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A bioassay for ascidian-specific antifouling activity

Patrick L. Cahill [a], Javier Atalah [a], Andrew I. Selwood [a], & Jeanne M. Kuhajek [a]

[a] *Cawthron Institute, 98 Halifax St East, Nelson 7010, New Zealand*

Corresponding author: Patrick Cahill; Cawthron Institute, 98 Halifax St East, Nelson 7010, New Zealand; Phone: +643 548 2319; Email: patrick.cahill@cawthron.org.nz

Abstract

Discovering and characterizing marine antifouling agents requires robust and repeatable bioassay protocols. In this study, a bioassay that tracked larval development of the solitary ascidian *Ciona savignyi* was developed. The effects of temperature, photoperiod, substrate, larval density, vessel size, and carrier solvent were investigated. Maximal larval development and bioassay reproducibility were achieved 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 the culture vessels to capacity further increased settling rates; 87 ± 5% of larvae completed metamorphosis within 5 days compared to 45 ± 5% in control wells. The performance of the bioassay was assessed using the reference compounds polygodial, portimine, oroidin, chlorothalonil, and tolylfluanid. These compounds were bioactive in the low ng mL^{-1} to high µg mL^{-1} range, and, when polygodial positive controls were included in eight independent runs of the bioassay, repeatability and intermediate precision were ± 14 and 17%, respectively. However, enrichment with an environmental extract decreased the potency of polygodial. Overall, the bioassay developed here represents a sensitive and repeatable measure of ascidian-specific antifouling activity.

Key Words

Antifouling; bioassay; *Ciona savignyi*; settlement; metamorphosis

1 Introduction

Chemical interactions between marine organisms are diverse and complex, ranging from induction of conspecific settlement (Pawlik 1992) to deterrence of grazers or predators (Pawlik 1993). One area of marine chemical ecology that has been a particular focus in recent years is provisioning of antifouling defenses. A variety of chemical antifouling agents have already been described from the sea (Clare 1996), with sessile invertebrates presenting a particularly rich source of these secondary metabolites because they often contend with intense competition for space (Branch 1984; Wahl 2009). Not only are natural antifouling agents important drivers of marine community dynamics (McClintock & Baker 2010), these compounds have potential commercial uses for preventing biofouling on manmade structures or in drug discovery (Fusetani 2011). Regardless of whether the ultimate aim is enhanced ecological understanding or commercial application, studies investigating natural antifouling agents typically rely on bioassays (for a comprehensive overview see Dobrestov et al. 2014).

Bioassay protocols based on marine bacteria, diatoms, barnacles, mussels and seaweeds are well established (for reviews see Bressy et al. 2014; Briand 2009) but there are a plethora of other biofouling taxa (Dürr & Thomason 2010) that are rarely applied in this context. Ascidians are a prime example of a group of biofoulers that are underutilized in bioassay protocols. These sessile filter-feeders comprise major components of biofouling communities in tropical and temperate environments (Dürr & Thomason 2010), and include several invasive pest species (Lambert 2001; Lambert 2007). The availability of a standardized ascidian-based bioassay would reduce the risk

of ‘overlooking’ antifouling agents specific towards these ecologically and commercially relevant biofoulers, plus facilitate the development of targeted antifouling technologies.

In this study, a semi-quantitative bioassay for screening compounds and natural product extracts for antifouling activity against ascidians was developed. The bioassay was based on inhibition of settlement and metamorphosis of the Pacific transparent ascidian *Ciona savignyi* Herdman (Cionidae). This Japanese native is an invasive species with a rapidly expanding distribution that currently includes British Columbia, California, New Zealand, and Puget Sound (Fofonoff et al. 2003; Lambert & Lambert 1998). *Ciona savignyi* is well suited for use in a bioassay because it is a hermaphroditic spawner with a short conditioning period, high fecundity, and defined progression of metamorphic changes (Jiang & Smith 2005; Kourakis et al. 2010; Moody et al. 1999). Initially, the effects on the larvae of temperature, photoperiod, substrate, larval density, vessel size, and carrier solvent were investigated, and experimental end-points evaluated. The combination of parameters yielding maximum larval development and bioassay reproducibility were identified, then the performance of the bioassay was assessed using reference compounds.

