Larval metamorphosis of Phestilla spp. in response to waterborne cues from corals

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ABSTRACT
Many marine invertebrates depend on their larvae for dispersal and to settle and metamorphose in the appropriate habitat for adult survival, yet the mechanisms of habitat selection remain poorly understood. In Hawaii, the nudibranch Phestilla sibogae only feeds on Porites compressa and requires a water-soluble cue from this coral for metamorphosis. On Guam, we tested three different species of Phestilla to determine if their larvae require water-soluble compounds from corals to induce metamorphosis. Larvae of P. sibogae metamorphosed at high rates to waterborne cues from multiple species of corals in the genus Porites. Larvae of Phestilla minor could distinguish among waterborne compounds from different species of Porites, but also had high rates of metamorphosis in filtered seawater and in response to corals that adults did not eat. Larvae of Phestilla sp. 2 could distinguish among water-soluble cues from different species of Goniopora and consistently had the highest rates of metamorphosis in response to waterborne cues released from Goniopora fruticosa. P. minor was the only species studied that did not require waterborne cues for larval metamorphosis.

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1. Introduction
Larval recruitment is a critical process that can determine the structure of marine communities (Caley et al., 1996; Gaines and Roughgarden, 1985; Grosberg and Levitan, 1992; Roughgarden et al., 1988; Underwood and Fairweather, 1989). Marine invertebrate larvae can use physical, chemical and biological cues to choose appropriate settlement substrata (Pawlik, 1992; Scheltema, 1974; Zimmer and Butman, 2000). Specialist predators need to find their prey species for survival, and in some cases their larvae have been shown to choose the appropriate settlement substrates for adult survival (Pawlik, 1992). Larvae of specialist predators often use chemical signals from their host to find the appropriate habitat and stop their dispersal phase, termed settlement. Some larvae also require chemical cues to induce metamorphosis, which is the physiological and morphological transformation from a larva to a juvenile.

Larvae of many marine molluscs can settle and metamorphose in response to chemical cues released by their prey, including sea hares (Switzer-Dunlap and Hadfield, 1977), the queen conch Strombus gigas (Boettcher and Targett, 1996), the dorid nudibranch Adalaria proxima (Lambert and Todd, 1994; Lambert et al., 1997), and the sacoglossan Alderia modesta (Krug, 2001; Krug and Manzi, 1999; Krug and Zimmer, 2000). Phestilla sibogae is a specialist aeolid nudibranch that has been studied for its larval ecology, physiology and behavior during the last 30 years (Hadfield, 1977; Hadfield and Paul, 2001). Larvae of P. sibogae in Hawaii metamorphose in response to a water-soluble cue released from the host coral Porites compressa (Hadfield and Scheuer, 1985; Hadfield and Pennington, 1990). Larvae of P. sibogae are capable of detecting the inductive cue in the water column, and in the presence of the inducer they stop swimming and settle to the benthos (Hadfield and Koehl, 2004). A better understanding of larval behavior has led to the development of a model which predicts the spatial patterns of recruitment of individual larva (Koehl et al., 2007), the conclusions of which were validated by field surveys for recruitment of P. sibogae (Hadfield et al., 2006). Even though the larval ecology of P. sibogae has been well studied, the larval behavior and ecology of other species in this genus have only recently been studied.

On the tropical Pacific island of Guam, five genetically distinct species of nudibranchs in the genus Phestilla were found, each with different host specificity and life history characteristics (Faucci et al., 2007; Ritson-Williams et al., 2003, 2007). How do these differences in life history characteristics influence the larval ecology of these specialist predators? In this study we compared three species of Phestilla to determine if they all metamorphosed in response to water-soluble cues from their preferred prey and tested the diversity of coral species that produce metamorphic inducers.

2. Materials and methods
2.1. Species studied
P. sibogae (Bergh, 1905) is an aeolid nudibranch that feeds on at least six species of corals in the genus Porites on Guam (Ritson-Williams et al., 2003). P. sibogae has been synonymized under the
name *P. lugubris* (Rudman, 1981); however, to be consistent with a large body of literature we use the name *P. sibogae*. Larvae of *P. sibogae* are known to metamorphose in the presence of water-soluble compounds from *P. compressa* in Hawaii and will metamorphose in response to other corals in the genus *Porites*. Larvae of *P. sibogae* will also metamorphose in response to some coral species it will not eat, but at lower rates than in response to corals of the genus *Porites* (Ritson-Williams et al., 2003).

