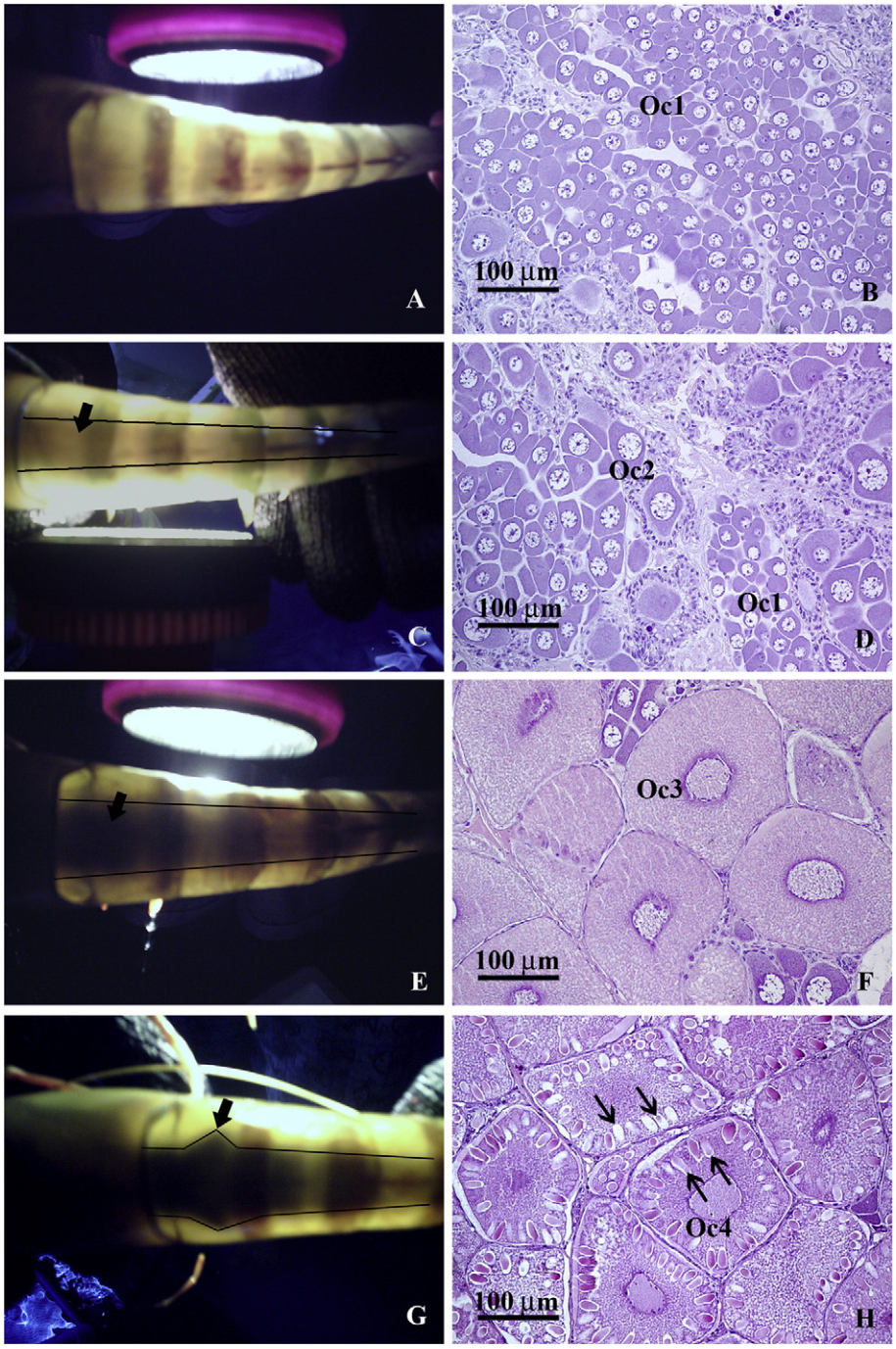

Fig. 1. The external appearances shown by transmitted light and correlative histology of ovaries at various stages of development. (A) The external appearance of stage I ovary, showing clear dorsal surface and (B) the histology, showing predominating step 1 oocytes (Oc1). (C) The external appearance of stage II ovary as a thin dense midline (arrow). This stage of ovary contained mostly step 2 oocytes (D, Oc2). (E) Stage III ovary appeared as a thick band (arrow) and contained mostly step 3 oocytes (F, Oc3). (G) Stage IV ovary appeared as a thick, broad band with wing-like structure representing lateral lobes (arrow), and it contained a large number of step 4 or mature oocytes (H, Oc4), with numerous cortical rods (arrows) in their peripheries.

boosted twice with either NS or GnRH at 10 day-intervals (on days 1, 11, 21). Ovarian maturation and spawning in all groups were carefully observed on the daily basis throughout a 60 day period after the final injection.

