of 5 – 30% (e.g., Piazza et al. 2012, Ross and Bidwell 2001, Stronkhorst et al. 2004). The larvae were unaffected by a complex environmental extract produced from the culture waters of green-lipped mussels but the same extract reduced the potency of polygodial to almost half. This result highlights the potential for changes in potency when working with complex chemical samples, as is common when attempting to isolate natural products (Colegate and Molyneux 2007).

Overall, this study yielded insights into the environmental factors impacting metamorphosis of *C. savignyi* larvae. This increased understanding of the interplay between the environment and the biology of *C. savignyi* is vital to understanding, and perhaps counteracting, the invasive tendencies of this organism. The findings also provide context for the growing body of research examining the biochemical and genetic determinants of *C. savignyi* metamorphosis. Demonstrating that *C. savignyi* larvae are sensitive to a range of natural and synthetic bioactive agents highlighted one potential application for these larvae. Using *C. savignyi* larvae to assess the toxicity of compounds under optimised laboratory conditions presents a potentially useful preliminary screening tool but the outcomes of such experiments must be carefully

interpreted and should be followed by ecologically relevant investigations. Nevertheless, chemicals targeting *C. savignyi* metamorphosis could potentially be used to control this pest species.

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Figure 1. Stages of *Ciona savignyi* larval development. At the moment of settlement, metamorphosis is triggered (Stage A). Tail resorption is completed in a short time (Stage B). The outer tunic layer is cut off (Stages C & D), and then two lateral siphons and a pair of functional stigmata develop (Stage E). The number of stigmata on each side of the branchial basket increases (Stage F), completing metamorphosis. Scale bars = 300 μm.

Figure 2. Effects of (A) temperature, (B) photoperiod, and (C) substrate on *Ciona savignyi* larvae. Metamorphic progress was calculated based on the sum of the number of individuals reaching each metamorphic stage converted to numerals (A = 1, B = 2, C = 3, D = 4, E = 5, F = 6). Values are means (n = 3) ± 95% confidence interval.

Figure 3. Proportion of *Ciona savignyi* larvae completing Stage E of metamorphosis within 5 days for various combinations of larval density and vessel size. Values are means (n = 3) ± 95% confidence interval.

Figure 4. Relative bias from eight independent experiments for the effects of polygodial treatments on *Ciona savignyi* metamorphosis. Values are means (n = 8) relative to a known dose-response relationship for polygodial ± 95% confidence interval.