*P. minor* (Rudman, 1981) is a small (max. 7 mm) aeolid nudibranch that feeds on *Porites annae* and *Porites lutea* on Guam (Ritson-Williams et al., 2003). The larvae of *P. minor* can be competent for metamorphosis as soon as or before 1 day after hatching, and previous experiments showed that approximately 10 to 20% of the larvae can spontaneously metamorphose in 0.2 µm filtered seawater (FSW) (Ritson-Williams et al., 2007).

*Phestilla* sp. 2 is morphologically similar to *P. sibogae* but is genetically distinct (Faucci et al., 2007). Adults of *P. sp. 2* feed on some but not all species of corals in the genus *Goniopora* on Guam and Palau (Ritson-Williams et al., 2003). Larvae of *P. sp. 2* are lecithotrophic and are competent for metamorphosis approximately 4 days after hatching (Ritson-Williams et al., 2007).

### 2.2. Larval cultures

Adult nudibranchs were collected from the field and maintained in the laboratory in flow-through seawater aquaria on their prey corals. *P. sibogae* was fed *Porites* (Synaraea) rus, *P. minor* was fed *P. annae*, and *P. sp. 2* was fed *Goniopora fruticosa*. Egg masses from each species of *Phestilla* were collected daily and maintained in larval chambers with daily changes of 0.2 µm filtered seawater (FSW) as described in Ritson-Williams et al. (2003). When the veliger larvae were fully formed and moving (typically 5 days after fertilization), they were hatched from multiple egg masses and placed in antibiotic FSW (90 µg ml⁻¹ penicillin G and 75 µg ml⁻¹ streptomycin sulfate) in two to four larval chambers. The larval chambers were surrounded by flow-through seawater to maintain ambient seawater temperatures (approximately 28–32 °C), and antibiotic FSW was exchanged from the larval chambers daily. After hatching, larvae were maintained without feeding in antibiotic FSW in these chambers until they were competent for metamorphosis, which was 4 days for *P. sibogae* and *P. sp. 2* and 1 day for *P. minor*.

### 2.3. Preparation of waterborne cues

For the larvae of *P. sibogae* a variety of coral species in the genus *Porites* were tested to determine if there was a difference in metamorphic inducers for as many species of *Porites* as possible. For the larvae of *P. sp. 2* initial experiments showed that these larvae would respond to a water-soluble metamorphic inducer from *G. fruticosa*. Subsequent experiments tested coral water prepared from different species of *Goniopora* to determine if water-soluble metamorphic inducers were found in the corals that *P. sp. 2* was known to feed on (Ritson-Williams et al., 2003) and at what dilutions waterborne cues from these corals could induce metamorphosis. Initial experiments with larvae of *P. minor* showed no difference in the inductive activity of water-soluble cues from multiple corals species. *P. minor* feeds preferentially on two corals in the genus *Porites*; therefore, water-soluble cues from its preferred prey *P. annae* were compared to water-soluble cues from corals that it would not feed on (Ritson-Williams et al., 2003) to determine if larvae of *P. minor* would metamorphose at the same rates to waterborne cues from preferred and non-preferred prey.

All of the coral fragments (approximately 2 cm long) were broken off of their colony at least 24 h before their use in an experiment or for preparing coral waterborne cues. They were held in flow-through seawater to allow them to recover from the fragmentation. Waterborne cues (hereafter termed “coral water”) were prepared by soaking fragments of each species of coral in seawater. Fragments of the corals were placed as densely as possible in a glass beaker with seawater and aerated with an air stone for 24 h. Coral water conditioned with *P. compressa* was made with the same methods during a visit to Hawaii. Corals in the genus *Goniopora* were conditioned in seawater for 48 h to prepare coral water because the 24 hour incubations did not induce metamorphosis in *Phestilla* sp. 2 (Ritson-Williams et al., 2007). After 24 or 48 h, the conditioned coral water was decanted from the coral fragments and filtered through a paper coffee filter. The coral water was then frozen for use in subsequent experiments to prevent decomposition of active components. Before the coral water was added to any bioassay it was thawed to room temperature and then added to the larval experiments. The coral water prepared in this manner was considered 100% in the dilution assays and was combined with FSW at 1:1 and 1:3 volumes for the 50% and 25% concentrations.