2.5. Examination of ovarian maturation

The light transmission method is a simple technique routinely used to verify the stages of ovarian maturation by shining a torch light through the shrimp body. The developmental stages of ovarian cycle were judged according to the criteria shown in Results, Fig. 1. Prior histological study was performed in other groups of shrimp to ascertain correlation between the external appearances of ovarian stages as determined by the light transmission method and the ovarian histology. The stages of ovarian development were determined by a person who had an expertise in using the light transmission method but did not know the treatment conditions of various experimental groups. For histological examination, the ovaries were removed from shrimp at each stage of ovarian development, fixed in Bouin's fixative overnight, and processed conventionally for paraffin embedding. The sections were placed on gelatin-coated slides, processed for staining with hematoxylin and eosin, and viewed under an optical microscope.

In the GnRH-primed animals, the development of ovaries from stage I to stage IV (mature stage) was checked every 24 h post-injection using the light transmission method as previously mentioned. The number of days for the ovaries to reach the final maturation stage were recorded and compared among each treatment and control groups. The ovarian maturation was confirmed by allowing the shrimp to spawn at the following night after full ovarian maturation.

2.6. Assessment of egg quantity and quality after spawning

The quantity and quality of spawned eggs were evaluated and expressed as the number of eggs per spawn and the percentage of fertilized eggs. Each shrimp with a fully mature ovary was allowed to spawn in a tank containing 400 L of sea water. After spawning, the eggs were randomly sampled (from 100 mL of sea water, 3 times) and