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 50-mL glass Petri dish (90 mm dia.) filled with 20 mL of 0.3-µm filtered and UV-sterilized seawater (FSW). Sperm were then harvested and transferred to glass Petri dishes (90 mm dia.) 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

The temperature, photoperiod, and substrate preferences of larvae were assessed in three separate experiments. Firstly, the effects of exposing larvae to 15, 18, 21, and

25°C were evaluated. For the photoperiod experiment, larvae were exposed to the following lighting regimes: 24:0, 12:12, 0:24 h intense (100 ± 10 μmol m^{-2} s^{-1}) or subdued (10 ± 5 μmol m^{-2} s^{-1}) light:dark. Lastly, substrate type (polystyrene, acrylic, or glass) and texture (smooth or rough) were varied individually. Treatment wells were unlined (polystyrene) or lined with either acrylic discs (35-mm diameter) or glass cover slips (35-mm diameter, Gerhard Menz GmbH, Saarbrücken, GE). Untreated unlined wells, acrylic discs, and glass cover slips comprised smooth treatments. Sandblasted acrylic and sanded (800-grit sandpaper) glass and polystyrene comprised rough treatments; it should be noted that these surface treatments likely resulted in different feature sizes for each of the three substrate types and that the walls of the wells remained untreated in all cases.

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

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

2.3. Vessel size and larval density

Vessel size was assessed in conjunction with larval density. Polystyrene Petri dishes (LabServ, Thermo Fisher Scientific, AU; 90 mm dia.), six-well plates (36 mm dia.), and twelve-well plates (Corning® Costar®; 23 mm dia.) 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).

2.4 *Fill volume*

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 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). Temperature, photoperiod, and experimental end-point matched those for the vessel size and larval density experiment (Section 2.3) but culture waters were not renewed for the duration of the experiment.

2.5 Carrier solvent

The sensitivity of larvae to two common carrier solvents was assessed for the final format of the bioassay: 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 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.

142 ***2.6 Indicative performance of the bioassay***

143 To provide an indication of the effective range of the bioassay, polygodial (ENZO Life
144 Sciences, Farmingdale, NY), portimine (Cawthron Natural Compounds, Nelson, NZ),
145 oroidin (ENZO Life Sciences), chlorothalonil (Sigma-Aldrich, St Louis, MO), and
146 tolylfluanid (Sigma-Aldrich) were screened using the protocol outlined in Section 2.5.
147 These compounds were selected because they represent a range of both known natural
148 antifouling agents (i.e., polygodial and oroidin; Cahill & Kuhajek 2014; Tsukamoto et al.
149 1996), synthetic antifouling agents (i.e., chlorothalonil and tolyfluanid; Voulvoulis et al.
150 1999), and natural compounds with unexplored antifouling activity (i.e., portimine;
151 Selwood et al. 2013). With the exception of polygodial (1, 2.5, 5, 10, 15, 20, 40, 80 ng
152 mL^{-1}) and portimine (0.05, 0.1, 0.5, 1, 5, 10, 20 ng mL^{-1}), concentrations tested against
153 the bioassay were 0.001, 0.005, 0.01, 1, 5, and 10 µg mL^{-1}. Stock solutions prepared in
154 20% (v/v) ethanol (polygodial), RSW (portimine), or 20% (v/v) DMSO (oroidin,
155 chlorothalonil, tolylfluanid) were added to wells to yield the desired test concentrations;
156 control wells contained solvent only (n = 3).

157 Polygodial treatments (5, 10, and 50 ng mL^{-1}) were subsequently included in
158 eight independent runs of the bioassays performed over a 1-year period, allowing
159 repeatability (i.e., intra-run variability) and intermediate precision (i.e., inter-run
160 variability) to be estimated (n = 3). In a separate experiment to assess the potential
161 implications of fractionating complex samples, the potency of pure polygodial was
162 compared to that of polygodial enriched with an environmental extract. The extract was
163 produced by eluting 10 L seawater that ~ 400 g of green-lipped mussels, *Perna*
164 *canaliculus* Gmelin, had been cultured in for 1 day mussel through a 20-cm^3 column of

Diaion HP20 resin (Mitsubishi Chemical, Tokyo, JP). The column was flushed with 50 mL ethanol, and the resulting extract dried and re-suspended in 1 mL 20% (v/v) ethanol. Polygodial stock solutions prepared in 1 mL of 20% (v/v) ethanol or 1 mL of extract were added to wells to yield final polygodial concentrations of 2.5, 5, 10, and 50 ng mL^{-1} (n = 3).

2.7 Data Analyses

Temperature, photoperiod, and substrate data were analyzed using linear mixed-effects modelling (Bolker et al. 2009), with metamorphic progress as the response variable, time as a continuous covariate, and replicate 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 & Bates 2006). Principal response curves (PRC), a redundancy analysis for multivariate responses in repeated-measures design (Van den Brink & Ter Braak 1998; Van den Brink & Ter Braak 1999), were used with 999 permutations to identify the metamorphic stages that were driving treatment effects. Mean absolute PRC coefficients were calculated by combining the values from the temperature, photoperiod, and substrate datasets. The effects of larval density and vessel size on settlement and metamorphosis were assessed using a two-way factorial ANOVA. The effect of fill-volume 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 completing metamorphosis by 50% relative to blank controls (EC_{50}) calculated (Kuo et al. 1993). Repeatability (relative bias)

and intermediate precision 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

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). Larval development 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). The number of larvae completing metamorphosis within 7 days 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). Larvae reached stages A and B 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.

