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REPORT

Settlement induction of *Acropora palmata* planulae by a GLW-amide neuropeptide

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Abstract Complex environmental cues dictate the settlement of coral planulae in situ; however, simple artificial cues may be all that is required to induce settlement of ex situ larval cultures for reef re-seeding and restoration projects. Neuropeptides that transmit settlement signals and initiate the metamorphic cascade have been isolated from hydrozoan taxa and shown to induce metamorphosis of reef-building *Acropora* spp. in the Indo-Pacific, providing a reliable and efficient settlement cue. Here, the metamorphic activity of six GLW-amide cnidarian neuropeptides was tested on larvae of the Caribbean corals *Acropora palmata*, *Montastraea faveolata* and *Favia fragum*. *A. palmata* planulae were induced to settle by the exogenous application of the neuropeptide Hym-248 (concentrations $\geq 1 \times 10^{-6}$ M), achieving 40–80% attachment and 100% metamorphosis of competent planulae (≥6 days post-fertilization) during two spawning seasons; the remaining neuropeptides exhibited no activity. Hym-248 exposure rapidly altered larval swimming behavior (<1 h) and resulted in >96% metamorphosis after 6 h. In contrast, *M. faveolata* and *F. fragum* planulae did not respond to any GLW-amides tested, suggesting a high specificity of neuropeptide activators on lower taxonomic scales in corals. Subsequent experiments for *A. palmata* revealed that (1) the presence of a biofilm did not enhance attachment efficiency when coupled with Hym-248 treatment, (2) neuropeptide-induced settlement had no negative effects on early life-history developmental processes: zooxanthellae acquisition and skeletal secretion occurred within 12 days, colonial growth occurred within 36 days, and (3) Hym-248 solutions maintained metamorphic activity following storage at room temperature (10 days), indicating its utility in remote field settings. These results corroborate previous studies on Indo-Pacific *Acropora* spp. and extend the known metamorphic activity of Hym-248 to Caribbean acroporids. Hym-248 allows for directed and reliable settlement of larval cultures and has broad applications to the study and rehabilitation of threatened *Acropora* populations in the Caribbean.

Keywords *Acropora palmata* · Caribbean coral reefs · Restoration · Settlement · Metamorphosis · Neuropeptides

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Introduction

Global declines of coral reef ecosystems continue in response to multiple and accumulating stressors from both anthropogenic and natural sources (Wilkinson 2008). Coral reef conservation efforts currently target both the mitigation and alleviation of global stressors (Aronson and Precht 2006), as well as local restoration projects designed to stimulate reef health and larval recruitment (Omori and Fujiwara 2004; Yeemin et al. 2006). Such rehabilitation efforts commonly utilize asexual reproduction methods (i.e., transplantation of coral fragments) to enhance coral abundance at affected reef sites (Becker and Mueller 2001; Okubo et al. 2005; Rinkevich 2005). However, transplant projects can result in reduced genetic diversity (Baums

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2008) and cause damage to donor colonies. Expanding knowledge of coral sexual reproduction and larval development (e.g., Harrison et al. 1984; Szmant 1986; Harrison and Wallace 1990; Richmond 1997; Baird et al. 2009) has allowed for mass culturing of sexually produced coral propagules for rehabilitation (Heyward et al. 2002; Miller and Szmant 2006; Omori et al. 2008).

Successful re-seeding of coral populations with sexual propagules requires that larvae are induced to settle onto artificial substrates transplanted to impacted sites or directly onto substrates at the impacted sites. The natural settlement process consists of various steps: substrate exploration, attachment and metamorphosis (reviewed in Harrison and Wallace 1990). Once developmentally competent, larvae exhibit complex and variable behaviors in response to abiotic and biotic factors that determine their swimming direction (reviewed in Ritson-Williams et al. 2009), but generally swim toward the benthos (e.g., Szmant and Meadows 2006) and actively probe substrate surfaces with putatively chemosensory cells located in their aboral ends (Vandermeulen 1974; Harrison and Wallace 1990). Directed by environmental cues, larvae select appropriate habitats and attach to the substrate, a process that may occur over several hours depending on the species and their attraction to the substrate (Morse et al. 1988; Miller and Mundy 2003; Harrington et al. 2004). Once firmly attached, the larvae begin a differentiation process (commonly referred to as metamorphosis) that initially involves flattening of the oral-aboral axis and development of radial mesenteries (Harrison and Wallace 1990; Heyward and Negri 1999). Metamorphosis leads to the formation of a primary polyp, which may require one or more days for complete pharynx and tentacle formation (Heyward and Negri 1999; Negri et al. 2001; Miller and Mundy 2003). The completion of the settlement process yields a permanently attached and fully differentiated juvenile coral polyp (Harrison and Wallace 1990).

Numerous factors may influence settlement and metamorphosis of coral larvae. Larval can be included to settle and metamorphose by bacteria (Negri et al. 2001), microbial biofilms (Webster et al. 2004; Erwin et al. 2009), calcified red algae (Morse et al. 1988, 1996; Heyward and Negri 1999; Negri et al. 2001; Harrington et al. 2004; Ritson-Williams et al. 2010), calcified green algae (Nugues and Szmant 2006), and bare carbonate skeletons (Golbuu and Richmond 2007). Studies characterizing natural larval cues are essential to understanding which reef biota stimulates natural larval settlement; however, the inherent variability in the activity, composition and availability of such cues often limit their application to large-scale induction of larval settlement during mass culturing efforts. Further, some cues emanate from unsuitable substrata, ultimately resulting in high post-settlement mortality of coral spat via competitive overgrowth, epidermal sloughing or substrate disintegration (Harrington et al. 2004; Nugues and Szmant 2006; Szmant and Miller 2006).