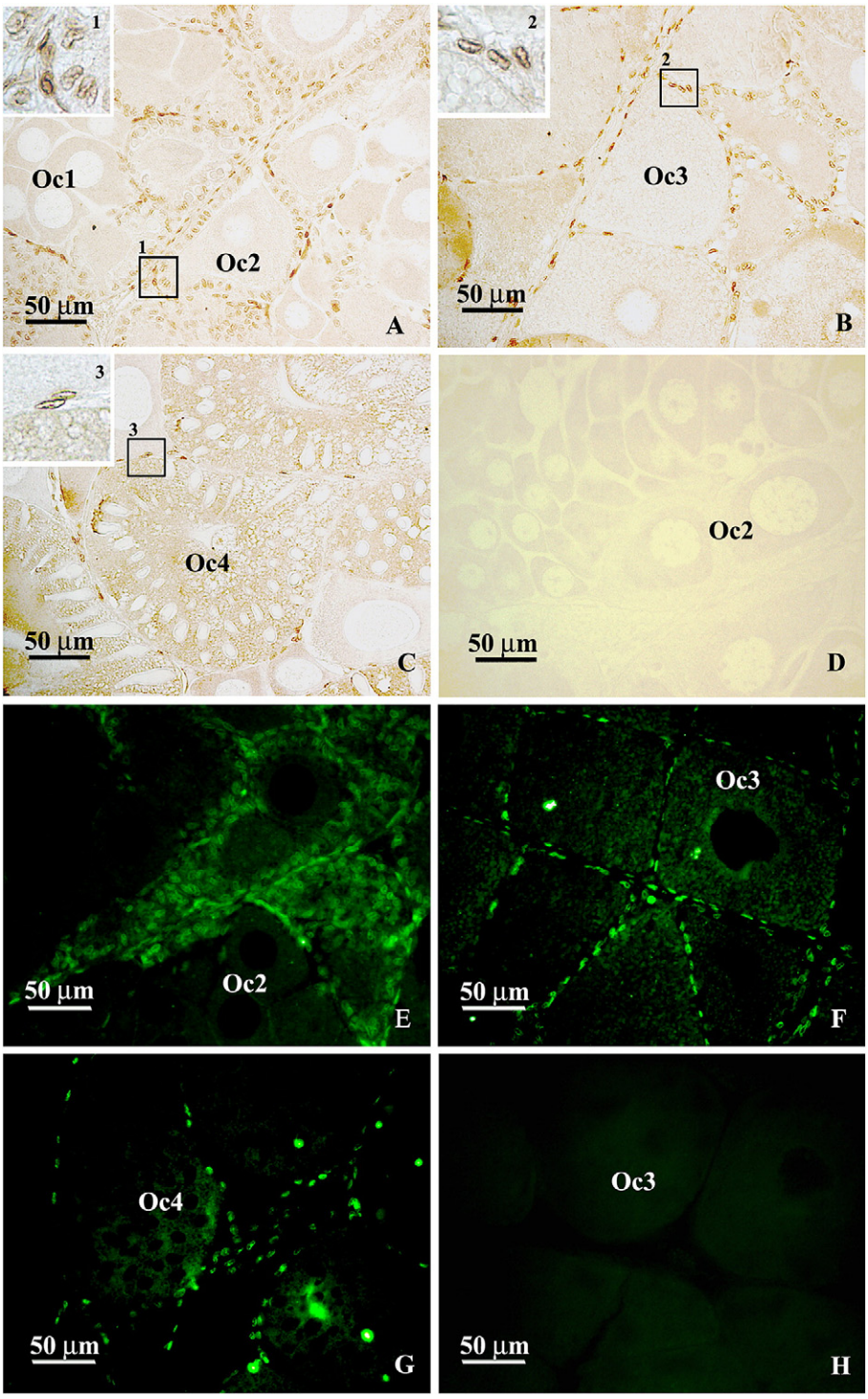

Fig. 2. (A–C) Immunoperoxidase localization of lGnRH-I-like substance in the shrimp ovaries, demonstrating the ir-lGnRH-I in the follicular cells surrounding oocytes in different ovarian stages: proliferative (A), vitellogenic (B), and mature (C). At higher magnification, the ir-lGnRH-I was observed in the cytoplasm of the follicular cells (A–C, insets). (D) The section incubated with anti-lGnRH-I preadsorbed with l\GnRH-I peptide showed no staining. (E–G) Immunofluorescence of lGnRH-I-like substance in the shrimp ovaries. The follicular cells surrounding step 2 (E, Oc2), step 3 (F, Oc3), and step 4 (G, Oc4) oocytes displayed ir-lGnRH-I. (H) Section incubated with PBS instead of the primary antibody showed no fluorescence.

counted to assess the total number of spawned eggs. To determine the percentage of fertilization, at least 200 eggs were observed under an optical microscope and only the normal developing embryos (gauged from the appearance of gastrulation at 6–8 h after spawning) were indicated as successful fertilization. This embryonic stage allowed us to easily distinguish between fertilized and non-fertilized eggs. Data were expressed as mean±S.D from triplicates. The duration for the ovaries to reach maturation, the number of eggs spawned, and the percentage of fertilizations were compared among GnRH-treated and NS-treated groups using one-way analysis of variance (ANOVA), followed by a Tukey Post Hoc multiple comparison. The probability value less than 0.05 ($P<0.05$) indicated a significant difference.

3. Results

3.1. Correlation of ovarian stages by external observations with ovarian histology

According to King (1948) and Yano et al. (1988), the ovarian cycle in the shrimp can be classified into 4 stages: spent, proliferative, vitellogenic, and mature. From observations of the external appearance; in stage I (spent stage) the ovary was transparent with no distinguishable ovarian outline (Fig. 1, A); in stage II (proliferative stage) the ovary appeared as a thin opaque line along the dorsal axis of the body (Fig. 1, C); in stage III (vitellogenic stage) the ovary appeared as a thick band covering approximately the middle half of the dorsal surface (Fig. 1, E); and in stage IV (mature stage) the ovary appeared turgid, broad with wing-like lateral lobes, and was intensely dark and occupied almost the whole dorsal surface of the shrimp (Fig. 1, G). Histologically, stage I ovary was characterized by the predominance of step 1 oocytes (Oc1) surrounded by connective tissue (Fig. 1, B). Stage II ovary was marked by the abundance of step 2 oocytes (Oc2) with basophilic cytoplasm (Fig. 1, D). Stage III ovary contained mostly step 3 or yolky, vitellogenic oocytes (Oc3) (Fig. 1, F). Stage IV ovary was distinguished by the presence of numerous step 4 or mature oocytes (Oc4) which contained rod-like bodies (cortical rods) in their peripheral cytoplasm (Fig. 1, H). These observations confirmed that light transmission method is a reliable technique which could be used to predict the stages of ovarian development, as the ovarian stages gauged from external appearance corresponded well with the histological characteristics.

