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Larval metamorphic competence in four species of *Phestilla* (Gastropoda; Opisthobranchia)

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Abstract

Many marine invertebrates depend on their larvae for dispersal and to find the appropriate habitat for adult survival, yet their larval ecology remains poorly known. In this study we test the time required until metamorphic competence in the veliger larvae of four species of *Phestilla* nudibranch. Larvae of *Phestilla melanobrachia* are planktotrophic and had the highest percentage of metamorphosis in response to the prey coral *Tubastraea aurea*. Twelve days after hatching these veligers had approximately 30% metamorphosis in response to *Tubastraea aurea* polyps and to waterborne compounds released from *T. aurea*. Larvae of the facultative lecithotroph *Phestilla sibogae* had the highest rates of metamorphosis in response to *Porites cylindrica* after five days. However, *P. sibogae* also had approximately 50% metamorphosis in response to the non-prey coral *Turbinaria reniformis*. *Phestilla minor* had significantly more metamorphosis in response to *Porites annae* than to filtered seawater. *Phestilla minor* was the only species to have spontaneous metamorphosis in response to filtered seawater; 27% metamorphosis was observed after four days. The lecithotroph *Phestilla* sp. 2 had 80% metamorphosis in response to its host coral *Goniopora fruticosa* and to waterborne cues from *G. fruticosa* five days after hatching. This species also had approximately 40% metamorphosis in response to non-prey corals *Porites cylindrica* and *Turbinaria reniformis*. These different larval development times make *Phestilla* spp. an ideal group to study the ecological and evolutionary consequences of different life history strategies in marine invertebrates.
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Keywords: Evolutionary ecology; Larval competence; Metamorphosis; *Phestilla*

1. Introduction

Recruitment of larvae to the appropriate adult habitat is a key stage for survival in many benthic marine organisms. Throughout marine invertebrates there are many different larval forms and dispersal strategies for finding the appropriate adult habitat (Thorson, 1950; Jablonski and Lutz, 1983; Levin and Bridges, 1995). Most marine invertebrates undergo a complex physiological and morphological metamorphosis from larvae to juveniles when they settle. Larval metamorphic competence refers to the capability of a developing larva to settle and undergo the morphological and developmental changes associated with metamorphosis (reviewed in Bishop et al., 2006). Species that require longer larval periods and feed in the plankton (planktotrophs) to reach metamorphic competence are generally thought to have greater dispersal potential

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than larvae with non-feeding, short planktonic periods (lecithotrophs) before metamorphosis (Levin and Bridges, 1995).

Some molluscan genera have developed a wide variety of larval strategies. Within the gastropod genus *Conus*, *C. lividus* releases larvae that feed in the plankton for up to 50 days before they are competent for metamorphosis. On the opposite end of the spectrum *C. pennaceus* has larvae that metamorphose into juveniles hours after hatching (Perron, 1981). The opisthobranch *Alderia modesta* has a range of larval types including planktotrophic and lecithotrophic veligers within the same egg clutches (Krug, 1998), and the potential biogeographic and genetic consequences of planktotrophic versus direct-development larval ecology is illustrated in three species of *Crepidula* (Collin, 2001).

Within the opisthobranch genus *Phestilla* we found a wide range of larval strategies. The veliger larvae of the nudibranch *Phestilla sibogae* have been well studied as a model for larval ecology (Harris, 1975; Hadfield 1977; Rudman, 1981; Miller and Hadfield, 1990; Hadfield and Koehl, 2004). This species has facultative lecithotrophic veligers that settle in response to host corals of the genus *Porites* (Kempf and Hadfield, 1985). A water soluble compound(s) released by corals in the genus *Porites* is the metamorphic inducer for *P. sibogae* (Hadfield and Pennington, 1990). The other described species in this genus, *Phestilla melanobrachia* and *P. minor*, are known to eat corals (Harris, 1975; Rudman, 1981), but their larval ecology is poorly known.

In this study we document the time required to reach metamorphic competence of *Phestilla melanobrachia*, *P. sibogae*, *P. minor*, and one undescribed species (*Phestilla* sp. 2) in response to preferred and non-preferred prey. These *Phestilla* spp. offer an unusual opportunity to study the relationship of host specificity with larval duration until metamorphic competence, and highlight the importance of understanding larval life histories to better understand speciation in marine invertebrates.