Other studies have investigated the internal endocrine pathway induced by external cues (Iwao et al. 2002), thereby targeting a more predictable means to induce settlement and metamorphosis in larval cultures. Neuropeptides are internal signal molecules known to regulate numerous physiological processes in cnidarians, including metamorphosis, and have been described from hydrozoan, scyphozoan and anthozoan taxa (Grimmelikhuijzen et al. 2002; Müller and Leitz 2002). In particular, a family of cnidarian neuropeptides sharing an amidated glycine-leucine-tryptophan C-terminus motif (GLW-amide family) is especially diverse in structure and function, regulating metamorphosis in *Hydractinia* (Takahashi et al. 2008) and neuron differentiation and tentacle formation in *Hydra* (Takahashi et al. 2000, 2003). While these studies have focused primarily on hydrozoans, the *Hydra*-derived GLW-amide neuropeptide Hym-248 (Takahashi et al. 1997) also induces metamorphosis in cultured planulae of nine Pacific *Acropora* species (Iwao et al. 2002) and mixed *Acropora* coral slicks (Hatta and Iwao 2003) with remarkable consistency. These results suggest that although significant intra-specific variability exists in settlement responses to external cues, the underlying neurotransmission signal induced by these cues is conserved and can be exogenously manipulated. A reliable and efficient means to stimulate attachment and metamorphosis of coral larvae under laboratory conditions has significant implications for developmental studies (Hirose et al. 2008) as well as reef restoration efforts (Omori and Fujiwara 2004; Petersen et al. 2005a).

Acropora palmata (Lamarck 1816) was historically a dominant reef-building coral in the Caribbean that has suffered recent and drastic population declines throughout the region (Bruckner 2002). This has prompted conservation and restoration efforts through federal protection as a threatened species under the U.S. Endangered Species Act (NMFS 2006), local rehabilitation projects (Becker and Mueller 2001) and the establishment of ex situ populations in aquaculture (Petersen et al. 2008). To date, no study has reported the metamorphic activity of Hym-248 and related GLW-amide neuropeptides on *A. palmata* or any Caribbean reef coral. The present study tested the effect of six cnidarian neuropeptides from the GLW-amide family on the metamorphosis and attachment of planulae from the broadcast spawning reef-building corals *A. palmata* and *Montastraea faveolata* and the brooding coral *Favia fragum*. More detailed studies were conducted with the active inducer (Hym-248) on *A. palmata*, characterizing the development of competency, the optimal neuropeptide concentration for attachment and metamorphosis, and the

time course of larval response to Hym-248. To test the applicability of Hym-248 metamorphosis induction to restoration efforts, the stability of Hym-248 in solution at room temperature and the post-settlement survival and development of primary polyps induced with Hym-248 were also examined.

Methods

Gamete collection, fertilization and larval rearing

Gamete bundles released by multiple *Acropora palmata* colonies were collected on 19 August 2008 (3 days after the full moon, AFM) and 10 August 2009 (5 days AFM) at the Tres Palmas Reserve in Rincón, Puerto Rico. Gamete bundles released by multiple *Montastraea faveolata* colonies were collected on 23 August 2008 (7 days AFM) at Turrumote Reef in La Parguera, Puerto Rico. Spawned gametes were cross-fertilized for 1 h, and the eggs were subsequently rinsed of excess sperm. Concentrated fertilized eggs were added to 6 and 12-l polycarbonate culture bins containing 5 μm filtered seawater (FSW). Daily water changes were conducted on all bins to maintain temperature and remove accumulated debris and mucous. In 2009, larvae were transferred to 6-l polycarbonate bins containing 0.2 μm FSW as larvae developed into motile planulae (4 days post-fertilization) and cleaned daily to prevent microbial film build-up on the container surfaces. Planulae released by 20 adult *Favia fragum* colonies were collected on 27 August 2009 (8 days after the new moon) and maintained in a 12-l polycarbonate culture bin with flow-through 5 μm FSW.

Neuropeptide induction experiment: *M. faveolata* and *F. fragum*

Six previously characterized cnidarian neuropeptides purchased from GenScript USA Inc. were tested for induction of attachment and metamorphosis of coral larvae (Table 1).

Table 1 Name, primary structure and purity level of six cnidarian GLW-amide neuropeptides tested for settlement and metamorphosis induction of Caribbean coral larvae

Peptide	Primary structure	Purity (%)	Citation
Hym-53	NPYPGLW-NH_2	98.8	Takahashi et al. (1997)
Hym-54	GPMTGLW-NH_2	96.6	Takahashi et al. (1997)
Hym-248	EPLPIGLW-NH_2	94.4	Takahashi et al. (1997)
Hym-249	KPIPGLW-NH_2	90.3	Takahashi et al. (1997)
Hym-331	GPPPGLW-NH_2	97.3	Takahashi et al. (1997)
MMA	pEQPGLW-NH_2	88.2	Leitz et al. (1994)

Twenty *M. faveolata* planulae (4 days post-fertilization) or 10 *F. fragum* planulae (1 days post-release) were added to each well of sterile, polystyrene 6-well culture plates (Falcon®) with 10 ml 0.2 μm FSW. Each of the six neuropeptides was tested individually at final concentrations of 1×10^{-5} M, with three replicate wells per treatment. Since GLW-amide neuropeptides exhibit activity at concentrations $\geq 1 \times 10^{-6}$ M (Takahashi et al. 1997, 2003; Iwao et al. 2002), peptides were initially tested at 1×10^{-5} M. Activity at lower concentrations cannot be ruled out; however, previous reports for GLW-amide neuropeptides report activity at and above a threshold concentration (Takahashi et al. 1997, 2003; Iwao et al. 2002), with no inhibition of activity at higher concentrations, similar to the results for Hym-248 reported herein. Control treatments received no neuropeptide addition. Culture plates were floated in water baths to maintain temperature, and censused under light microscopy after 24 h. Both attachment (differentiated polyps permanently affixed to substrate) and metamorphosis (differentiated polyps whether attached or not) were recorded because of the high frequency of free-floating yet fully differentiated polyps. In addition, survivorship of differentiated polyps and undifferentiated planulae were recorded to document any potential toxicity effects of the neuropeptide inducers. Except for the Hym-248 tile experiment, all experiments were conducted without additional substrate for attachment, thus all attachment reported occurred on the polystyrene surfaces of the 6-well plates.