3.2 Larval density and vessel size

Both density (ANOVA, $F = 21.4$, $p < 0.005$) and vessel size (ANOVA, $F = 13.3$, $p < 0.005$) significantly influenced settlement and metamorphosis. The proportion of larvae completing metamorphosis 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 metamorphosed larvae was observed in twelve-well plates (Figure 3).

3.3 Fill volume

The proportion of larvae that successfully settled and metamorphosed increased 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.

3.4 Carrier solvent

No detectable effects on larval development were observed when carrier solvent was added to the bioassay at or below 20 µL mL^{-1} for ethanol or 10 µL mL^{-1} for DMSO. When dosed above these concentrations, the number of larvae that completed metamorphosis was significantly reduced (ethanol ANOVA: $F = 12.6$, $p < 0.005$; Dunnett's $p = 0.009$; DMSO ANOVA: $F = 16.0$, $p < 0.005$; Dunnett's $p = 0.02$).

3.5 Indicative performance of the bioassay

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 runs of the bioassay had negative dose-dependent effects on metamorphosis, with repeatability and intermediate precision of ± 14 and 17%, respectively (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

235 enriched *vs.* pure polygodial represents an approximately 80% increase in EC_{50} (7.8 vs.

236 4.3 ng mL^{-1}).

4 Discussion

Initially identifying environmental parameters that favour development of *C. savignyi* larvae provided a foundation upon which to progressively build a bioassay protocol. Larval settlement and metamorphosis were enhanced at 18°C, 12:12 h subdued light:dark, and smooth polystyrene substrate. Exceeding 21°C reduced the number of larvae completing metamorphosis to almost zero, but no observable differences were found in the proportion of larvae that settled and metamorphosed between 15 and 21°C. A similarly broad optimal temperature range has previously been reported for *C. savignyi* embryogenesis (12 – 20°C; Nomaguchi et al. 1997), thus the moderate value of 18°C was used in the final bioassay protocol. Likewise, enhanced development of 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 select a surface upon which to settle. Compared to temperature and photoperiod, substrate had only limited impact on the larvae and the default substrate of untreated polystyrene was deemed the most practicable. Even though larvae were effectively presented with a substrate choice in treatments where the base and sides of the wells were of dissimilar composition, no obvious settlement preferences were observed. This apparent insensitivity to substrate type and texture is unsurprising given that ascidians are one of the least discerning marine taxa in relation to surface selection (Aldred & Clare 2014).

The temperature, photoperiod, and substrate datasets were also used to establish an appropriate end-point for the bioassay. In selecting an end-point, emphasis

was placed on minimizing the total duration of the bioassay while ensuring adequate power to detect effects. The earliest developmental stage of metamorphosis to show strong treatment response was stage E, which is reached within 5 days under the conditions of the bioassay. A 5 day experimental timeframe with a single response variable (i.e., stage E larvae) represents a relatively rapid and labour-efficient bioassay.

When larval density and vessel size were subsequently evaluated, settlement and metamorphosis were enhanced above 5 or 10 larvae mL^{-1}, with twelve-well plates having the greatest proportion of metamorphosed larvae overall. Density-dependent settlement is a common phenomenon among marine larvae; examples include the gregariousness of larvae of the barnacle *Balanus amphitrite* Darwin (Head et al. 2003), the tubeworm *Hydroides dianthus* Verrill (Toonen & Pawlik 1996), and the oyster *Ostrea edulis* L. (Bayne 1969). Enhanced larval development in smaller vessels could be a result of conspecific settlement cues or reduced surface area to volume ratio. Under the later scenario, larvae are more likely to contact, and thus settle on, the internal surfaces of a smaller vessel. Although 5 larvae mL^{-1} appeared sufficient to maximise settlement rates in twelve-well plates, the final bioassay protocol used 10 larvae mL^{-1} due to the more pronounced effect of larval density seen for the Petri dish treatments.

Filling wells to capacity further increased the proportion of settled and metamorphosed larvae. This finding is in keeping with observation that some *C. savignyi* larvae tend to settle at the air-water interface (pers. obs.), a phenomenon previously reported for other ascidians (e.g., Fletcher & Forrest 2011). Culture waters contact the underside of the lid in wells that were filled to capacity, eliminating the air-water interface and forcing larvae to settle on the internal surfaces of the well.