2. Materials and methods

2.1. Species studied

Phestilla melanobrachia is an aeolid nudibranch described by Bergh in 1874. The distribution, adult diet breath, and larval competence of this species were studied by Harris (1968, 1975). *Phestilla melanobrachia* feeds on at least 8 species of corals from 4 genera, all in Dendrophyllidae. Harris (1975) found a maximum of 13.4% larval metamorphosis in response to *Dendrophyllia elegans*.

Phestilla sibogae (Bergh, 1905) is the best studied species in this genus as it has been the focus of extensive research by Dr. Michael Hadfield during the last 30 years. This species has been synonymized under the name *P. lugubris* by Rudman (1981); however, to be consistent with previous literature we use the name *P. sibogae*. *P. sibogae* has lecithotrophic larvae that are competent for metamorphosis 4 days after hatching (=9 days after fertilization) (Miller and Hadfield, 1986). *Phestilla sibogae* adults feed on at least 6 *Porites* species, but not *Turbinaria reniformis* or *Goniopora fruticosa*, which are eaten by other *Phestilla* spp. (Ritson-Williams et al., 2003).

Phestilla minor is a small (max 7 mm) species that was described by Rudman (1981). *P. minor* also have veliger larvae, but the time required to reach metamorphic competence is previously unreported. On Guam *P. minor* eats two species of corals, *Porites annae* and *Porites lutea*. *Phestilla* sp. 1 from Guam eats two different species of corals, *Porites (Synerea) rus* and *Porites cylindrica*. *Phestilla minor* and *Phestilla* sp. 1 have the narrowest diet breadth of the *Phestilla* spp. studied (Ritson-Williams et al., 2003). Due to limited larvae we were unable to conduct competence experiments with *Phestilla* sp. 1; however, our preliminary observations suggest that it has a similar larval strategy as *Phestilla minor*.

Phestilla sp. 2 is morphologically similar to *Phestilla sibogae* and is briefly mentioned in the literature, (Gosliner, 1992; Gosliner et al., 1996) but remains undescribed. The time until metamorphic competence of its veliger larvae has not been previously studied. Previously we found that the adults did eat some but not all *Goniopora* corals but would not eat *Porites cylindrica* (Ritson-Williams et al., 2003).

2.2. Larval cultures

Adult nudibranchs were maintained in laboratory aquaria on their respective host corals, and their egg masses were collected daily. Egg masses were maintained in larval chambers (described below and in Miller and Hadfield, 1986) with daily water changes using 0.2 μm filtered seawater (FSW). After the larvae were fully developed (when the veligers were completely formed and moving), typically 5 days after fertilization, the egg masses were hatched by tearing them with forceps. *Phestilla minor* veligers were maintained in plastic petri dishes as described below. *Phestilla melanobrachia*, *P. sibogae*, and *P.* sp. 2 veligers from

5–10 egg masses were placed in antibiotic FSW (90 μg ml^{-1} Penicillin G, and 75 μg ml^{-1} streptomycin sulfate) in 2–4 larval chambers. The larval chambers consisted of an inner pvc pipe with 80 μm nitex screen covering one end which was raised above the bottom of a 1 L plastic beaker. In this way the water in the beakers could be changed without removing the larvae from the inner pvc chamber. The plastic beakers were partially submerged in a flow-through seawater tank to maintain ambient ocean temperature (approx 28–32 °C). Antibiotic FSW was changed daily for each chamber. The larvae of *Phestilla melanobrachia* and *P. sibogae* were fed a combination of the microalgae *Isochrysis* sp. and *Rhodomonas* sp., larvae of *Phestilla* sp. 2 and *P. minor* were not fed.

2.3. Larval bioassays

For all of the species except for *Phestilla minor* a subset of the larvae were removed from their chambers and then tested for metamorphosis in response to different treatments at different times after hatching. *Phestilla sibogae* and *Phestilla*. sp. 2 larvae were removed from the same larval culture (which consisted of a combination of egg masses from different parents all laid on the same day) at each time tested. Due to the limited availability of *Phestilla melanobrachia* larvae the data presented is from multiple larval cultures; different days of testing used different larval cultures. *Phestilla melanobrachia* larvae were assessed on day 8 to determine if they were competent to metamorphose but this was not replicated so is excluded from statistical analysis. After day 8, six or seven replicates were used for each treatment on each day except only 3 replicates were used to test *Tubastraea aurea* polyps on day 11. Waterborne compounds were obtained from *Tubastraea aurea* by soaking coral fragments in seawater for 48 h and then filtering the seawater through a paper coffee filter.