Hym-248 induction experiment: *A. palmata*

From two to 7 days post-fertilization in 2008 and 2009, 10 *A. palmata* larvae were added per well to sterile 6-well plates with 10 ml of 0.2 μm FSW. Experimental treatments received Hym-248 at a final concentration of 1×10^{-5} M. Control treatments received no neuropeptide addition. Six replicate wells were conducted per treatment. Experimental plates were floated in water baths to maintain temperature and scored under light microscopy after 24 h. Developmental progression was qualitatively assessed daily, following the nomenclature of Randall and Szmant (2009). In 2008, the experiment was not conducted on day 7, due to extensive spontaneous metamorphosis and attachment of larvae in the cultures.

Statistical significance was determined by 2-way ANOVAs using SigmaPlot v11 (Systat Software Inc.), with larval age (days post-fertilization) and treatment (with or without neuropeptide) as independent variables and survivorship, attachment and metamorphosis as dependent variables. Significant effects were followed by Bonferroni post hoc comparisons to determine pair-wise significance within factors.

Hym-248 concentration experiment: *A. palmata*

To assess the dose–response of Hym-248 activity, 10 *A. palmata* planulae (8 days post-fertilization in 2008 and 2009) were added per well to sterile 6-well plates with 10 ml of 0.2 μm FSW. Hym-248 was added to wells at the following final concentrations: 1×10^{-8} M (10 nM), 1×10^{-7} M (100 nM), 1×10^{-6} M (1 μM), 1×10^{-5} M (10 μM) and 1×10^{-4} M (100 μM). Control treatments received no neuropeptide addition. Three replicate wells were conducted per treatment. Experimental plates were floated in water baths to maintain temperature and censused under light microscopy after 24 h. Statistical significance was determined using two-way ANOVAs with treatment and year as independent variables and survivorship, attachment and metamorphosis as dependent variables.

Hym-248 response time experiment: *A. palmata*

To assess the response time of *A. palmata* planulae to Hym-248, 10 *A. palmata* planulae (10 days post-fertilization in 2008) were added per well to a sterile 6-well plate with 10 ml of 0.2 μm FSW. Experimental treatments received Hym-248 at a final concentration of 1×10^{-5} M. Control treatments received no neuropeptide addition. Three replicate wells were conducted per treatment. Experimental plates were floated in water baths to maintain temperature and scored under light microscopy after 1, 3 and 6 h.

Hym-248 stability experiment: *A. palmata*

To assess the stability of Hym-248 in solution at room temperature, 10 *A. palmata* planulae (7 days post-fertilization in 2009) were added per well to sterile 6-well plates with 10 ml of 0.2 μm FSW. Experimental treatments received Hym-248 incubated at 4°C for 10 days or Hym-248 incubated at 26°C for 10 days to a final concentration of 1×10^{-5} M. Control treatments received no neuropeptide addition. Six replicate wells were conducted per treatment. Experimental plates were floated in water baths to maintain temperature and scored under light microscopy after 24 h. Statistical significance was determined using one-way ANOVAs with treatment as the independent variable and survivorship, attachment and metamorphosis as dependent variables.

Hym-248 tile experiment: *A. palmata*

To assess whether tile pre-conditioning (i.e., biofilm formation) increased settlement rates of *A. palmata* larvae exposed to Hym-248, 10 cm × 10 cm porcelain tiles ($n = 8$) were conditioned in a back-reef of San Cristobal Reef, Puerto Rico at 2 m depth for 11 days. Tiles were collected simultaneously in individual containers on the day of the settlement experiment. Non-conditioned tiles ($n = 8$) were soaked in 5 μm FSW for 2 h prior to the experiment to remove excess dust and debris. Four treatments ($n = 4$ tiles per treatment) were established: (1) Hym-248 + Biofilm, (2) Hym-248, (3) Biofilm and (4) Control (no Hym-248, no biofilm). Fifty *A. palmata* planulae (7 days post-fertilization) were added to 1-l plastic bins containing 500 ml of 0.2 μm FSW and 1 tile. Hym-248 was added to the designated bins at a final concentration of 2×10^{-6} M. Bins were incubated at room temperature for 24 h and scored under light microscopy. Statistical significance was determined using two-way ANOVAs with neuropeptide and biofilm treatment as the independent variables and survivorship, metamorphosis, total settlement and tile settlement as dependent variables. Following settlement scoring, the eight tiles with the most settled larvae were returned to the back-reef and attached horizontally to wire racks. After 12 and 36 days, the tiles were transported back to the lab and viewed under light microscopy to assess post-settlement survival and juvenile development.

Results

Neuropeptide induction experiment: *M. faveolata* and *F. fragum*

No attachment or metamorphosis induction was observed by *M. faveolata* planulae for any of the six neuropeptides tested. No consistent attachment or metamorphosis was observed in *F. fragum* planulae, with only two larvae metamorphosing and settling in response to Hym-54 and Hym-248. Therefore, no additional experiments were conducted for these species.