3.2. The presence of a GnRH-like factor in the ovarian tissue

Using antibodies against five GnRH isoforms to determine the presence of ir-GnRH in the shrimp ovary, only ir-lGnRH-I was found to be localized in the cytoplasm of follicular cells which surrounded developing and mature oocytes in proliferative, vitellogenic and mature stages of ovaries (Fig. 2, A–C). However, positive immunostained follicular cells were more abundant in proliferative and vitellogenic stages (Fig. 2, A–C). There was no ir-lGnRH-I detected in the cytoplasm of oocytes at any stages, as well as in the surrounding connective tissue. Similar findings were observed using immunofluorescence technique in which no false positive could occur as in immunoperoxidase stain where the effect of endogenous peroxidase might not be totally suppressed (Fig. 2, E–G). In contrast, there were no ir-GnRHs in any of the ovarian stages using the other four antibodies, i.e., anti-LHRH, anti-sGnRH, anti-lGnRH-III, and anti-octGnRH (negative data not shown). Both controls of immunoperoxidase and immunofluorescence were unstained (Fig. 2, D and H).

3.3. The shortening of ovarian maturation period by GnRH administration

There are cumulative data indicating that several isoforms of GnRH share a common receptor (Millar et al., 2004). Therefore, we hypothesized that the three GnRH isoforms used in this bioassay experiment may be able to have reproductive effects, although the immunoreactivities of LHRH and sGnRH were not detectable in the ovarian tissue. Intramuscular administration of exogenous LHRH, sGnRH, and lGnRH-I into female shrimp appeared to shorten the ovarian maturation period when compared to the control shrimp injected with NS only (Table 1). After eyestalk-ablation, the ovary of the NS-treated shrimp reached maturation from stage I to IV within 7.43±0.98 days. At a dose of 10 ng/g BW, both sGnRH and LHRH shortened ovarian maturation period to 6.00±0.82 and 6.14±1.21 days, respectively, but were not significantly different from that of the control. Notably, eyestalk-ablated shrimp which were administered 50 ng/g BW of sGnRH, LHRH, and lGnRH-I developed fully mature ovaries within 4.29±1.38, 4.43±0.79, and 4.00±0.82 days, respectively. Statistical analysis revealed a significant shortening of ovarian maturation period by all GnRH isoforms at this dose of 50 ng/g BW. All of the controls, and GnRH-induced shrimp whose ovaries were determined as “mature” by external observation method, underwent spawning on the following night. The results are summarized in Table 1.

Administration of exogenous GnRHs also shortened the duration of ovarian maturation in non-ablated shrimp (Table 1). The ovaries of the eyestalk-intact, NS-treated shrimp developed into fully mature stage within 34.50±5.00 days. Upon treatment with 500 ng/g BW of both sGnRH and lGnRH-I, the shrimp developed fully mature ovaries within 23.20±2.59 and 22.80±2.17 days, respectively. These durations were significantly shortened when compared to that of the control group. However, 71.43% of the eyestalk-intact shrimp spawned after sGnRH and lGnRH-I treatments, but only 57.14% of the control shrimp underwent spawning.

Table 1
Effect of GnRHs on the period of ovarian maturation, quantity of spawned eggs, and fertilization

Groups	Treatments	Number of shrimp	Doses (ng/g BW)	Period of maturation (days)	% of spawners	Number of % eggs/spawn	% Fertilization
Eyestalk-ablated groups							
1	NS	7	–	7.43±0.98	100	$0.98\times10^6\pm4.59\times10^5$	84.53±3.55
2	sGnRH	7	10	6.00±0.82	100	$0.94\times10^6\pm2.26\times10^5$	-
3	sGnRH	7	50	4.29±1.38*	100	$0.95\times10^6\pm2.59\times10^5$	79.91±7.09
4	LHRH	7	10	6.14±1.21	100	$0.86\times10^6\pm1.53\times10^5$	-
5	LHRH	7	50	4.43±0.79*	100	$0.91\times10^6\pm4.24\times10^5$	81.57±4.51
6	lGnRH-I	7	50	4.00±0.82*	100	$0.89\times10^6\pm1.41\times10^5$	82.07±7.17
Eyestalk-intact groups							
7	NS	7	-	34.50±5.00	57.14	$1.15\times10^6\pm1.37\times10^5$	80.13±6.16
8	sGnRH	7	500	23.20±2.59*	71.43	$1.06\times10^6\pm2.81\times10^5$	81.26±4.78
9	lGnRH-I	7	500	22.80±2.17*	71.43	$1.01\times10^6\pm1.37\times10^5$	82.00±3.98

Note: * means statistically difference between control and GnRH-treated groups at $P<0.05$.
NS, normal saline.