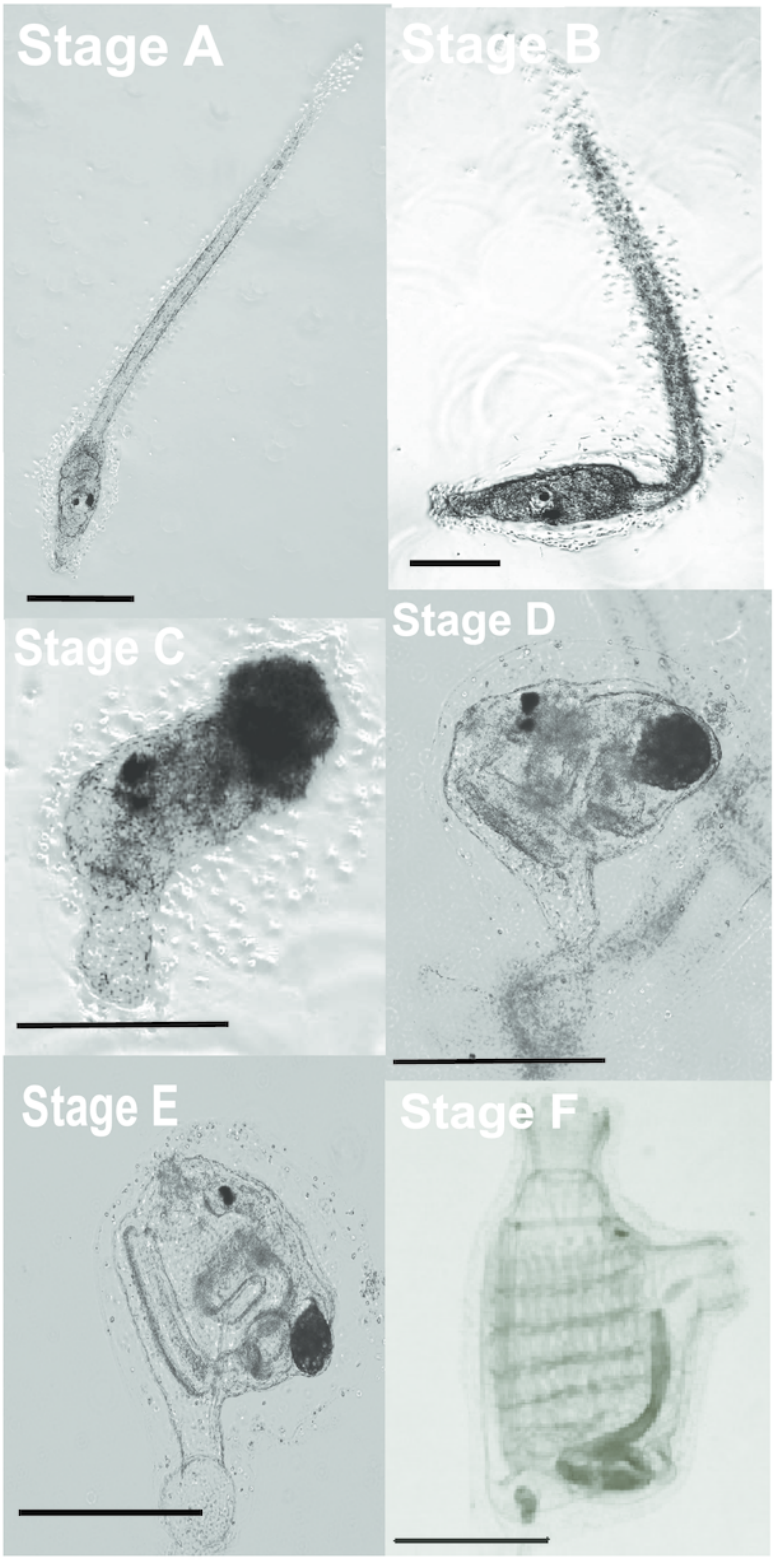
Figure 5. Relative bias for the effects of polygodial enriched with an environmental extract on *Ciona savignyi* metamorphosis. Values are means (n = 3) relative to pure polygodial ± 95% confidence interval.

545 **Table 1.** Variance component estimates and overall variability for the effects of
546 polygodial treatments on *Ciona savignyi* metamorphosis.

1

Stages of *Ciona savignyi* larval development.