Effects of Hym-248 on *A. palmata* larval survivorship

No significant effect of the neuropeptide Hym-248 on survivorship of *A. palmata* larvae (i.e., toxicity effects) was observed in any of the experiments. In the induction experiments, survivorship increased significantly with larval age ($F_{4,55} = 10.10$, $P < 0.001$ in 2008; $F_{5,66} = 14.60$, $P < 0.001$ in 2009), due to lower survivorship in larvae undergoing gastrulation (2 days post-fertilization, 75.0 ± 4.0% in 2008, 48.3 ± 5.1% in 2009; mean ± SE) compared to post-gastrulation planula larvae (3–7 days post-fertilization; 80.8–93.3% in 2008, 85.8–94.2% in 2009); however, no significant differences in survivorship of larvae treated with Hym-248 and control larvae were observed ($F_{1,58} = 2.55$, $P = 0.116$ in 2008; $F_{1,70} = 0.09$,

$P = 0.765$ in 2009). In the neuropeptide concentration experiments, a significant effect of year on survivorship was observed ($F_{1,34} = 6.87$, $P = 0.015$), with higher survivorship in 2008 (96%) than 2009 (83%), but again, no significant effect of Hym-248 treatment on larval survivorship ($F_{5,30} = 1.46$, $P = 0.241$). Similarly, the stability experiment and tile settlement experiment revealed no significant toxicity effects of Hym-248 treatment on *A. palmata* larvae ($F_{2,15} = 1.94$, $P = 0.177$ and $F_{1,14} = 0.93$, $P = 0.355$, respectively).

Hym-248 induction experiment: *A. palmata*

No attachment or metamorphosis response was observed by *A. palmata* gastrulae (2 days post-fertilization) or early planula larvae (3 days post-fertilization) in response to Hym-248 treatment. Beginning 4 days post-fertilization, larvae became competent, with 40% of planulae metamorphosing in response to Hym-248 treatment in 2008 and 64% in 2009 (Fig. 1). By 5 days post-fertilization, the percentage of larvae induced to metamorphose increased to 92% in 2008 and 100% in 2009, and on day 6 and 7 days post-fertilization, there was 100% metamorphosis in both years (Fig. 1). Of the metamorphosed planulae, only a portion attached to the substrate while the remainder formed floating (i.e., unattached) polyps. Attachment frequencies were highly variable among replicates but generally increased with larval age. Significant statistical interactions between treatment and age were observed in both years for attachment ($F_{4,55} = 12.13$, $P < 0.001$ in 2008; $F_{5,66} = 18.10$, $P < 0.001$ in 2009) and metamorphosis induction ($F_{4,55} = 65.32$, $P < 0.001$ in 2008; $F_{5,66} = 218.18$, $P < 0.001$ in 2009). In 2008, approximately 40% of induced planulae attached in assays conducted between days four to six post-fertilization. In 2009, attachment increased with larval age, reaching a maximum of 79% on day seven (Fig. 1).

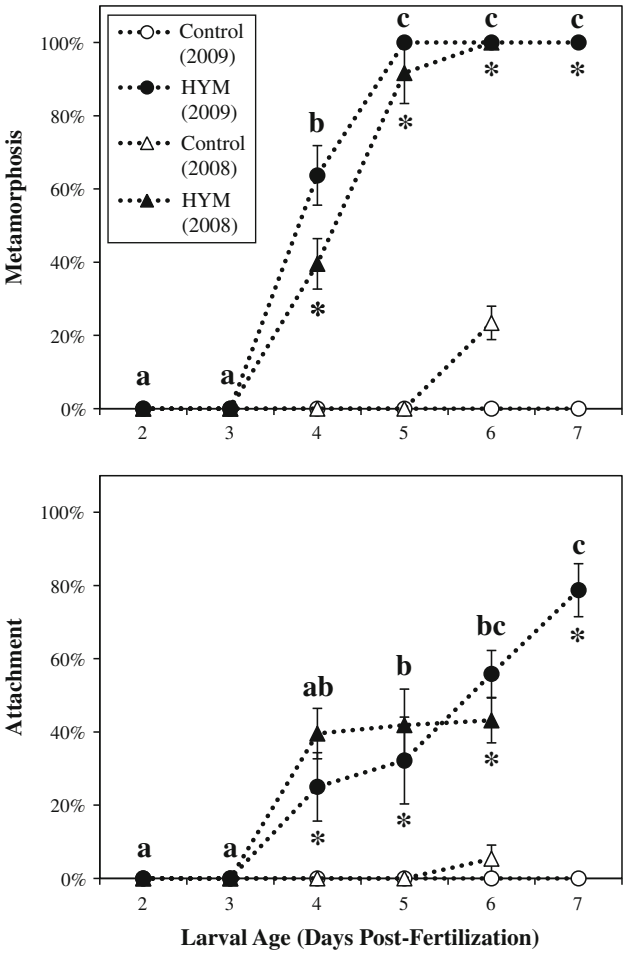

Fig. 1 Attachment and metamorphosis of *Acropora palmata* larvae in response to the neuropeptide Hym-248 from 2 to 7 days post-fertilization ($n = 6$, 10 larvae per replicate). Data from two spawning seasons (2008 and 2009) are shown, with *filled symbols* indicating Hym-248 treatment (1×10^{-5} M) and *open symbols* controls. *Asterisks* denote significant differences ($P < 0.05$) between treatments and controls for each larval age (2008 and 2009 data). *Different letters* above symbols depict significant differences ($P < 0.05$) among larval ages within Hym-248 treatments (2009 data)

Hym-248 concentration experiment: *A. palmata*

Hym-248 induced 100% metamorphosis of *A. palmata* planulae at concentrations $\geq 1 \times 10^{-6}$ M. Treatments at 1×10^{-7} M induced a smaller proportion of larvae to metamorphose (37% in 2008, 55% in 2009) and concentrations $\leq 1 \times 10^{-8}$ M did not induce metamorphosis (Fig. 2). Attachment occurred at the same neuropeptide concentrations as metamorphosis, although frequencies of attachment exhibited higher variability among replicates. In 2008, attachment steadily increased with higher peptide concentrations, reaching a maximum of 53% in the highest dosage (1×10^{-4} M). In 2009, the proportion that attached was more erratic, reaching a maximum of 63% in the second highest dosage (1×10^{-5} M; Fig. 2).