283 Fortuitously, when wells are filled to capacity the majority of larvae settle on the
284 underside of the plate lid where, compared to those attached to the bottom or sides of
285 the well, they are easier to count (pers. obs.).

286 Once the final protocol had been established, reference compounds were used
287 to assess the performance of the bioassay. The bioassay detected antifouling activity at
288 low ng mL^{-1} to high µg mL^{-1} ranges for the natural antifouling agent polygodial (Cahill &
289 Kuhajek 2014), the algal biotoxin portimine (Selwood et al. 2013), and the synthetic
290 antifouling biocides chlorothalonil and tolyfluanid (Voulvoulis et al. 1999). Tolerance of
291 *C. savignyi* larvae for the carrier solvents ethanol (20 µL mL^{-1} max.) and DMSO (10 µL
292 mL^{-1} max.) facilitated screening these compounds, which, with the exception of
293 portimine, have only limited water solubility. The results reported here meet or exceed
294 potency estimates previously determined for these compounds using other bioassay
295 systems. For example, polygodial is effective against fungal pathogens at approximately
296 1 µg mL^{-1} (Kubo & Himejima 1991), chlorothalonil kills water fleas and fathead minnows
297 at 0.03 − 0.2 µg mL^{-1} (Sherrard et al. 2002), and tolylfluanid controls seaweed
298 zoospores at 0.03 µg mL^{-1} (Wendt et al. 2013).

299 Due to its high potency against *C. savignyi* and limited human toxicity
300 (Metugriachuk et al. 2005), polygodial was selected as a reference compound for
301 routine application of the bioassay. When polygodial treatments were included in eight
302 independent runs of the bioassay, repeatability and intermediate precision were ± 14
303 and 17%, respectively. Polygodial positive controls (5, 10, and 50 ng mL^{-1}) provide a
304 reference against which to estimate the potency of samples. Although only semi-
305 quantitative, such potency estimates will be useful when comparing the effectiveness of

antifouling agents or tracking bioactive recovery rates from fractionated samples. Nevertheless, enrichment with an environmental extract reduced the potency of polygodial by 80%. This result highlights the potential for potency changes associated with complex samples, an important factor to note when fractionating natural product extracts.

Overall, the bioassay provides a sensitive measure of ascidian-specific antifouling activity that can be applied to both pure compounds and natural product extracts and fractions. The bioassay is not only rapid and repeatable, it is based on a globally relevant invasive fouling organism. By implementing this protocol as part of a suite of bioassays, antifouling agents effective against ascidians should become apparent. Such compounds are of commercial interest for controlling biofouling or in drug discovery and, when derived from sessile marine organisms, have implications for understanding marine invertebrate community dynamics.

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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 for polygodial treatments from eight independent runs of a *Ciona savignyi* bioassay. Values are means (n = 8) relative to a known dose-response relationship for polygodial ± 95% confidence interval.

Figure 5. Relative bias in a *Ciona savignyi* bioassay for polygodial enriched with an environmental extract. Values are means (n = 3) relative to pure polygodial ± 95% confidence interval.

