



A true test of colour effects on marine invertebrate larval settlement

Veronica Ells^{a,c}, Natalia Filip^{a,c}, Cory D. Bishop^{a,c}, M. Edwin DeMont^{a,c},
Truis Smith-Palmer^{b,c}, Russell C. Wyeth^{a,c,*}

^a Biology Department, St Francis Xavier University, 2321 Notre Dame Ave, Antigonish, Nova Scotia B2G 2W5, Canada

^b Chemistry Department, St Francis Xavier University, 2321 Notre Dame Ave, Antigonish, Nova Scotia B2G 2W5, Canada

^c Centre for Biofouling Research, St Francis Xavier University, 2321 Notre Dame Ave, Antigonish, Nova Scotia B2G 2W5, Canada

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ABSTRACT

Past tests of settlement by marine invertebrate larvae on different colours were not designed to distinguish between responses to substrate colour and substrate brightness. Using colour vision testing methods, the design of this study provided a true test for responses to colour by including both coloured and grayscale settlement plates. The dominant fouling taxa at the test sites (the solitary ascidian, *Ciona intestinalis*; the colonial ascidians *Botryllus schlosseri* or *Botrylloides violaceus*; and a bryozoan *Bugula* sp.) showed no significant differences in settlement between blue, red or green plates. In contrast, the ascidians responded to substrate brightness with significantly lower settlement on lighter plates, while the bryozoans showed no significant preference relative to substrate brightness. The methods used here are a model for future tests of responses to colour during settlement. In addition, the contrasting responses to brightness are at odds with previous laboratory experiments that showed larvae of both ascidians and bryozoans are negatively phototactic at settlement. These results suggest that other cues must supersede any phototactic responses in the bryozoans, or alternatively that the range of light intensities near darker and lighter substrata were not part of the tests in the earlier reports.

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1. Introduction

Settlement plates are used widely in marine biology, including studies of larval biology, invasive species, and biofouling (e.g., Collin et al., 2013; Ellrich et al., 2015; Humanes and Bastidas, 2015; Krishnan et al., 2015). Experiments place the plates in marine habitats and subsequently monitor the abundance or diversity of organisms or species that settle on the plate. Many of these experiments have not explicitly controlled for the effect of colour. Often, this is simply because all plates deployed are constructed from the same material, and thus any differences in colour once the plates are deployed (due to differences in incident light) are reasonably assumed to be negligible. Other experiments, however, particularly those dealing with applied aspects of biofouling and antifouling coatings, involve plates with different materials which may be different colours (as perceived by humans). Unfortunately, it is hard to assess the prevalence of this situation, since colour is rarely reported for coatings (e.g., Vucko et al., 2012; Watson et al., 2015; Zargiel and Swain, 2014). Regardless, if coating colours do vary, controls for the effect of colour (as perceived by the settling animals) are needed to

properly test the effect of the other differences among the settlement plate surfaces. A few studies have been conducted to test responses to colour, however, these have not been designed appropriately given the complicated nature of colour perception.

Tests of behavioural responses to colour must take into account the complexities of light stimuli and the proximate mechanisms underlying the responses of animals to those stimuli (Kelber et al., 2003; Skorupski and Chittka, 2011). Responses to light stimuli depend on the spectra of the stimuli, the number and spectral sensitivities of photoreceptor types, and the sensory processing circuits of the nervous system that ultimately control the behavioural responses to light. To the authors' knowledge, no previous studies of settlement responses to colour have fully articulated what aspect of colour responses was being tested (Dahlem et al., 1984; Dobretsov et al., 2013; Finlay et al., 2008; Guenther et al., 2009; Hodson et al., 2000; James and Underwood, 1994; Robson et al., 2009; Satheesh and Wesley, 2010; Su et al., 2007; Swain et al., 2006; Yule and Walker, 1984). Among studies of the sensory biology of vision, the usual starting point is to establish whether an animal has colour vision. In particular, the test must attempt to differentiate between responses driven by spectral composition differences (i.e. colour) and intensity (i.e. brightness) (Crisp, 1976). Animals can differentially respond to different colours without having colour vision because of how light spectra may or may not match the spectral sensitivity of photoreceptor pigments. Crucially, however, an animal without colour vision should show the same differential response to achromatic light

* Corresponding author at: Biology Department, St Francis Xavier University, 2321 Notre Dame Ave, Antigonish, Nova Scotia B2G 2W5, Canada.