Hym-248 response time experiment: *A. palmata*

After 1 h of exposure to Hym-248, treated larvae changed from elongated, actively swimming planulae to rounded planulae spinning about their axis. After 3 h of exposure, 73.3 ± 5.1% (mean ± SE, $n = 3$) of treated larvae had metamorphosed and 31.5 ± 11.3% had attached. After 6 h of exposure, nearly all treated larvae had metamorphosed (96.3 ± 3.7%) and over one-third had attached (38.5 ± 11.2%). None of the control planulae underwent

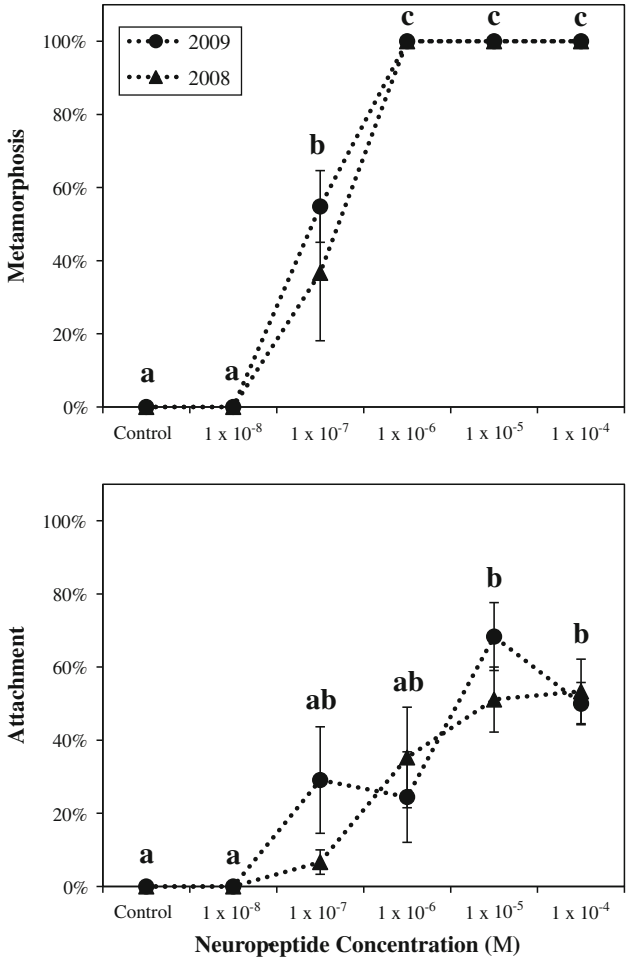

Fig. 2 Attachment and metamorphosis of *Acropora palmata* planulae in response to different concentrations of the neuropeptide Hym-248 ($n = 3$, 10 larvae per replicate). Data from two spawning seasons (2008 and 2009) are shown. *Different letters* above *symbols* depict significant differences ($P < 0.05$) among treatment dosages (combined data)

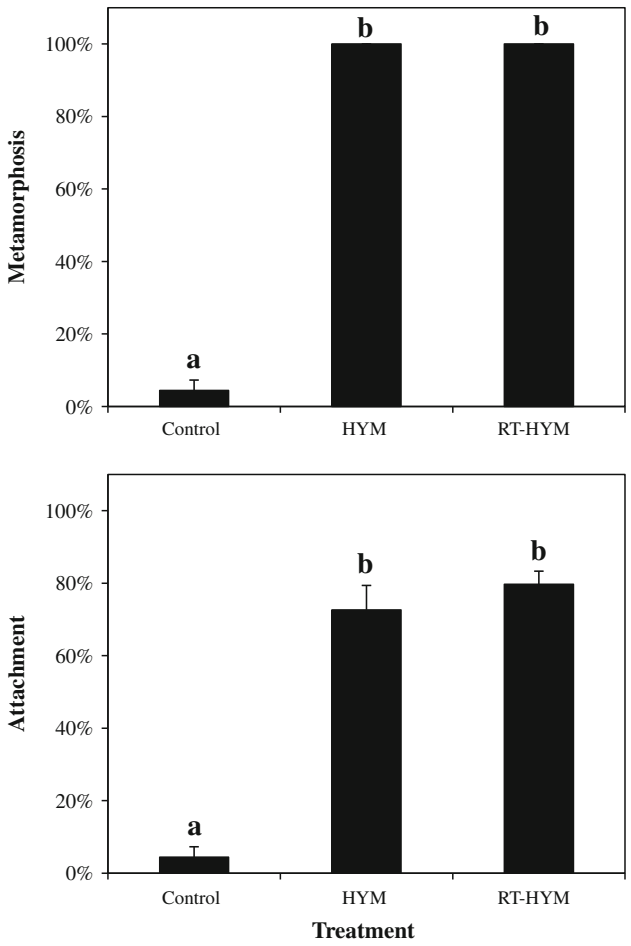

Fig. 3 Stability of attachment and metamorphic activity of Hym-248 on *Acropora palmata* planulae ($n = 6$, 10 larvae per replicate), showing larval response to neuropeptide solutions (1×10^{-5} M) stored at 4°C (HYM) and room temperature (RT-HYM). *Different letters* above *bars* depict significant differences ($P < 0.05$) among treatments

attachment or metamorphosis; rather, they remained elongate and swam actively throughout the experiment.