### 2.4. Larval bioassays

Metamorphosis bioassays were conducted to determine the amount of larval metamorphosis in response to a variety of treatments. Ten to fifty larvae were placed in individual 5 or 9 ml wells of Costar® media culture well plates (nos. 3513 and 3516). The larvae were placed in each well in as little filtered seawater as possible (approximately 0.3 ml) to minimize the dilution of the coral water treatments. After the addition of the larvae the wells were filled to 5 ml with one of the following treatments: FSW, a coral fragment
Larvae of *P. sibogae* had more metamorphosis in response to the fragments of *Porites* spp. and their coral water than in response to FSW (Fig. 1A). When tested with coral water made from *P. annae*, *Porites cylindrica*, *P. lichen*, and *P. rus*, larvae of *P. sibogae* had the same amount of metamorphosis in response to waterborne cues as in response to the fragments of different *Porites* spp. (Tukey’s post-hoc test). The full strength coral water of *P. compressa*, *P. cylindrica*, *P. lutea* and *P. rus* induced significantly more metamorphosis than FSW (Fig. 1B). Except for *P. lutea* at 25% concentration, there was no difference in the rates of metamorphosis among the full strength and subsequent dilutions of coral water, indicating that the larvae can detect waterborne cues from these corals at reduced concentrations.

Larvae of *P. minor* consistently had relatively high rates (20–60%) of metamorphosis in FSW during these bioassays (Fig. 2A, B, C). In a bioassay with fragments of *P. annae* (used as a positive control for metamorphosis), coral water made from a variety of coral species and FSW, larval metamorphosis in response to the various treatments differed (Fig. 2A), but Tukey’s test did not discriminate among the treatments. In a second assay (Fig. 2B), the larvae of *P. minor* had higher rates of metamorphosis in response to coral water conditioned with *P. annae* (the preferred prey for adults) than to coral water from *Porites* (*Synaraea*) *rus*, but neither of these induced more metamorphosis than FSW (Tukey’s post-hoc test). There were also significantly different rates of metamorphosis in response to coral water prepared from *P. annae*, which was greater than the metamorphosis in response to coral water from *P. cylindrica*, but again neither of these significantly differed from FSW (Fig. 2C).

Like *P. sibogae*, larvae of *Phestilla* sp. 2 did not metamorphose at high rates to FSW and required coral water-soluble cues for metamorphosis (Fig. 3A, B, C). *Phestilla* sp. 2 had more metamorphosis in response to fragments of *P. cylindrica*, *G. fruticosa* and the water-soluble cues from *G. fruticosa* than in response to FSW (Fig. 3A). There were different rates of metamorphosis in response to coral water.
prepared from different corals in the genus *Goniopora* (Fig. 3B). Coral water from *Goniopora minor*, *G. lobata* and *G. fruticosa* induced more metamorphosis than FSW or coral water from *G. tenuidens* and *G. eclipensis*, with the highest response to coral water from *G. fruticosa*. In the dilution assays, all of the dilutions of coral water induced the same amount of metamorphosis as the full strength coral water (Fig. 3C). Only the three concentrations of *G. fruticosa* coral water and the full strength *G. minor* coral water induced significantly more metamorphosis than FSW.

4. Discussion

In this study, the larvae of both *P. sibogae* and *Phestilla* sp. 2 required water-soluble cues released from their preferred prey for metamorphosis; however, *P. minor* did not require water-soluble cues for metamorphosis. *Phestilla melanobrachia* also metamorphosed in response to water-soluble cues released from its preferred prey, *Tubastraea aurea* (Ritson-Williams et al., 2007). Water-soluble cues are an important mechanism for pelagic larvae to detect their prey, mediating critical life history events such as settlement and metamorphosis. These experiments show that both *P. sibogae* and *P. sp. 2* can metamorphose in response to multiple coral species and required water-soluble cues, not necessarily the coral itself, to induce metamorphosis.