Ten individual egg masses of *Phestilla minor* larvae were maintained in 25 ml plastic petri dishes (pre-soaked in freshwater for 24 h) containing FSW. Individual egg masses were hatched with forceps into a petri dish, and only the veligers themselves were transferred to a new petri dish of FSW without any egg case material. Each egg case was used as an individual replicate with at least 10 larvae per dish. Just after hatching a fragment of live *Porites annae* was added to five petri dishes, in five other dishes FSW was added as a control. The replicate dishes were scored for cumulative metamorphosis on a daily basis for 5 days. This data was arcsin square-root transformed and analyzed with a repeated measures ANOVA.

For the other species each metamorphosis assay consisted of 10–60 larvae which were placed in individual 5 or 9 ml wells of Costar® media culture well plates (nos. 3513 and 3516). The larvae in one well were exposed to one treatment of a coral fragment, waterborne compounds from coral, or the control of FSW. Five replicates were used for each treatment, unless otherwise noted. Coral fragments approximately 2 cm long were broken from their colony at least 24 h before the assay. For *Phestilla* sp. 2 assays, waterborne compounds were obtained from *Goniopora fruticosa* by soaking coral fragments in seawater for 48 h and then filtering through a paper coffee filter.

In each well plate veligers and empty shells (the juvenile slugs are transparent and difficult to count precisely) were counted 24 and 48 h after the addition of the treatment. Percent metamophosis was calculated as the number of empty shells / total number of larvae in the well × 100. *Phestilla sibogae* and *P. melanobrachia* metamorphose from veliger larvae into juvenile slugs 24 h after exposure to host corals (Hadfield, 1977). We found the highest rates of metamorphosis in the larvae of *Phestilla minor* and *Phestilla*. sp. 2 after 48 h. For *Phestilla* sp. 2 we present the data for metamorphosis after 24 and 48 h; however, since there were consistently higher rates of metamorphosis after 48 h, we only used this data for statistical comparisons.

For every species except for *P. minor* the rates of metamorphosis in response to different potential prey corals were compared on the last day tested. To assess when a species reached metamorphic competence the percent metamorphosis in response to its preferred prey was compared for all of the times tested. Data were arcsin square-root transformed and analyzed with a one-way ANOVA, followed by a Tukey's post-hoc test to determine significant groupings. If the data were not normal or the variances were not equal the non-parametric Kruskal–Wallis test was used to determine if there was a significant difference between the rates of metamorphosis in response to the treatments or at different times. The Kruskal–Wallis test was followed by a comparison of mean ranks to determine significant groupings. All analyses were conducted using Statistix 7 (Analytical Software).

3. Results

Phestilla melanobrachia has planktotrophic larvae that require time and food in the plankton to reach metamorphic competence (Harris, 1975). Eight days after hatching, only 8% of the larvae metamorphosed in response to polyps of their prey coral, *Tubastraea aurea*

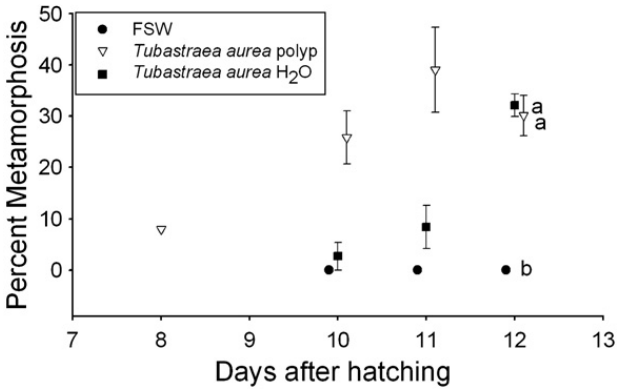

Fig. 1. *Phestilla melanobrachia* larval competence. Symbols represent the mean percentage of larvae to metamorphose in response to 0.2 μm filtered seawater (FSW), the coral *Tubastrea aurea*, and *T. aurea* water soluble compounds. Percent metamorphosis was measured using larvae of different ages (days after hatching, symbols are offset for clarity). Error bars are ±1 SE. The same letters next to the symbols indicate treatments that are statistically the same.