Hym-248 stability experiment: *A. palmata*

Larvae treated with Hym-248 solutions stored at 4°C and Hym-248 solutions stored at room temperature exhibited no significant difference in attachment ($P = 0.909$) or metamorphosis ($P = 1.000$). In both neuropeptide treatments, larvae exhibited 100% metamorphosis and 70–80% attachment, compared to control treatments where only two larvae (4%) metamorphosed and attached (Fig. 3). Since Hym-248 exhibits activity at lower concentrations than tested in this experiment, partial degradation of the peptide molecules may have occurred without affecting the overall attachment and metamorphosis of planulae.

Hym-248 tile experiment: *A. palmata*

The neuropeptide treatment significantly increased larval metamorphosis ($F_{1,14} = 4283.43$, $P < 0.001$), whereas biofilm treatment had no significant effect ($F_{1,14} = 2.10$, $P = 0.173$) compared to unconditioned tiles. A significant interaction was observed between Hym-248 and biofilm treatments for attachment to tile surfaces and total attachment ($F_{1,14} = 13.16$, $P < 0.05$ and $F_{1,14} = 29.86$, $P < 0.001$, respectively). The majority of the attached larvae (66.9%) were located on the sidewalls of the plastic containers holding the tiles, rather than directly on the tiles. For treatments receiving Hym-248, the presence of a biofilm did not enhance metamorphosis (99.3% no biofilm and 95.6% with biofilm, $P = 0.643$), significantly decreased total attachment (57.4% no biofilm and 31.9% with biofilm, $P < 0.001$) and significantly increased attachment directly

on tile surfaces (11.8% no biofilm and 17.0% with biofilm, $P < 0.05$; Fig. 4). Due to low attachment directly on tile surfaces, low numbers of spat were available for monitoring post-settlement mortality (2–7 spat per tile; 36 spat total).

After 12 days on the reef, survivorship averaged 60.1 ± 7.9% (mean ± SE, $n = 8$) and ranged from 33.3 to 100% for individual tiles. No significant difference ($P = 0.846$) was observed between survivorship on unconditioned tiles (58.3 ± 14.4%) and conditioned (61.8 ± 9.1%). In addition, all surviving spat had acquired zooxanthellae and initiated skeletal secretion. After 36 days, survivorship averaged 16.7 ± 9.5% and ranged from 0 to 66.7% for individual tiles. Unconditioned tiles exhibited higher survivorship (29.2 ± 17.2%) compared to conditioned tiles (4.17% ± 4.2%), although the difference was not significant ($P = 0.486$). High variability among replicates and overall low survivorship limited statistical power; however, all surviving spat appeared healthy and had begun colonial growth, presenting multiple (2–3) polyps (Fig. 5).

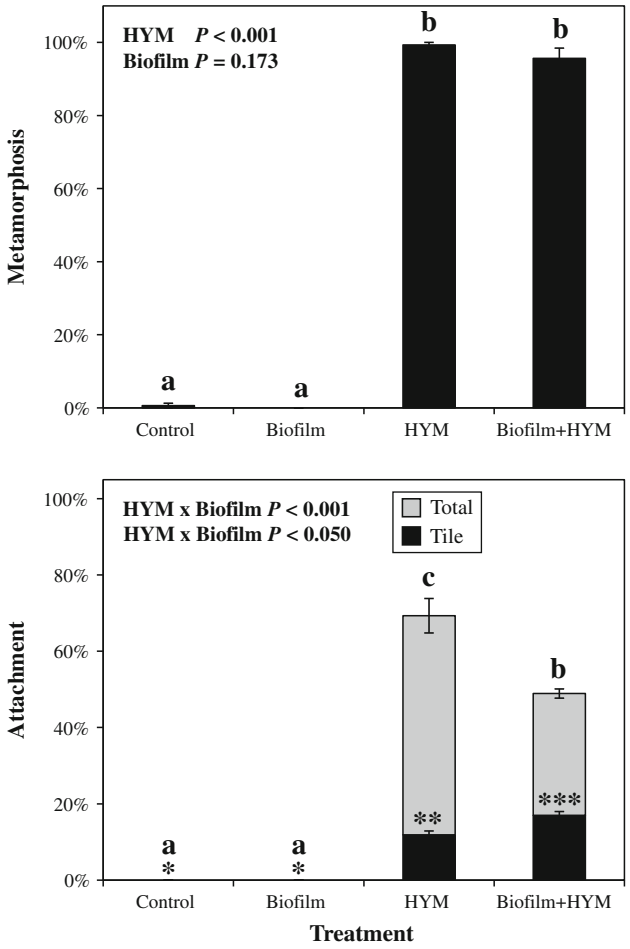

Fig. 4 Attachment and metamorphosis of *Acropora palmata* planulae in response to unconditioned and conditioned (biofilm) tiles treated with neuropeptide solutions (2×10^{-6} M, HYM; $n = 4$, 50 larvae per replicate). *P*-values for relevant treatment effects and interactions are shown in *bold*. *Different letters* above *bars* depict significant differences ($P < 0.05$) among treatments. In the *bottom panel*, *gray bars* refer to total attachment, while *black bars* indicate attachment on tile surfaces only. *Asterisks* (*) denote significant differences ($P < 0.05$) for tile attachment