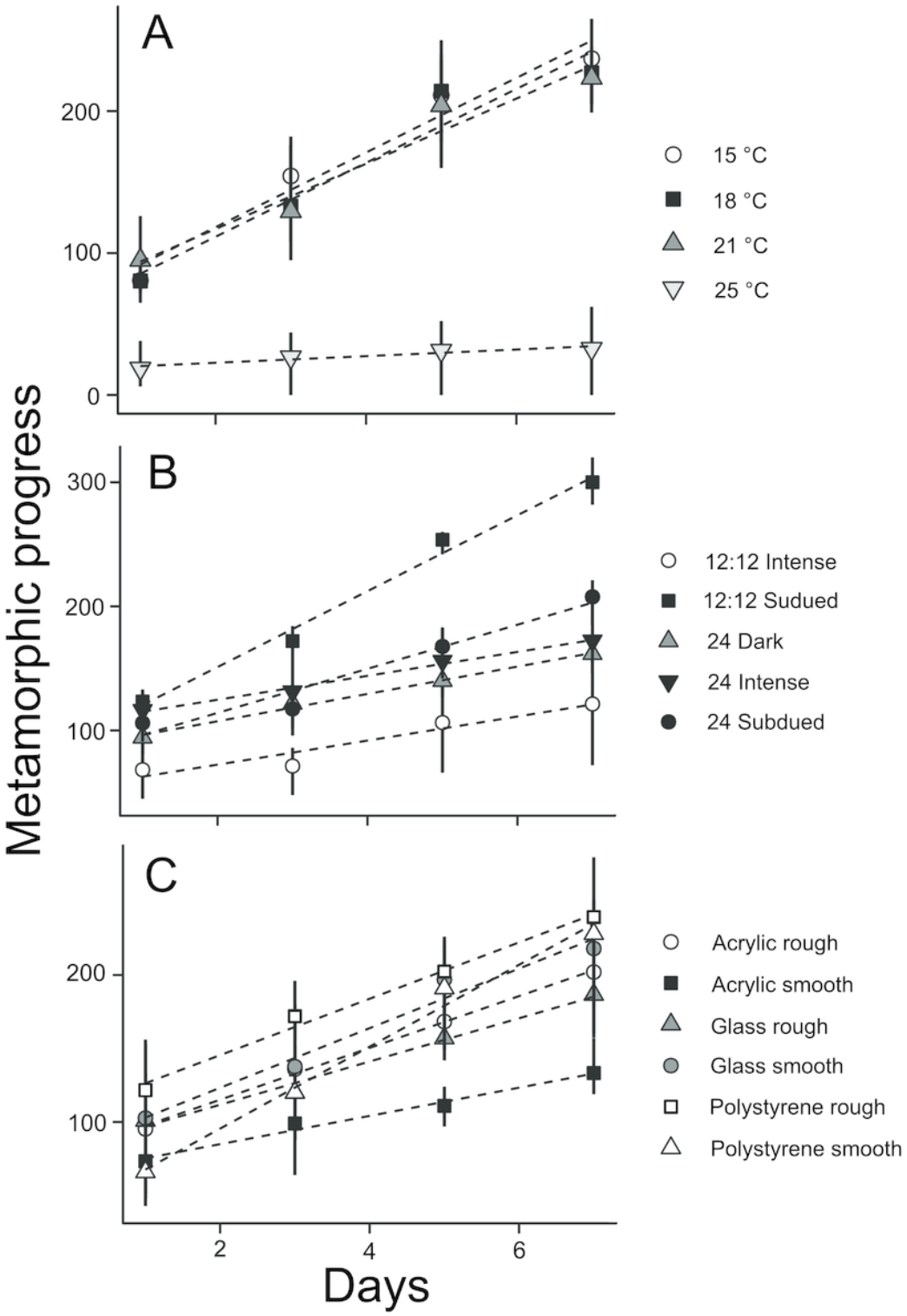
Stages of *Ciona savignyi* larval development. At the moment of settlement, metamorphosis is triggered (Stage A). Tail resorption is completed in a short time (Stage B). The outer tunic layer is cut off (Stages C & D), and then two lateral siphons and a pair of functional stigmata develop (Stage E). The number of stigmata on each side of the branchial basket increases (Stage F), completing metamorphosis. Scale bars = 300 μm.


Stage A
Stage B
Stage C
Stage D
Stage E
Stage F

2

Effects of (A) temperature, (B) photoperiod, and (C) substrate on *Ciona savignyi* larvae.