3.4. Quantity and quality of the spawned eggs following GnRH treatment

The effect of GnRH on the quantity and quality of spawned eggs were estimated by the number of eggs per spawn and the percent of successfully fertilized eggs as demonstrated in Table 1. The numbers of spawned eggs were not significantly different among LHRH-, sGnRH-, lGnRH-I-injected and NS-injected groups in both eyestalk-ablated and eyestalk-intact shrimp. The numbers of spawned eggs in GnRH-treated groups ranged between $0.86–0.95\times10^6$ and $\sim1.0\times10^6$ in eyestalk-ablated and eyestalk-intact shrimp, respectively. Similarly, the numbers of spawned eggs in NS-treated groups were 0.98×10^6 for eyestalk-ablated and 1.15×10^6 for eyestalk-intact. These results showed no significant difference in the number of eggs per spawn between eyestalk-ablated and eyestalk-intact groups.

The percentages of successful fertilization, as the indicator for determining the quality of spawned eggs, were not significantly different among all groups (Table 1). In NS-treated groups, the percentages of fertilization were 84.53 and 80.13% for eyestalk-ablated and eyestalk-intact, respectively. In the GnRH-treated groups, the percentages of fertilization ranged from 79.91 to 82.07% for eyestalk-ablated and eyestalk-intact, which were not significantly different when compared to the controls. This suggested that the doses of GnRH used in this study had no effect on egg number and their fertilizability.

4. Discussion

In the present study, we have demonstrated the presence of ir-lGnRH-I in the shrimp ovary. Many previous studies have also reported the presence of a GnRH or GnRH-like factor in the gonads of other invertebrates, for examples, a protochrodate, *Ciona intestinalis*, (Tsutsui et al., 1998; Terakado, 2001), mollusks, *Haliotis trivolvis, Lymnea stagnalis* (Young et al., 1999), and *Aplysia californica* (Zhang et al., 2000), and a prawn, *Macrobrachium rosenbergii* (Ngernsoungnern et al., 2008a). Notably, ir-GnRH in the shrimp ovaries was found exclusively in the follicular cells surrounding the developing and mature oocytes, and was similar to GnRH localization in the granulosa cells of *C. intestinalis* ovary. It was shown that the peptides purified from *C. intestinalis* gonadal extract stimulated sex steroid production from this organ (Di Fiore et al., 2000).

Follicular cells in the ovaries of invertebrates have been reported to secrete various substances. In crustaceans, the follicular cells have been shown to secrete vitellogenin-stimulating ovarian hormone (VSOH) which is believed to play a central role in ovarian maturation (Van, 1992). Serotonin or 5-HT was detected in ovarian follicular cells of *P. monodon*, and was shown to stimulate ovarian maturation (Wongprasert et al., 2006). Moreover, 5-HT receptor was expressed in cytoplasm of Oc3 and Oc4 of *P. monodon* (Ongvarrasopone et al., 2006). Egg-laying hormone (ELH), has also been observed in the ovarian follicular cells of abalones (Chanpoo et al., 2001; Saitongdee et al., 2005), as well as in *P. monodon* (Liu et al., 2006). The latter study also suggested a role of ELH in the induction of shrimp spawning. Therefore, it is possible that the follicular cells produce GnRH locally and this local GnRH may play an autocrine and/or paracrine role in the induction of ovarian maturation and spawning. It is also possible that the ovarian GnRH may act in concert with 5-HT, ELH or VSOH in controlling ovarian maturation. However, further studies are needed before a conclusion can be made.