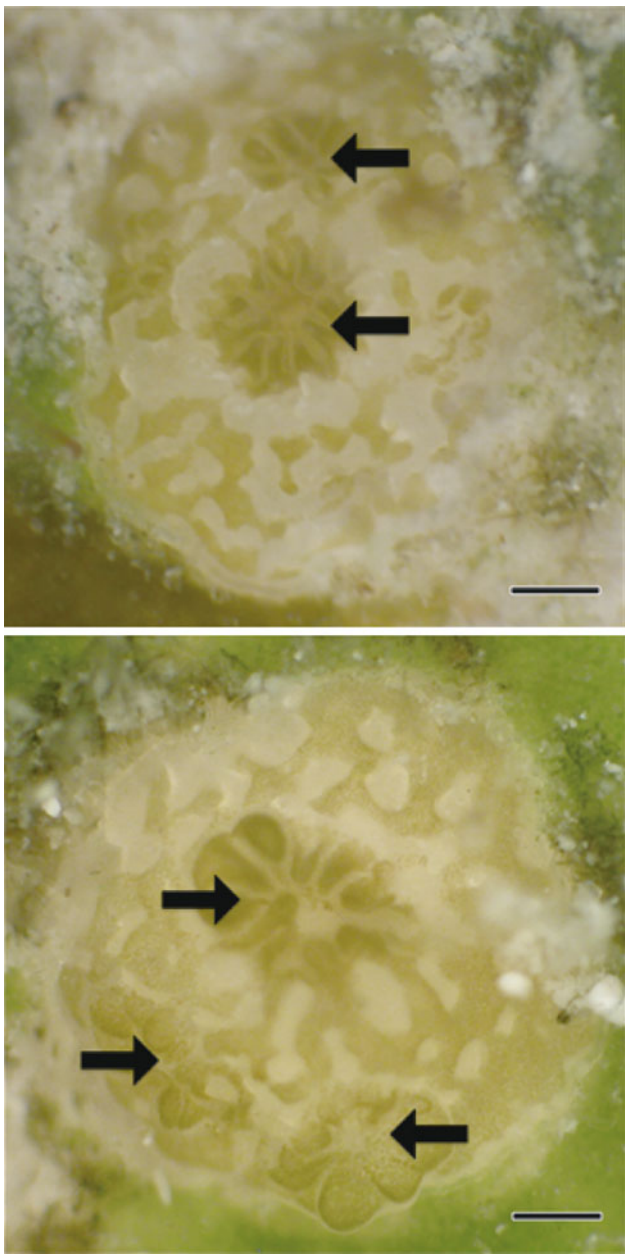

Fig. 5 *Acropora palmata* spat 36 days post-settlement following induction by the neuropeptide Hym-248. Acquisition of zooxanthellae symbionts (*brown coloration*) and development of a calcified skeleton are evident, along with the emergence of colonial polyps (*black arrows*). *Scale bar* 100 µm. Photo credit: P. Medina-Rosas

Discussion

Attachment and metamorphosis of laboratory-reared planulae from the reef-building Caribbean coral *Acropora palmata* were successfully induced by the exogenous application of neuropeptide Hym-248. Nearly 100% metamorphosis was achieved in planulae 5 days post-fertilization from two spawning seasons treated with Hym-248 at concentrations $\geq 1 \times 10^{-6}$ M. Hym-248 exposure rapidly altered the swimming behavior and resulted in >96% metamorphosis after only 6 h. These results are consistent with the previously reported effects of Hym-248 on metamorphosis of Pacific species of *Acropora* (Iwao et al. 2002; Hatta and Iwao 2003) and extend the known metamorphic activity of this neuropeptide to Caribbean acroporids, a monophyletic and putatively basal lineage of the genus (van Oppen et al. 2001).

Planulae of *A. palmata* did not respond to five other GLW-amide neuropeptides, again similar to reports from Pacific acroporids (Iwao et al. 2002), revealing a high specificity of the signal cascade to a particular neurotransmitter. In contrast, other cnidarian taxa respond similarly to a suite of related neuropeptides sharing an amidated GLW C-terminus, but varying in amino acid length and composition (Schmich et al. 1998; Takahashi et al. 2003). For these organisms, the amidated tripeptide appears to be the active site, with no response to synthetic LW-amide peptides or GLW peptides and retained activity in a synthetic, serially deleted GLW-amide peptide (Schmich et al. 1998; Takahashi et al. 2003). In only one case has specific activity been reported for a GLW-amide neuropeptide, notably Hym-248, that is not induced by related neuropeptides (Takahashi et al. 2003), suggesting some degree of functional diversity exists in the GLW-amide family. Results from acroporid corals provide further evidence for functional diversity among GLW-amide neuropeptides, as the metamorphosis pathway appears to require an active site beyond the GLW-amide C-terminus, presumably recognizing the N-terminus or overall amino acid sequence composition. Further studies involving the modification of Hym-248 are required to determine the exact recognition site for the coral endocrine system.

Shared metamorphic activity of GLW-amide neuropeptides across multiple cnidarian classes (Hydrozoa, Anthozoa) points to a conserved neurotransmission system that stimulates the genetic cascade leading to metamorphosis. However, Hym-248 and related GLW-amide neuropeptides did not induce metamorphosis in two additional species of Caribbean corals (this study) and five additional species of Pacific corals (Iwao et al. 2002), including three genera in the family Acroporidae. The conserved activity of Hym-248 across cnidarian classes, but not among related corals, suggests the inductive effects of this neuropeptide on *Acropora* species result from: (1) parallel evolution, or (2) successful mimicking of native *Acropora* neuropeptides. Evidence for the latter hypothesis exists in high-throughput DNA sequence databases, where gene fragments coding for putative GLW-amide neuropeptides have been recovered from *A. millepora* (Meyer et al. 2009) distinct from the amino acid sequence of Hym-248 (GenBank acc. nos. EZ038197 and EZ006786). Future genetic studies targeting sequencing and expression analysis of larval transcriptomes may reveal Hym-248 mRNA preproproteins in *Acropora* spp., as well as additional inductive neuropeptide candidates in other coral genera. In fact, a putative GLW-amide gene fragment has been isolated from the transcriptome of *Montastraea faveolata* larvae (Schwarz et al. 2008) and shown to be up-regulated (4.52-fold) in late stage compared to early stage larvae (Voolstra et al. 2009). Due to variable mRNA processing, chemical analyses are required to confirm the existence of neuropeptides deciphered from mRNA sequences; however, genetic signatures have been shown to accurately predict neuropeptide structure (Leviev et al. 1997; Takahashi et al. 1997).