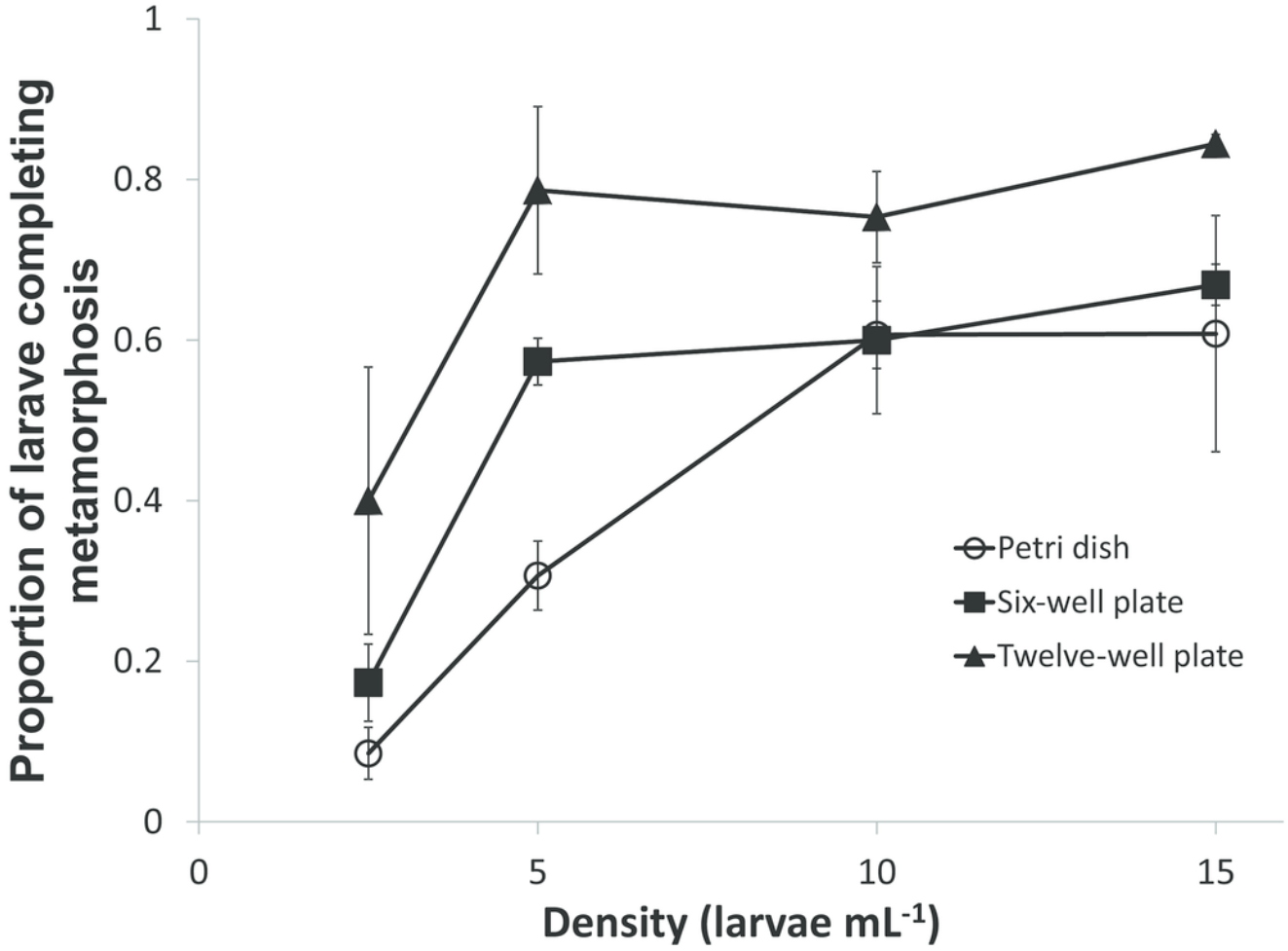
Effects of (A) temperature, (B) photoperiod, and (C) substrate on *Ciona savignyi* larvae. Metamorphic progress was calculated based on the sum of the number of individuals reaching each metamorphic stage converted to numerals (A = 1, B = 2, C = 3, D = 4, E = 5, F = 6). Values are means (n = 3) ± 95% confidence interval.


A
B
C
Metamorphic progress
Days
0
100
200
300
2
4
6
15 °C
18 °C
21 °C
25 °C
12:12 Intense
12:12 Sudued
24 Dark
24 Intense
24 Subdued
Acrylic rough
Acrylic smooth
Glass rough
Glass smooth
Polystyrene rough
Polystyrene smooth