*P. sibogae* is already well known to require a water-soluble cue from its host coral *P. compressa* for metamorphosis (Hadfield and Scheuer, 1985). This obligate relationship has been a valuable case study in larval ecology that has fostered a variety of interdisciplinary studies encompassing neurobiology (Kempf et al., 1992; Leise and Hadfield, 2000; Willows, 1985), larval behavior (Hadfield and Koehl, 2004; Miller and Hadfield, 1986), and modeling cue concentrations in relation to larval recruitment and transport (Hadfield et al., 2006; Koehl et al., 2007). The study presented here shows that multiple coral species in the genus *Porites* produce water-soluble compound(s) that induce metamorphosis in the larvae of *P. sibogae*. Larvae of *P. sibogae* can detect waterborne cues even at 25% concentrations that were tested during this study. The larvae of *P. sibogae* had similar rates of metamorphosis in response to all dilutions for most of the species of *Porites* studied (except for *P. lutea*). Research in Hawaii has shown that water collected from the reef induced the same amount of larval metamorphosis for *P. sibogae* as a 30% dilution of coral water from *P. compressa* prepared with the same methods as those used in this study (Hadfield and Koehl, 2004), suggesting that 25% is an ecologically relevant concentration for these experiments. Future experiments with field collected coral water would be an interesting comparison to our laboratory conditioned coral water. In the plankton, the larvae respond to inducer water by stopping their swimming and sinking to the benthos, which is probably a mechanism for larvae to settle adjacent to their host corals, ensuring adequate time for metamorphosis in a suitable habitat for growth and survival (Hadfield and Koehl, 2004). This is an immediate behavioral response to a short duration exposure to inducer water. A recent study showed that even a short exposure (2 h) to a metamorphic inducer was sufficient to induce settlement but not metamorphosis in the sacoglossan *Alderia* sp., and four day old larvae had higher rates of metamorphosis than one or two day old larvae in response to a 12 hour exposure to the inducer (Botello and Krug, 2006). A short term exposure of 2 h induced approximately 10% metamorphosis of larvae of *P. sibogae* after exposure to the metamorphic inducer from *P. compressa* (Hadfield, 1977). After 8 h of exposure to the inducer from *P. compressa* there was 30% metamorphosis and after 24 h there was 60% metamorphosis, showing that increased exposure time to the metamorphic inducer can increase rates of metamorphosis.

*Phestilla* sp. 2 also metamorphosed in response to waterborne cues from multiple corals in the genus *Goniopora*. The larvae of *P. sp. 2* were selective and did not metamorphose in response to all of the *Goniopora* spp. tested, they only metamorphosed in response to the coral species that they would eat (Fig. 3B, Ritson-Williams et al., 2003). In the field (on Guam) *P. sp. 2* was only found on *G. fruticosa*, which was the only coral water that consistently induced more metamorphosis than FSW (Fig. 3A, B, C). These larvae had lower rates of metamorphosis in response to *Goniopora minor* and *G. lobata* (Fig. 3B). *Phestilla* sp. 2 can eat these two species, but was never found on them in the field (Ritson-Williams et al., 2003). Why *P. sp. 2* does not eat all of the coral species in the genus *Goniopora* remains to be determined, but the larvae of *P. sp. 2* consistently metamorphosed at the highest rates in response to the water-soluble compound(s) from its preferred prey, *G. fruticosa* (Fig. 3B, C). Seawater had to be incubated for 48 h with *Goniopora* spp. to create inductive coral water suggesting that these corals release less of the chemical cue(s) or that larvae of *P. sp. 2* are less sensitive to inducer compounds than *P. sibogae*. Future experiments could test if larvae of *P. sp. 2* respond to natural concentrations of the cue(s) collected from corals in the field and what duration of exposure to the cue(s) is necessary to induce larval metamorphosis.

Significant rates of metamorphosis in response to non-host corals were also observed. The larvae of *P. sp. 2* metamorphosed at high rates in response to *P. cylindrica*, which it will not eat. High rates of settlement and metamorphosis onto non-preferred prey is unlikely to occur in the field (on Guam) as we never found an individual of *Phestilla* on a coral that it would not eat in the laboratory (Ritson-Williams et al., 2003). However, it is important to note that larval settlement does not perfectly match adult feeding preferences, and it may be that some metamorphosis can occur in poor habitats, but would be undetectable in the field if the juveniles crawled onto other coral species or had high rates of post-settlement mortality.