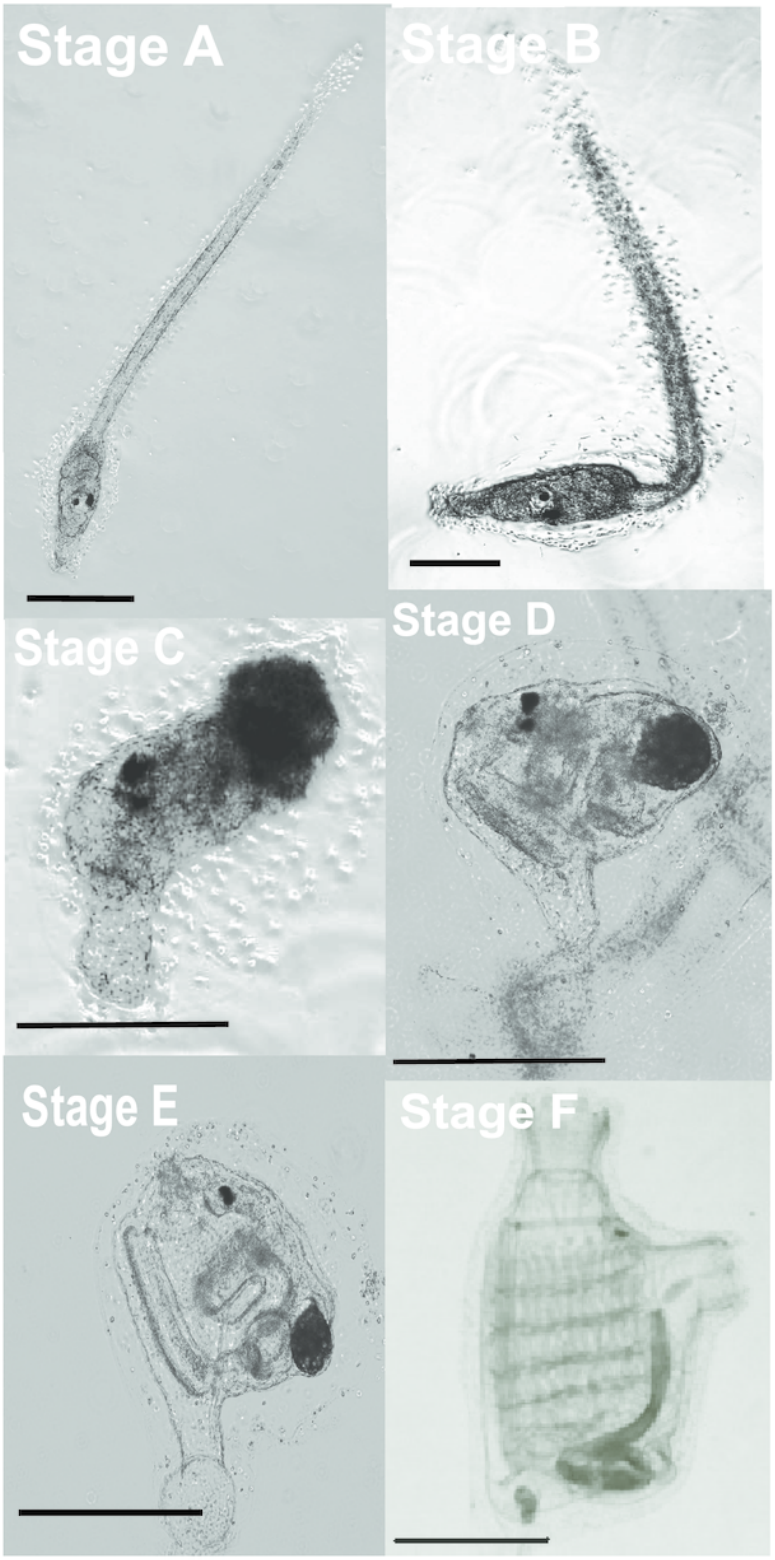
441 **Table 1.** Variance component estimates and overall variability for polygodial treatments
442 used to validate a larval ascidian bioassay.

443

1

Stages of *Ciona savignyi* larval development.

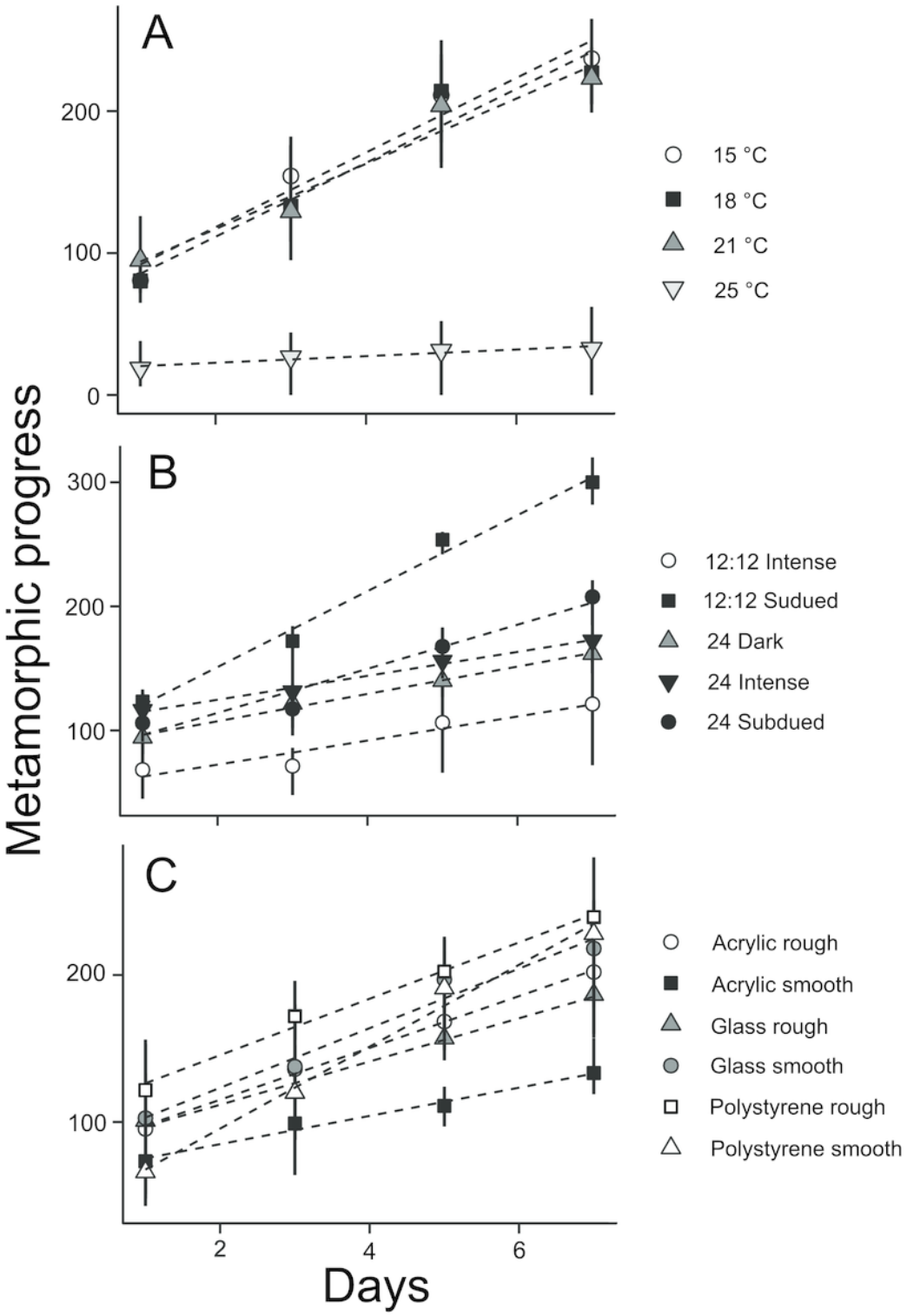
Stages of *Ciona savignyi* larval development. At the moment of settlement, metamorphosis is triggered (Stage A). Tail resorption is completed in a short time (Stage B). The outer tunic layer is cut off (Stages C & D), and then two lateral siphons and a pair of functional stigmata develop (Stage E). 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.