3

Proportion of *Ciona savignyi* larvae completing metamorphosis

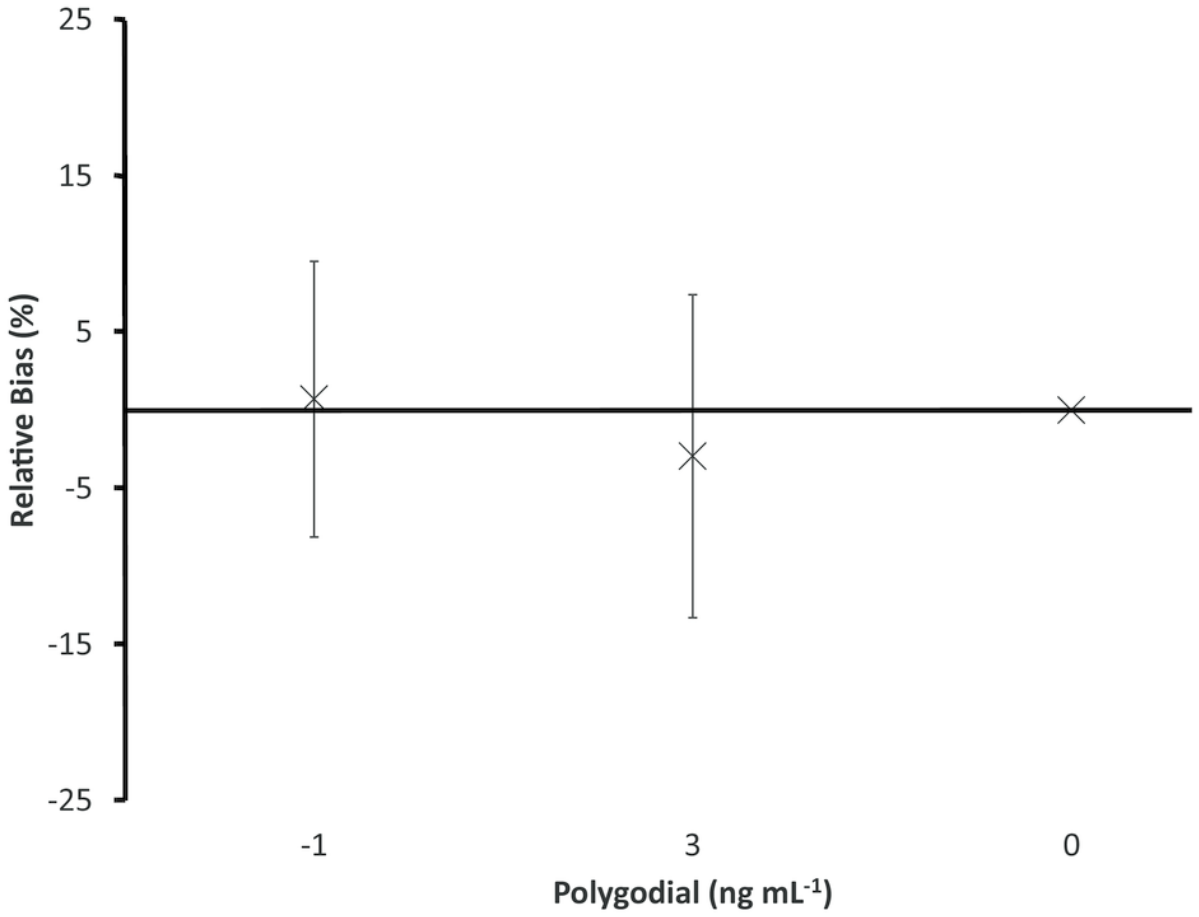
Proportion of *Ciona savignyi* larvae completing Stage E of metamorphosis within 5 days for various combinations of larval density and vessel size. Values are means (n = 3) ± 95% confidence interval.



4

Relative bias for polygodial treatments.

Relative bias from eight independent experiments for the effects of for polygodial treatments on *Ciona savignyi* metamorphosis. Values are means (n = 8) relative to a known dose-response relationship for polygodial ± 95% confidence interval.



5

Relative bias for polygodial enriched with an environmental extract.

Relative bias for the effects of polygodial enriched with an environmental extract on *Ciona savignyi* metamorphosis. Values are means (n = 3) relative to pure polygodial ± 95% confidence interval.