(Fig. 1). Twelve days after hatching *P. melanobrachia* larvae metamorphosed at the same rates to *T. aurea* polyps and coral water, both of which induced significantly greater rates of metamorphosis than FSW (Kruskal–Wallis, $H=11.989$, $p=0.0025$). Eleven days after hatching we observed 39% metamorphosis in response to *T. aurea* polyps, which is greater than the previously reported 13% metamorphosis for this species (Harris, 1975), however there was no significant difference between metamorphosis in response to *T. aurea* polyps on days 10, 11 and 12 (one-way ANOVA, $F=1.05$, $p=0.378$).

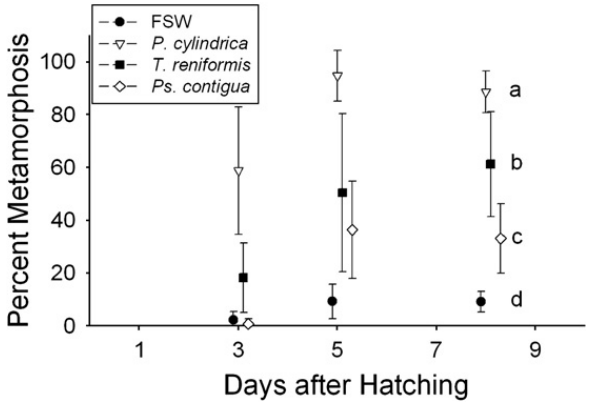

Fig. 2. *Phestilla sibogae* larval competence. Symbols represent the mean percentage of larvae of different ages (days after hatching) to metamorphose in response to 0.2 μm filtered seawater (FSW), the coral *Porites cylindrica, Turbinaria reniformis,* and *Psammacora contigua* (symbols are offset for clarity). For each time and treatment, $n=5$, error bars are ±1 SE. The same letters next to the symbols indicate treatments that are statistically the same. The same larval cultures were subsampled over time.

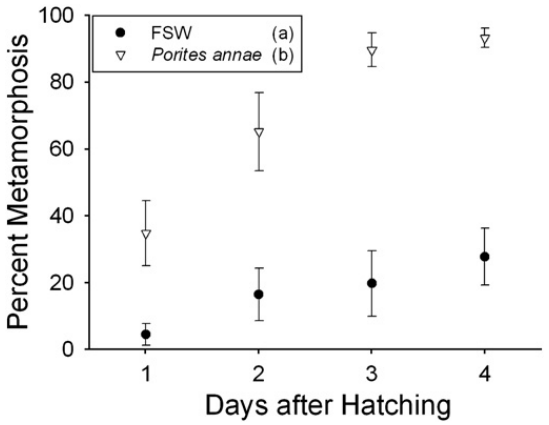

Fig. 3. *Phestilla minor* larval competence. Symbols represent the mean percentage of larvae of different ages (days after hatching) to metamorphose in response to 0.2 μm filtered seawater (FSW) and the coral *Porites annae*. Five replicates of each treatment were scored for cumulative metamorphosis, each replicate is an individual egg mass, error bars are ±1 SE. Significant groupings are shown as letters in parenthesis in the legend.

Phestilla sibogae is known to have facultative lecithotrophic larvae (Kempf and Hadfield, 1985). Eight days after hatching the highest percentage of metamorphosis was in response to *Porites cylindrica*, but *Turbinaria reniformis* and *Psammocora contigua* also induced significantly more metamorphosis than FSW (Fig. 2) (one-way ANOVA, $F=33.06$, $p<0.001$). *Porites cylindrica* induced more metamorphosis on day five compared to three days after hatching (one-way ANOVA, $F=7.66$, $p=0.007$).