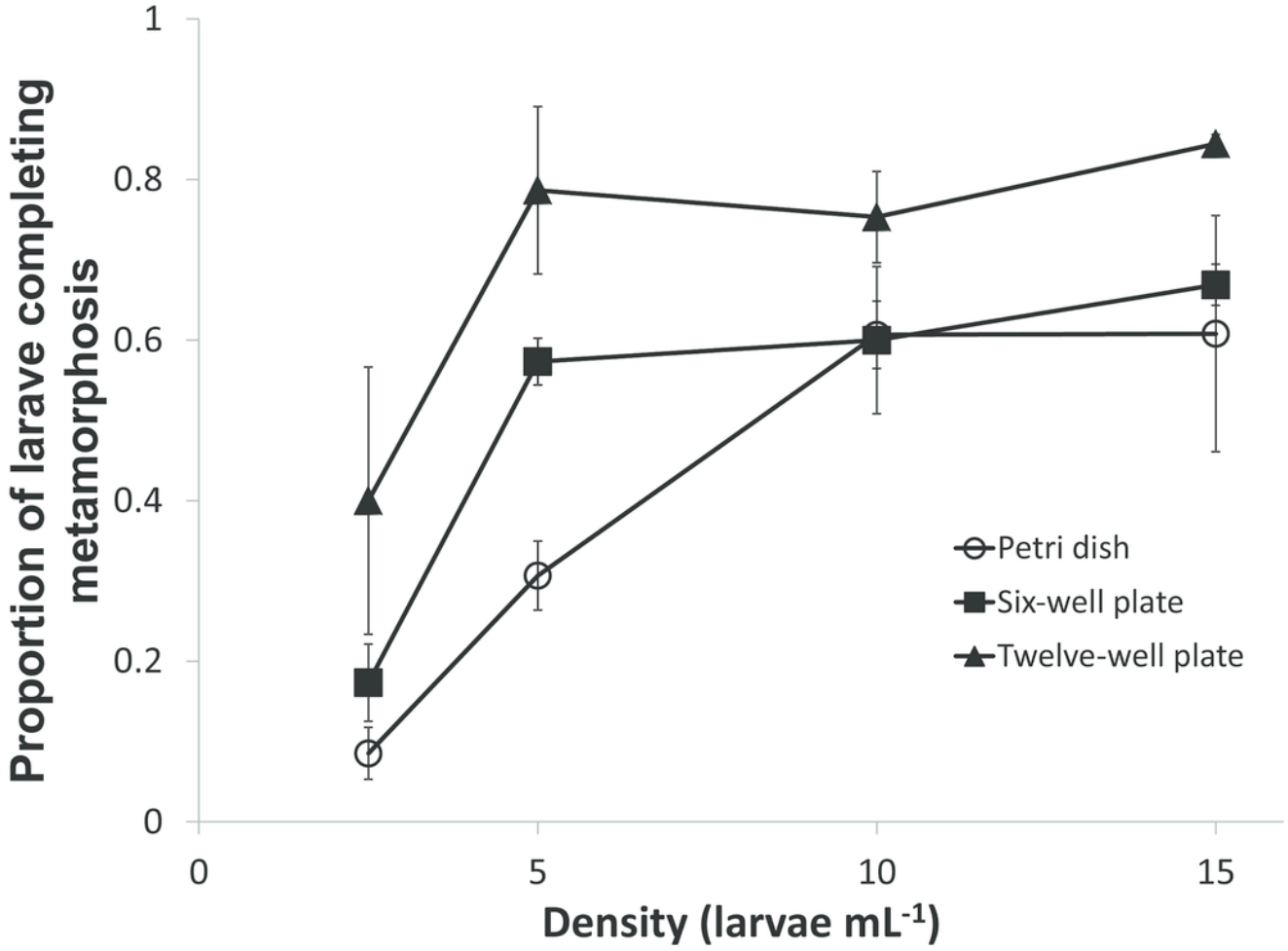
Effects of (A) temperature, (B) photoperiod, and (C) substrate on *Ciona savignyi* larvae. Metamorphic progress was calculated based on the sum of the number of individuals reaching each metamorphic stage converted to numerals (A = 1, B = 2, C = 3, D = 4, E = 5, F = 6). Values are means (n = 3) ± 95%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