It has been reported that most vertebrates GnRHs exert similarly high activities with all receptors of non-mammalian vertebrates (Sealfon et al., 1997). In chiton, lGnRH-I and tunicate (t)GnRH-II were found to be both capable of stimulating the release of ripe gametes (Gorbman et al., 2003). Likewise, different GnRH isoforms including mammalian (m)GnRH, sGnRH, chicken (c)GnRH-II, and tGnRH-I, injected into *C. intestinalis* have been shown to induce gamete release (Terakado, 2001). In the present study, we have demonstrated that LHRH, sGnRH, and lGnRH-I also exerted shortening of the ovarian maturation period, which may be due to the ability of different GnRH isoforms in binding to the same receptor, or that they could stimulate a common signal transduction pathway. However, the presence of GnRH receptor in the shrimp and its binding to different isoforms of GnRHs should be studied before a firm conclusion can be made. It is interesting to note that the amino acids which determine the conformation of the GnRH peptides and responsible for binding with the receptors are all located at the distal ends of both N and C termini, and that they are highly conserved among GnRH peptides (Sower et al., 2004). Thus, this might explain the similar effect of LHRH, sGnRH, and lGnRH-I on enhancing the shrimp ovarian maturation observed in the present study. Although octGnRH and lGnRH-III were not used in our study because of its limited availability, our previous research has demonstrated that ir-octGnRH and ir-lGnRH-III were present in the CNS of *P. monodon* (Ngernsoungnern et al., 2008b). We believe that octGnRH and lGnRH-III could also exert effects similar to the three GnRH isoforms used in the current study. We plan to test the effects of these GnRHs on the shrimp ovarian maturation in a further study.

The three GnRH isoforms exerted a strong effect on ovarian maturation in the eyestalk-ablated (treated at 50 ng/g BW) as well as in eyestalk-intact (treated at 500 ng/g BW) shrimp. The shorter ovarian maturation cycle in the eyestalk-ablated animals may be due in part to the elimination of inhibition by GIH and MOIH which are synthesized and released from the eyestalk X-organ-sinus gland system (Okumura and Aida, 2001). The exogenous administration of GnRHs in eyestalk-intact groups also significantly shortened the ovarian maturation period, but appeared to be less effective when compared to eyestalk-ablated group. This may be due to gonad-inhibiting hormone still being intact in the eyestalks, thus a much higher level of exogenous GnRH was needed to override the effects. Because a high dose of GnRH was required for exerting its effect on the eyestalk-intact animals, one question that could be raised and need to be addressed is whether the high concentration used herein could be due to the non-specific action of the administered hormone. Further research is being performed by using of combined doses of known reproductive stimulating hormones, such as ELH, in single or in combination with GnRH to test their effects at the lowest effective doses. In addition, The relationship between exogenous (circulating) and local (ovarian) GnRH, 5-HT, and ELH in induction of ovarian maturation in penaeid shrimp needs to be investigated further, as the research could provide basic knowledge as well as practical use of the hormonal control of ovarian maturation and spawning. With further studies to refine the effective doses of GnRH to elicit positive results, there is a high possibility that hormone-priming could be used instead of the eyestalk ablation technique. If possible, a stimulatory method will be more humane and acceptable in shrimp aquaculture.

Acknowledgements

This study was supported by the Thailand Research Fund (Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0249/2546) to A. Ngernsoungnern and P. Sretarugsa, and Senior Research Scholar Fellowship to P. Sobhon), and Commission on Higher Education (Research Team Development Grant to P. Sobhon). The authors would like to thank Dr. Stacia A. Sower, Dr. Pei-San Tsai, and Dr. Judy King for generously providing GnRH antibodies and peptides, and Dr. Peter Hanna for proof-reading of this manuscript.

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Journal of Experimental Marine Biology and Ecology 354 (2008) 182–191

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

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The presence of GABA in gastropod mucus and its role in inducing larval settlement

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Received 17 August 2007; received in revised form 31 October 2007; accepted 5 November 2007

Abstract

Chemical substances that induce larval settlement have been the focus of many gastropod studies due to the importance of wild stock recruitment and production within aquaculture facilities. Gamma-aminobutyric acid (GABA), GABA analogs, and GABA-mimetics associated with certain crustose coralline algae (CCA), are known to induce larval settlement in commercial abalone (*Haliotis*) species, and other gastropods. Furthermore, mucus secreted from these gastropods has been shown to induce larval settlement, but the stimulatory components of mucus have not been thoroughly investigated. We now present data confirming that GABA is the settlement-inducing effector molecule contained within abalone mucus. To do this, we initially generated anti-GABA for use in immunoenzyme and immunofluorescent microscopy. Using these techniques GABA was identified in the nerves and epithelial cells of the foot, including mucus. Dried mucus samples subject to HPLC analysis revealed a mean concentration of 0.68 mM GABA after sample rehydration. The presence of GABA in these samples was confirmed by time-of-flight mass spectroscopy (TOF-MS). In addition, GABA was detected in the mucus of several abalone species and other gastropods by immunocytochemistry. Subsequent bioassays using both dry and fresh mucus strongly promoted induction of larval settlement.