Larval development and metamorphic competence have not been previously described for *Phestilla minor*. Three days after hatching 90% of the larvae metamorphosed in response to *Porites annae*, and there was some metamorphosis in the FSW control (max 27% on day 4) (Fig. 3). This is different from the other species tested in this study, all of which had little to no spontaneous metamorphosis in FSW. Both the treatment ($F=25.30$, $p=0.001$) and the time after hatching ($F=23.72$, $p<0.001$) had significant effects on the percent metamorphosis (repeated measures ANOVA); however, there was also a significant interaction of treatment and time ($F=3.91$, $p=0.021$). *P. annae* induced more metamorphosis than FSW (one-way ANOVA, $F=40.16$, $p<0.001$), and after three days metamorphosis in response to *P. annae* was significantly different from the first day (one-way ANOVA, $F=9.36$, $p<0.001$).

Phestilla sp. 2 did not require food to reach metamorphic competence and had a similar larval duration to competence as *Phestilla sibogae* (Fig. 4). *Phestilla* sp. 2 typically requires 48 hours of exposure

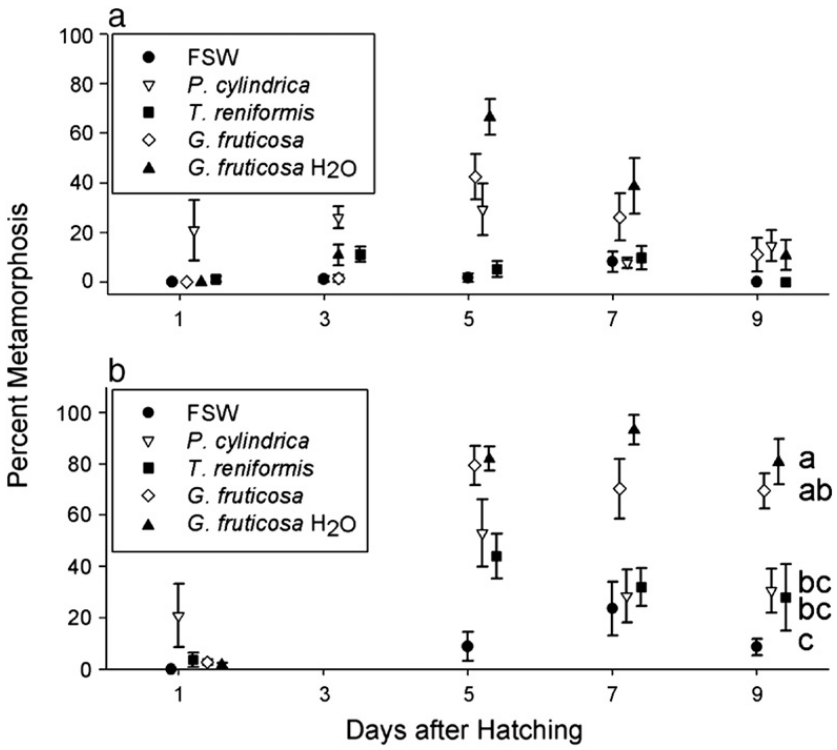

Fig. 4. *Phestilla* sp. 2 larval competence. Symbols represent the mean percentage of larvae of different ages (days after hatching) to metamorphose in response to 0.2 μm filtered seawater (FSW), the coral *Porites cylindrica, Turbinaria reniformis, Goniopora fruticosa,* and *G. fruticosa* coral water (symbols are offset for clarity). For each time and treatment $n=5$, except for day 9 when all treatments are $n=4$, error bars are ±1 SE. Significant groupings are shown as letters next to the treatment symbol. The same larval cultures were subsampled over time.

to its host coral to complete metamorphosis (Fig. 4b). Most of the larvae in our well plates died due to ciliates on day 3 after 48 h, thus these data were omitted from our analysis and graphs. Nine days after hatching *Goniopora fruticosa* and its waterborne cues induced significantly more metamorphosis than FSW (one-way ANOVA, $F=9.56$, $p<0.001$) (Fig. 4b). After five days, *Goniopora fruticosa* induced significantly more metamorphosis than one day after hatching (one-way ANOVA, $F=20.77$, $p<0.001$).

4. Discussion

Larval site selection is important for the survival and persistence of marine benthic organisms. All of the *Phestilla* species studied metamorphosed in response to their preferred prey although there was a range of larval duration until metamorphic competence (Table 1). Each *Phestilla* species has a different larval strategy which potentially influences their adult ecology, including host specificity, distribution and speciation.