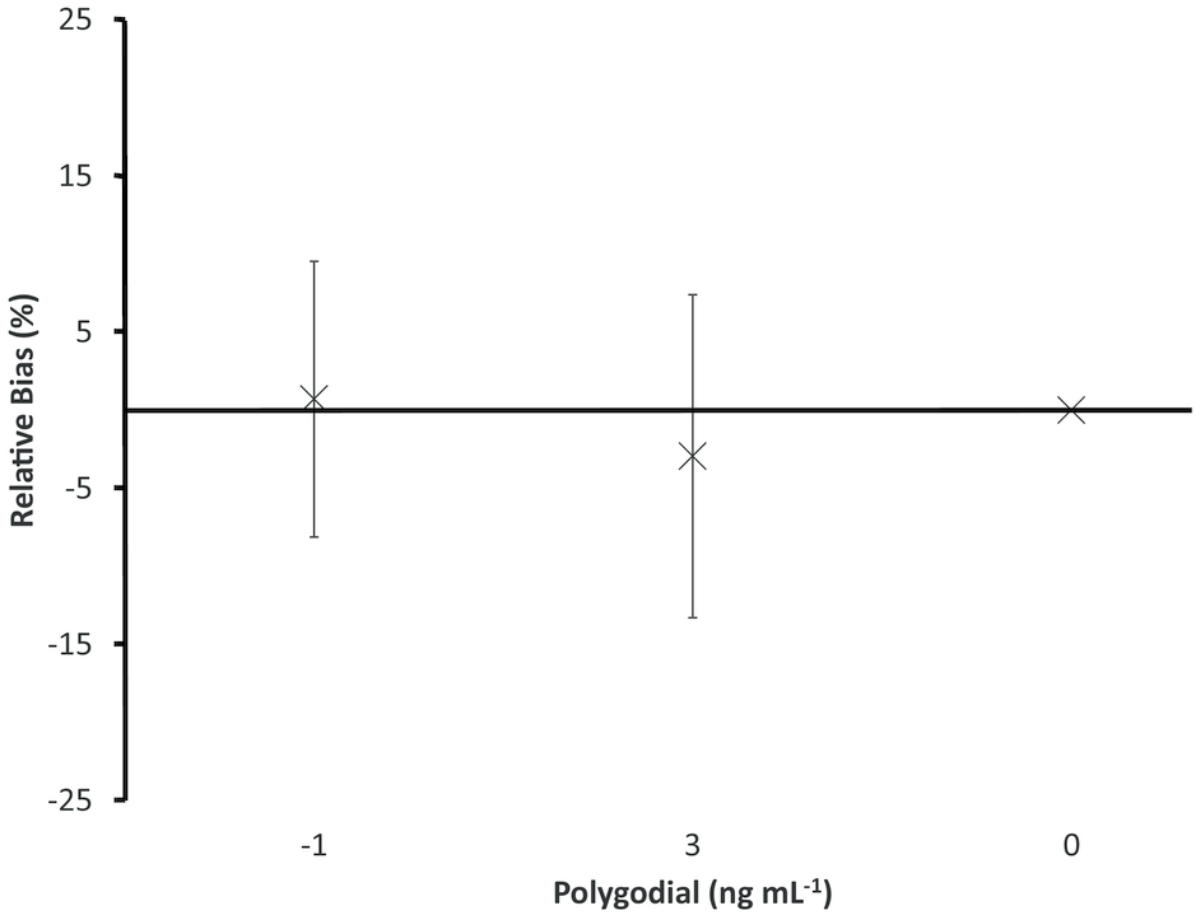
Proportion of *Ciona savignyi* larvae completing Stage E of metamorphosis within 5 days for various combinations of larval density and vessel size. Values are means (n = 3) ± 95% confidence interval.