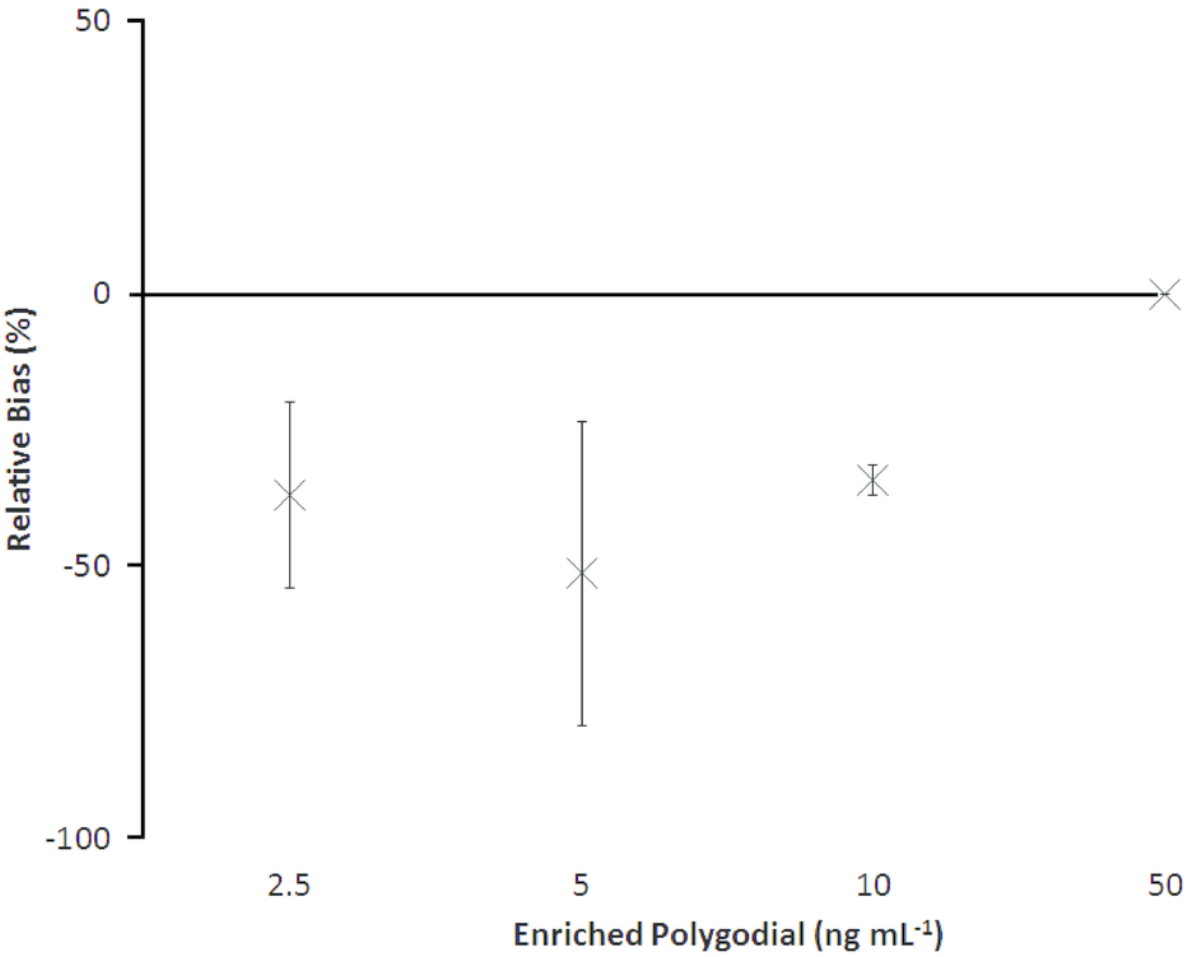


Table 1(on next page)

Overall variability for polygodial treatments.

Table 1. Variance component estimates and overall variability for the effects of polygodial treatments on *Ciona savignyi* metamorphosis.

	5 ng mL^{-1}	10 ng mL^{-1}	Mean
Var (Run)	-0.013	0.002	-0.005
Var (Error)	0.033	0.030	0.031
Overall	15%	20%	17%