Phestilla melanobrachia larvae had a maximum of 39% larval metamorphosis 11 days after hatching in response to *Tubastraea aurea* polyps, which *P. melanobrachia* eats on Guam (SMS, personal observation). Twelve days after hatching 30% of *P. melanobrachia* larvae also metamorphosed in response to *Tubastraea aurea* waterborne cues. A water soluble chemical cue produced by *T. aurea* is probably the metamorphic inducer for *P. melanobrachia*, which is consistent with *P. sibogae* and *Phestilla*. sp. 2 which require water soluble compound(s) produced by host

Table 1
Summary of *Phestilla* spp. metamorphic competence at different times and in response to different treatments

Species	Time until significant metamorphosis in response to preferred coral (days after hatching)	Treatments that induced significantly more metamorphosis than filtered seawater
Phestilla melanobrachia	>12 days	*Tubastraea aurea* polyps and waterborne metabolites
Phestilla sibogae	5 days	*Porites cylindrica* *Turbinaria reniformis* *Psammocora contigua*
Phestilla minor	3 days	*Porites annae*
Phestilla sp. 2	5 days	*Goniopora fruticosa* polyps and waterborne metabolites

corals to induce metamorphosis (Hadfield and Scheuer, 1985; Hadfield and Pennington, 1990). During this study we observed higher rates of metamorphosis (39%) than the 13.4% metamorphosis previously recorded for *P. melanobrachia* (Harris, 1975), however this is much less than the approximately 90% larval metamorphosis observed in the other three *Phestilla* spp. studied here. These larvae probably need more than 12 days in the plankton to fully develop; unfortunately we were unable to maintain our larval cultures longer. *P. melanobrachia* larvae might also require different nutrition to reach metamorphic competence; we were limited to *Isochrysis* sp. and *Rhodomonas* sp. for larval food, which is not representative of potential food in the ocean. With a larval period of greater than 12 days *P. melanobrachia* has the potential for a wide dispersal range, and has been observed from Hawaii to Australia (Harris, 1968; Rudman, 1981).

Phestilla sibogae larvae responded to *Porites cylindrica* with >90% metamorphosis five days after hatching. This is similar to the greater than 90% metamorphosis by day 5 reported in response to *Porites compressa* on Oahu (Miller and Hadfield, 1986). We also compared rates of metamorphosis in response to non-preferred prey. Throughout our studies *Turbinaria reniformis* (Dendrophylliidae) consistently induced higher rates of metamorphosis than FSW (Ritson-Williams et al., 2003). Unfortunately larval bioassays in standing water may expose larvae to unnatural concentrations of chemical cues (Zimmer and Butman, 2000). The water soluble compound(s) released by *T. reniformis* may be similar to the metamorphic inducer produced by *Porites* corals. *Phestilla sibogae* larvae in the ocean could also swim away from non-host corals. *P. sibogae* larvae were found to stop swimming when exposed to the *Porites compressa* waterborne cue (Hadfield and Koehl, 2004), and the larvae resumed swimming if they were moved out of water containing the inducer. *Phestilla sibogae* larvae can detect and behaviorally respond to their required host coral allowing them to settle near it and complete metamorphosis.

Phestilla sp. 2 larvae are most similar to *P. sibogae* in their development mode (lecithotrophic) and their age until metamorphic competence. *Phestilla* sp. 2 larvae may also be facultative lecithotrophs like *P. sibogae* since the larvae were observed feeding when offered food (RRW, personal observation). Five days after hatching there was approximately 80% metamorphosis in response to *Goniopora fruticosa* polyps and its water soluble cues. Five days after hatching 53% of the larvae metamorphosed in response to *Porites cylindrica*, and 44% in response to *Turbinaria reniformis*, both of which are not eaten by *Phestilla* sp. 2 (Ritson-Williams et al., 2003). It is important to note that these larvae were exposed to the treatment corals for 48 h. Future experiments could determine the minimum amount of exposure time required to induce metamorphosis (Botello and Krug, 2006). In light of the behavioral research on *P. sibogae* (Hadfield and Koehl, 2004), the minimum exposure time to metamorphic inducers is an important ecological consideration for planktonic larvae that can make behavioral choices. High rates of metamorphosis in response to non-preferred prey may result from high levels of exposure to waterborne metabolites in standing water or the presence of similar coral metabolite(s) as discussed above for *P. sibogae*. Variable metamorphosis in response to other corals could also indicate that larvae of *Phestilla* spp. may have some inherent variability in metamorphosis which could lead to host switching, a hypothesis proposed for other larvae (Hadfield and Strathmann, 1996).