Keywords: Abalone; GABA; Gastropod; *Haliotis*; HPLC; Immunohistochemistry; Larval settlement; Mucus; TOF-MS

1. Introduction

The amino acid, γ-aminobutyric acid (GABA), was the first molecule shown to induce synchronous larval settlement and metamorphosis of *Haliotis rufescens* into juveniles (Morse et al., 1979a). Later investigations revealed many isoforms of GABA (e.g. γ-hydroxybutyric acid, δ-aminovaleric acid and ε-aminocaproic acid), together with the accessory photosynthetic pigments in coralline algae and cyanobacteria, such as phycoerythrobilin, possess GABA-like settlement-inducing properties (Morse et al., 1979a, 1980, 1984; Morse and Morse, 1984a,b). The mechanism of action involved inhibition of cilia movement of swimming mollusc larvae (Akashige et al., 1981; Barlow, 1990).

It is now known that GABA can induce larval settlement in a number of molluscan species, including the mussel *Mytilus galloprovincialis*, the clam *Venerupis pullastra*, the oyster *Ostera edulis* (Garcia-Lavandeira et al., 2005), the black chiton, *Katharina tunicate* (Rumrill and Cameron, 1983), and in other various abalone species. The effective doses for the settlement of abalone species, viz, *H. rufescens*, *H. iris*, *H. discus hannai*, *H. virginea*, *H. australis*, *H. asinina* and *H. diversicolor* has been found to vary from 0.5 to 10 μM (Morse et al., 1979a,b; Moss and Tong, 1992; Yang and Wu, 1995; Roberts and Nicholson, 1997; Bryan and Qian, 1998; Moss, 1999; Roberts, 2001; Gapasin and Polohan, 2004), but it is apparent from these studies, that no single concentration of GABA has been optimal for settlement of all abalone species.

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doi:10.1016/j.jembe.2007.11.003

Apart from GABA, the presence of crustose coralline algae (CCA) has been confirmed as a contributing factor to settlement (Morse et al., 1979a,b, 1984; Morse and Morse, 1984a,b; Suenaga et al., 2004). In earlier analyses of CCA, precursors and metabolites of phycoerythrobilin were isolated. Later, algal photosynthetic accessory pigments were found that were structurally related to GABA (Morse et al., 1979a, 1984), and subsequently identified as inducers of settlement and metamorphosis of *Haliotis* larvae (Morse et al., 1979a,b, 1984; Morse and Morse, 1984a,b). Another study of larval settlement using the red crustose alga *Hydrolithon samoense* (Suenaga et al., 2004), showed GABA was not responsible, however, a GABA analog (δ-aminovaleric acid) was effective in settling *H. discus discus* and *H. discus hannai* larvae. CCA species can induce *H. rubra*, *H. laevigata*, *H. iris* and *H. virginea* larval settlement, but with a selective preference to CCA with thin crusts (Roberts and Nicholson, 1997; Daume et al., 1999a,b; Roberts et al., 2004). Several investigations have compared the roles of settlement-inducing agents (GABA, GABA derivatives, and CCA), using mixtures of diatoms and "mucus", as well as excluding them (Roberts, 2001; Gallardo and Buen, 2003).

Gastropod mucus has been shown to have many functions, such as aiding locomotion, feeding, protection, and settlement (Denny, 1983; Seki and Taniguchi, 1996; Davies and Hawkins, 1998). In an investigation of *H. discus hannai* larval settlement on the green alga, *Ulvella lens*, significant settlement occurred if mucus was present from previously grazing juveniles (Takahashi and Koganezawa, 1988). More recently, Slattery (1992) found that mucus alone was better than mucus plus diatoms, or diatoms plus 1 μM GABA, as an inductive cue for settlement of *H. rufescens* larvae. In a study of *H. asinina*, mucus together with benthic diatoms, *Navicula sp.*, or mucus mixed with diatoms, showed considerably higher larval settlement and metamorphosis than algae alone (Gallardo and Buen, 2003). In a comparative study using mucus, KCl or GABA, to induce larval settlement of *H. discus hannai*, mucus was most successful (Yang and Wu, 1995). This study also concluded that the amount of K^+ required was larval stage-dependent, and only the mucus was dose-independent. Overall, these studies support the earlier findings of Seki and Kan-no (1981) that larvae of *Haliotis* species preferentially settle on conspecific mucus trails.