*P. minor* has a different larval strategy than the other species of *Phestilla* studied here. The larvae of *P. minor* has a much shorter metamorphic competence time (Ritson-Williams et al., 2007), and in the laboratory its veliger larvae were often observed crawling on the benthos instead of swimming in the water (RRW and SMS, pers. obs.). The larvae of *P. minor* had the highest metamorphosis in response to their preferred prey *P. annae*; however, metamorphosis consistently occurred in the FSW and in response to water-soluble cues from other corals. In two of the experiments (but not the third) larvae of *P. minor* could distinguish between the water-soluble cues from their preferred coral (*P. annae*) and those of corals they would not eat such as *Porites rus* and *P. cylindrica* (Ritson-Williams et al., 2003). Since *P. minor* larvae have a short planktonic period and probably remain in the same habitat as their parents, settlement cues may be less important for finding suitable settlement substrata. The spontaneous metamorphosis in FSW observed in all of these assays (ranging from 20 to 60%) shows that these larvae have more flexibility in their metamorphosis than the other species of *Phestilla* studied here. This flexibility in metamorphosis has been described for other larvae (Hadfield and Strathmann, 1996), and might be a mechanism of host switching, which is a proposed process of diversification for *Phestilla* (Fauci et al., 2007).

There is a trend between diet breadth, larval competency time and the importance of water-soluble settlement cues in *Phestilla* spp. Previously, it was suggested that a greater diversity of prey corals is correlated with a longer planktonic phase (Ritson-Williams et al., 2007). This trend can also be linked to the diversity of corals that induce metamorphosis in these nudibranchs. Among *Phestilla* spp. only *P. minor*, and potentially *P. sp. 1*, do not require a waterborne metamorphic inducer. *P. minor* has the most restricted diet range and the shortest competency phase, all of which are strategies for recruiting into its natal habitat. Larvae of the sea hare *Aplysia californica* metamorphosed in response to a variety of macroalgal species but as juveniles would crawl onto preferred macroalgae (Fawlik, 1989). *A. californica* has planktotrophic larvae (that reach metamorphic competence 35 days after hatching), and larvae of
A. californica can metamorphose onto a variety of algal species after dispersal between habitats. This is a similar strategy as P. melanobrachia which eats corals in multiple genera and has planktrophic larvae. Pawlik (1989) describes the trade off between settlement specificity and larval dispersal “...larvae appear to be selective enough to increase the likelihood of juvenile survival, but sufficiently broad in their response so as to minimize larval mortality.” Some opisthobranchs have solved this trade off with a bet hedging strategy and have both lecithotrophic and planktotrophic larvae. (Krug 2001; Smolensky et al., 2009). Settlement cues and metamorphic inducers are required for many marine larvae; however, even within the genus Phestilla there is a range of strategies for settling in the appropriate habitat for post-settlement survival.

Settlement cues for marine invertebrates have the potential to influence benthic community structure (Grosberg and Levitan, 1992); however, relatively few settlement cues have been isolated and characterized. Histamine was found to be the settlement inducer for the sea urchin Holopneustes purpurascens (Swanson et al., 2004). The red alga Delisea pulchra produced higher concentrations of histamine than the other algae tested, and histamine was released into the water surrounding the alga at high enough concentrations to induce settlement in some of the larvae (Swanson et al., 2006). The data presented here are a first step in understanding the chemical nature of the settlement cues for nudibranchs in the genus Phestilla. Even still though there has been some recent progress on characterizing metamorphic inducers, water-soluble cues remain difficult to isolate and characterize (Hadfield and Paul, 2001; Paul and Ritson-Williams, 2008). These cues are often released at very low concentrations, which limits the material available for chemical characterization (Fine et al., 2006). Further work on isolation and characterization of the water-soluble cues that induce metamorphosis in Phestilla spp. will greatly increase our knowledge of how these nudibranchs find their host corals, and what types of compounds can influence larval settlement ecology. The data presented here show the importance of water-soluble cues from coral species that some, but not all, Phestilla spp. require for metamorphosis.

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Pawlik, J.R., 1989. Larvae of the sea hare Aplysia californica settle and metamorphose on water-soluble cues from coral species that some, but not all, Phestilla spp. require for metamorphosis.