Larval development and duration of *Phestilla minor* is quite different from the other species described above. Metamorphosis begins one day after hatching, and by three days after hatching 90% of the larvae metamorphosed in response to *Porites annae*. Immediately after hatching, the veliger larvae of *P. minor* were observed crawling along the bottom of our dish instead of swimming like the larvae of the other species. *P. minor* larvae have a short planktonic stage that probably leads to limited dispersal. Three days after hatching 20% of the larvae also metamorphosed in the FSW control. *Phestilla minor* is the only species studied that had increasing spontaneous metamorphosis over the duration of the experiment. Spontaneous metamorphosis of these larvae probably leads to limited dispersal and subsequent genetic isolation. *P. minor* has a greater documented distribution (Hawaii to Africa) than *P. melanobrachia* (Hawaii to Australia), which could be due to greater availability of host corals, or more likely a lack of search effort for *Phestilla* spp. in the Indian Ocean.

In *Phestilla* nudibranchs there is a correlation between diet breadth and the larval duration until metamorphic competence. These nudibranchs have a range of diet breadth from eight coral species eaten by *P. melanobrachia* to only two *Porites* spp. eaten by *Phestilla minor* and *P.* sp. 1 (Ritson-Williams et al., 2003). *Phestilla melanobrachia* has the widest diet breadth and larvae with the longest dispersal potential. With a greater dispersal potential *P. melanobrachia* has more chances of dispersing between islands that have a larger variation of coral species. On the other side of the continuum is *P. minor*, which only eats two species of *Porites* corals and has a short larval duration and

limited dispersal potential. Physiological constraints are most often used to explain the degree of specialization in individual species; however, abundance and availability of a host species after dispersal between oceanic islands could be an important factor in diet breadth. Wide dispersal ability and a wide diet breadth could be an adaptive strategy when host species are rare or ephemeral. *Phestilla melanobrachia* and *P. minor* have very different larval strategies for adult survival.

The potential planktonic duration is not only important for larval ecology and dispersal, it probably plays a role in *Phestilla* speciation. Over geologic time scales survival from extinction events has favored widely distributed molluscan species (Jackson, 1974; Hansen, 1978, 1980). Now molecular genetic techniques can be used to see patterns of speciation across oceanic islands in the Pacific (Palumbi, 1994, 1997; Benzie, 1999; Paulay and Meyer, 2006). Studies of other marine phyla have found that larvae with long dispersal are genetically homogeneous across large geographical scales (Duffy, 1993; Williams and Benzie, 1996; Duran et al., 2004a), while species with short larval durations show higher levels of genetic variation over small geographic scales (Hunt, 1993; Duran et al., 2004b; Kirkendale and Meyer, 2004). In a comparison of two nudibranchs that have different dispersal potentials, the planktotroph had no genetic differentiation on a coastline of 1600 km, while the species with lecithotrophic larvae had distinct populations with genetic differentiation in less than 10 km (Todd et al., 1998). However, there are confounding factors that can influence genetic exchange in the marine environment, which cause exceptions to this theory (Paulay, 1997; Hellberg, 1998; Benzie, 1999; Ayre and Hughes, 2000; Levin, 2006; Paulay and Meyer, 2006). Faucci et al. (2007) recently found that *P. melanobrachia* and *P. sibogae* are genetically connected between Hawaii, Guam and Palau, but *P. minor* has unique genotypes between these islands. Since *P. minor* larvae can spontaneously metamorphose there is potential for host switching which could have led to sympatric speciation, i.e. the evolution of *Phestilla* sp. 1 which inhabits a different niche by eating different *Porites* species (Ritson-Williams et al., 2003; Faucci et al., 2007). In terrestrial studies, some insects evolve into genetically distinct species between different hosts (Feder and Bush, 1989; Leebens-Mack et al., 1998; Johnson et al., 2002). *Phestilla* spp. have a range of larval strategies which offer an opportunity to study the importance of allopatric processes (dispersal and genetic isolation) and sympatric processes (niche partitioning) in the evolution of marine diversity.

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