4

Relative bias for polygodial treatments.

Relative bias for polygodial treatments from eight independent runs of a *Ciona savignyi* bioassay. 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 in a *Ciona savignyi* bioassay for polygodial enriched with an environmental extract. Values are means (n = 3) relative to pure polygodial ± 95% confidence interval.

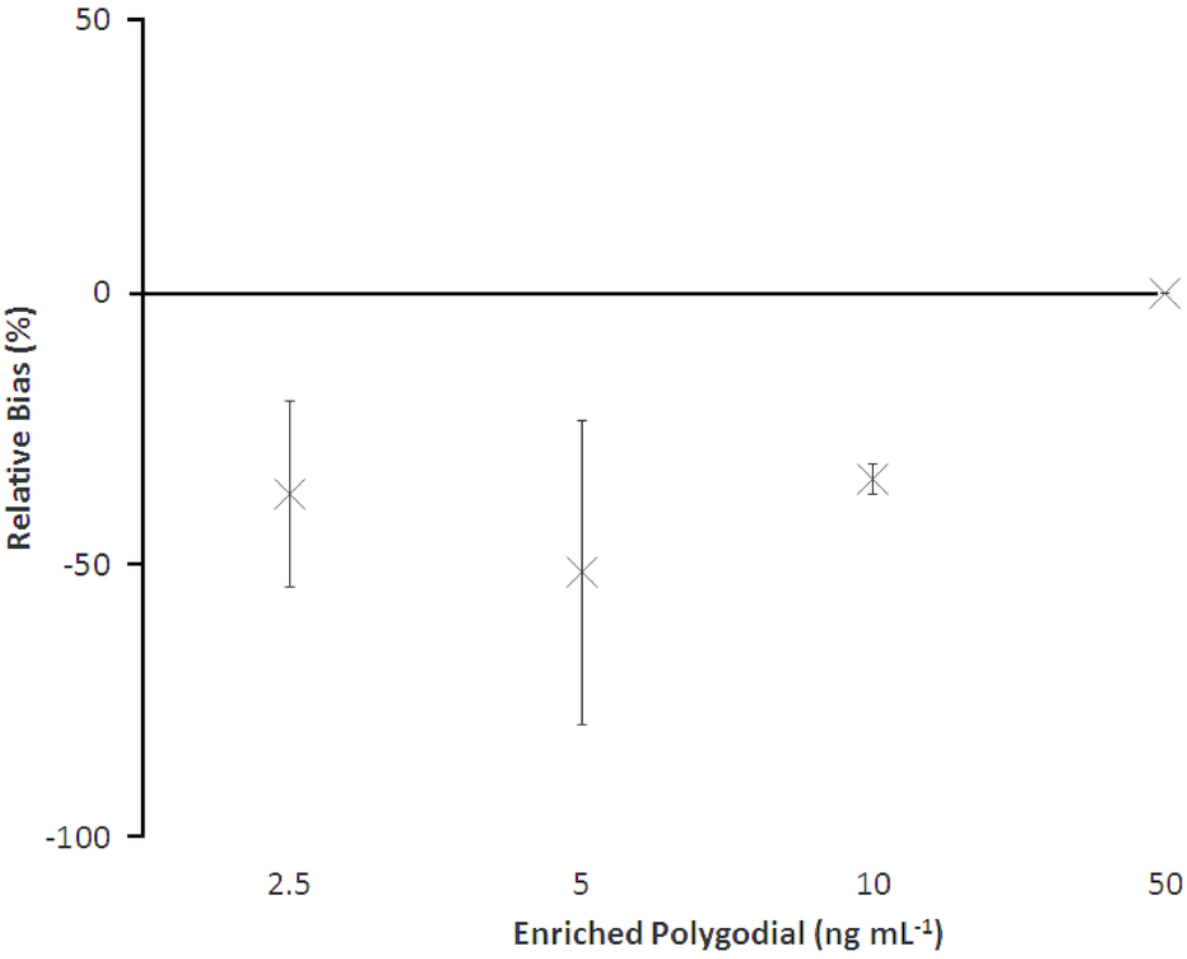


Table 1(on next page)

Overall variability for polygodial treatments.

Table 1. Variance component estimates and overall variability for polygodial treatments used to validate a larval ascidian bioassay.

	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%