E-mail addresses: x2012ajv@stfx.ca (V. Ells), nfilip@gmail.com (N. Filip), cbishop@stfx.ca (C.D. Bishop), edemont@stfx.ca (M.E. DeMont), tsmithpa@stfx.ca (T. Smith-Palmer), rwiyeth@stfx.ca (R.C. Wyeth).

of differing overall intensities (i.e., brighter or darker light without any variation in intensities with respect to wavelength). Thus, the basic experimental design for testing for responses to colour requires treatments that can be compared to determine if differential responses arise due to differences in intensity or spectral composition. A simple approach is to test neutral gray stimuli with varying intensities that establish whether or not responses to the coloured stimuli can also be produced by stimuli that vary in intensity (as will the coloured stimuli) but not in spectral composition (unlike the coloured stimuli). This method has not been a part of previous tests of settlement responses to colour. Some experiments have included black and white stimuli, but no previous tests have included a set of gray stimuli to properly assess differential responses due to intensity alone.

Fortunately, the results of some previous studies of some taxa have not found strong responses to colour (Guenther et al., 2009; Sathesh and Wesley, 2010; e.g., Visscher and Luce, 1928), and thus the conclusions are not compromised by omitting the gray stimuli which can help distinguish colour versus intensity responses. Nonetheless, a systemic bias exists in these previous studies that lack the gray stimuli. The experiments would never have been able to conclude that any differential settlement was due to colour, and not intensity. Indeed, some studies have concluded an effect of colour on some taxa, with barnacles and tubeworms reported as having differential settlement as a consequence of colour, without including grayscale controls for intensity (Robson et al., 2009; Sathesh and Wesley, 2010; Yule and Walker, 1984). Moreover, at least one other study has concluded that responses were due to differences in intensity while only testing coloured surfaces (Su et al., 2007). Finally, yet other studies have tested for an effect of “colour” when in fact only black and white or grayscale treatments were tested, presenting a problem of semantics rather than evidence (Dahlem et al., 1984; Darbyson et al., 2009; Dobretsov et al., 2013; Hodson et al., 2000; James and Underwood, 1994; Swain et al., 2006). Thus, given this right-royal-mess, the first motivation for this study was to introduce the practice of an unbiased experimental design, including grayscale and colour treatments as an initial step to understanding whether or not the animals in question use more than light intensity to guide settlement behaviour. The methods also attempt to remove all other potential biases, including the possibility that different coloured backgrounds could affect a human observer’s ability to measure settlement.

The second motivation was more practical. In a recent study, differences were observed in settlement rates by ascidians on potential anti-fouling treatments with different colours (Filip et al., 2016). Thus, the second goal was empirical verification that colour does not affect settlement of the ascidians (and other dominant taxa) at the testing sites in Nova Scotia, Canada. In the current experiment, ascidians and bryozoans were the two taxa that dominated settlement on the plates. Larvae of most benthic marine invertebrates typically become photo-negative as they age, presumably to promote settlement on the benthos (Thorson, 1964). In ascidians, Svane and Young (1989) have suggested the behavioural ontogenies may actually be more complicated and vary substantially between species. Nonetheless, among the ascidians present at the sites in this study, the solitary *Ciona intestinalis* Linnaeus has been shown to follow the typical pattern (Nakagawa et al., 1999). No data is available for the two colonial species present (*Botryllus schlosseri* Pallas or *Botryllodes violaceus* Oka). To the authors’ knowledge, only one previous study has considered differential settlement rates by an ascidian (*Didemnum* sp.) in response to colour, and no significant responses were found (Sathesh and Wesley, 2010). Results have been largely similar for bryozoans. Either of the two bryozoan species that may have settled on the plates in the current study (*Bugula fulva* Ryland and *B. turrita* Desor) have been recorded as initially positively phototactic, switching to negative phototaxis later in larval life (Ryland, 1977). Similar patterns have been found for a number of other bryozoans (Ramirez and Cancino, 1991; Ryland, 1977; Wendt and Woollacott, 1999). Bryozoans have not been studied with respect