The application of GLW-amide neuropeptides to reef restoration efforts may improve mass culturing efforts by increasing the efficiency of induced larval attachment and metamorphosis. Laboratory attachment of cultured larvae is typically conducted by exposing competent planulae to conditioned (i.e., biofilmed) settlement tiles, crustose coralline algae (CCA), or calcified reef substrata (e.g., coral rubble). Indeed, high rates of attachment and metamorphosis can be achieved by these methods (for *A. palmata*, see Erwin et al. 2009; Ritson-Williams et al. 2010) and such studies offer valuable insight into natural recruitment processes. However, the scalability of 'naturally'-induced larval attachment and metamorphosis to mass coral seedling production is limited, due to the inherent variability (e.g., microbial biofilms) and availability (e.g., CCA) of environmental cues. Further, natural induction of the complete settlement sequence generally requires 24–72 h of exposure to reach high activity levels. In contrast, neuropeptide ('artificial') induction of larval attachment is reliable, convenient, rapid and scalable (Hatta et al. 2004). Artificial induction by Hym-248 also represents an economical alternative to natural cues, with US$1 worth of neuropeptide able to induce 147 coral larvae (a conservative estimate assuming 1 larvae/ml of 1×10^{-6} M solution and 33% attachment). In addition, Hym-248 was stable in solution at room temperature, with no loss of attachment or metamorphic activity after 10 days, indicating the potential utility of this cue in remote field settings. Notably, peptide concentrations above the threshold level were used in the stability experiment, thus partial degradation may have occurred but remained undetected.

Hym-248 induced variable levels of attachment among *A. palmata* larvae, ranging from 40 to 80% in larvae 6 days or more post-fertilization. In the 2009 spawn season, cultured larvae exhibited more active swimming behaviors, and percent attachment increased with larval age. Similar effects of Hym-248 have been reported for Pacific acroporids, where near 100% attachment was reported in later stage larvae (exact time unspecified; Hatta et al. 2004). These results suggest that additional testing of well-developed *A. palmata* planulae (>7 days post-fertilization) may increase the attachment rates reported herein. Presumably, the rapid inhibitory effect of neuropeptide application on larval swimming limits their ability to search substrata and initiate attachment prior to metamorphosis, thereby resulting in the commonly observed side-wall attached polyps or free-floating, fully differentiated polyps. Future assays with Hym-248 should cater to the observed trend by providing settlement tiles on all sides of assay dishes. Contrary to expectations, biofilmed tiles did not induce metamorphosis in isolation and decreased overall attachment rates when coupled with Hym-248, compared to Hym-248 treatment alone. The biofilms may potentially inhibit Hym-248 activity by reducing peptide availability to coral planulae, via adsorption into the biofilm matrix or direct degradation by biofilm microbes. Notably, all of the surviving juvenile polyps transplanted to the reef established symbiotic algae populations and initiated skeletal secretion within 12 days and produced multiple polyps within 36 days, indicating no short-term effect of artificial induction on early life-history developmental processes.

Following induction of attachment and metamorphosis, post-settlement survival of juvenile corals is the second key process currently limiting mass cultivation output. Among the numerous factors affecting post-settlement survival, competitive interference and indiscriminate grazing are considered particularly detrimental to juvenile corals (Sammarco 1980; Sammarco and Carleton 1981; Edmunds and Carpenter 2001). While settlement tile conditioning may provide attractive cues to stimulate the attachment and metamorphosis of coral larvae, the formation of biofilms may also increase the competition for the young spat with other organisms colonizing the substrate, and attract invertebrate grazers. In contrast, Hym-248 settlement induction on bare tiles may enhance survivorship, allowing newly settled corals to become established prior to competitive interference. Recent studies have reported increased post-settlement survival in corals that settle in tile micro-crevices (Petersen et al. 2005b; Nozawa 2008), suggesting that cryptic refugia provide increased protection from grazers. Induction by Hym-248 allows for directed settlement in tile crevices, potentially decreasing subsequent exposure to invertebrate grazing. In fact, planulae of *A. palmata* can be attached and metamorphosed in small volumes of Hym-248 solution (100 µl) specifically applied to desired tile locations (P. M. Erwin, personal observation). Accurate and strategic settlement of coral larvae may therefore be a means to enhance survivorship upon transplantation to the reef environment. Although the scalability of such methods is currently limited, large increases in coral spat survivorship may justify the additional laboratory time and effort.

Neuropeptide-induced attachment and metamorphosis has implications in reef restoration, as well as studies of early life-history stages of coral larvae and juvenile polyps. Among Pacific acroporid corals, the metamorphic capacity of Hym-248 has been utilized to establish ex situ populations in aquaculture (*A. tenuis*; Petersen et al. 2005a), to study metamorphosis and zooxanthellae acquisition (*A. nobilis* and *A. microphthalma*; Hirose et al. 2008), and to characterize the transcriptome of coral larvae (*A. millepora*; Meyer et al. 2009). Our results report that Hym-248 also induces attachment and metamorphosis of planulae from the Caribbean congener *A. palmata* and suggest this neuropeptide will be a key tool for similar studies on endangered Caribbean acroporids. Further, the activity of GLW-amides is counteracted by a second family of cnidarian neuropeptides, the RF-amides (Katsukura et al. 2003), suggesting these neuropeptides may be utilized to prolong the larval stage in corals for experimental purposes or shipment to off-site aquaculture facilities. Neuropeptide activators of the metamorphosis cascade appear to be highly specific on lower taxonomic scales in corals, and future genetic and chemical studies may reveal novel GLW-amides that induce different coral species. Additional study of coral neuropeptides will offer further insight into the regulation of the primitive nervous system of cnidarians and the biotechnological application of neuropeptides to global coral reef restoration efforts.

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