to colour. Thus, given these past results, fouling by both ascidians and bryozoans was expected to be higher on darker plates. In addition, a preference for colour was not expected, based primarily on the general trend that colour vision is often of lesser importance in marine habitats (Marshall et al., 2015).

2. Materials and methods

2.1. Colour treatments

Colour and grayscale settlement plates were created by laminating commercial paint sample cards (Table 1; Fig. 1A). The three colours were chosen to span the visible spectrum (red, green, and blue). Without information on the absorption spectrum of the photopigments of the animals tested, it is not possible to choose grays that will match the intensity of the colour treatments, and thus grays were similarly chosen to span intensities from white to black. For each plate, two cards were trimmed to include only the coloured area, and laminated end-to-end inside clear Scotch Self Laminating Pouches (LS854WS) using a Scotch Thermal Laminator (TL902-C). The colour treatment area was 7 × 20 cm, with the laminate cut to provide a 1 cm clear border to match a 9 × 25 cm polyvinyl chloride back plate. An additional clear plastic laminate (same material as above, but with nothing inside the laminate pouch) was placed over top of the colour treatment, and the three layers (clear settlement laminate, colour laminate and back plate) were cable tied tightly together (Fig. 1B). Thus, the different colour treatments all had identical surface chemistry, and the outer clear layer could be removed for quantification of settlement without bias from differences in the background colour.

2.2. Deployment

Deployment frames were constructed from 2 in. diameter acrylonitrile butadiene styrene pipe (inside dimensions of 82 × 39 cm). Plates from each treatment (in random order) were secured with cable ties inside each frame, leaving a 1 cm gap between each of the plates and a 7 cm gap between plates and the top and bottom of the frame. Eight frames (each with the eight treatments) were deployed from docks at each of two sites (Fig. 1C). All frames were suspended at 1 m depth, with the treatment plates facing the dock (to minimize inter-frame variation in illumination due to shadows from boats and other structures). At Port Hawkesbury (Strait of Canso Yacht Club, Nova Scotia, Canada; 45.6134, –61.3653), frames were deployed on July 3rd, 2014 and sampled twice, 28 and 37 days later. At Cribbons, (Cribbons Point Marina, Nova Scotia, Canada; 45.7558, –61.8971), frames were deployed on August 12th, 2014 and sample twice, 11 and 18 days later. Sampling days were chosen based on regular monitoring of settlement and growth on the frames and an attempt to record settlement at approximately 50% coverage and just before the plates reached coverage saturation. All frames, back plates and colour treatments were reused at the second site, with new clear laminate settling surfaces attached.

Table 1

Normalized total reflectance intensity for the colour and grayscale treatments created from Pittsburgh Plate Glass Company paint cards. The five shades of gray cards (from white through black) provide a range of brightness values that span the brightness values of the three colour treatments. See Fig. 1 for spectroscopic methods and reflectance spectra.

Colour	Paint card	Brightness
White	Delicate White NT33	1.00
Light gray	Flagstone NT36	0.43
Medium gray	Dover Gray NT37	0.20
Dark gray	Knight’s Armor NT38	0.08
Black	Black Magic NT39	0.03
Red	Candy Corn CC16	0.26
Green	Wistful Walk EB15	0.14
Blue	Kimono CC4	0.25

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