In a recent immunocytological study of *H. asinina,* GABA was localized within neuron bundles, as well as in the epithelial cells of the cephalic and epipodial tentacles (Wanichanon et al., 2004). In the current study we have used immunocytochemistry to examine the presence of GABA in epithelial cells of the foot muscle, mantle mucus secreting cells, as well as mucus of the Australian blacklip abalone, *H. rubra*. We also established the presence of GABA in mucus using high performance liquid chromatography (HPLC) and time-of-flight mass spectroscopy (TOF-MS). In addition, we investigated the mucus of other haliotids and gastropods for the presence of GABA, and conducted a series of bioassays showing that mucus contains the settlement inducer.

2. Materials and methods

2.1. Animals

H. rubra were obtained from Port Phillip Bay and Bay City Sea Farm, Victoria, Australia. *H. asinina*, *H. diversicolor*, *Babylonia areolata*, *Chicoreus ramosus*, and *Strombus canarium*, were obtained from the Coastal Aquaculture Research and Development Center, Prachaubkirikun Province, Thailand.

2.2. Preparation of samples and immunolocalization

2.2.1. Specimen preparations

For section preparations, animals were anesthetized in seawater containing 5% $MgCl_2$, their shells removed and the foot muscle dissected. For light microscopy, dissected tissue was fixed in 4% paraformaldehyde overnight at 4 °C and subsequently dehydrated in ascending concentrations of ethyl alcohol for 30 min each. This was cleared with two changes of dioxane and a final dioxane-paraplast mixture for 30 min. Following paraffin wax embedding, 5 μm serial sections were prepared with a rotary microtome.

Mucus for immunocytological tests was obtained by wiping the foot muscle of individual gastropods using glass slides pre-coated with (3-aminopropyl) triethoxy-silane, then dried. Mucus for dot blot analysis, HPLC and TOF-MS analyses was prepared according to the method of Donovan and Carefoot (1997). Briefly, individual males and females (100±10 mm) were left for 15 min to attach to large glass petri dishes. The animals were then removed so that remaining mucus samples could be collected and dried at 60 °C for 1 h. A new razor blade was used to scrape and collect each mucus sample, which was then separated into 0.02 g aliquots and stored at 4 °C. When required for dot blot analyses, HPLC, and TOF-MS, samples were rehydrated with 100 μL of sterile deionised water (Millipore, Milli Q water System, Bendford MA), then centrifuged at 10,000 rpm (Model 13 Biofuge, Heraeus Instruments ™) for 5 min and the supernatant collected.

2.2.2. Polyclonal antibody production against GABA

Six-week-old imprinting control region male mice were obtained from the Animal Care Unit, Mahidol University and used with the approval of the Animal Ethics Committee to produce antiserum. GABA was linked to bovine serum albumin (BSA) by mixing 100 μM of GABA and 12 mg BSA (fraction V, Sigma), as well as 100 μM of glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Animal immunizations were conducted following adapted procedures of Matute and Streit (1986). Sera was collected every alternate week and kept at −20 °C. Antibody titer against GABA was tested using ELISA (Polak and Noorden, 1997) and the optical density (OD) of each well read at 492 nm using a spectrophotometer (Titertex Multiscan Flow Laboratories, Australia Pty. Ltd,) followed by BSA pre-absorption. Negative control samples included mouse pre-immune serum in place of the primary antibody (mouse anti-GABA).

2.2.3. Immunohistochemical detection of GABA in abalone tissues and gastropod mucus

Endogenous peroxidase activity detected in dewaxed tissue sections, as well as dried mucus, was inactivated by treatment with 0.3% H_2O_2 in 30% methanol for 30 min. Sections were washed in PBS containing 0.4% Triton X-100 (PBST), and non-specific antibody binding blocked by incubating sections in blocking buffer (2% normal goat serum, 4% bovine serum albumin in PBST) for 2 h. Preparations were then incubated with primary antibody, overnight in a moist chamber (at 4 °C), washed 3 times for 10 min each in PBS, and incubated with secondary antibody (1:200; goat anti-mouse-Biotin Ig, Sigma) in blocking solution for 45 min at room temperature (RT). After rinsing in PBS, tissue sections and mucus preparations were incubated in streptavidin-HRP (1:200 in PBST) for 45 min. Samples were then exposed to NovaRED substrate solution (Vector laboratory) containing H_2O_2 for 2-5 min (i.e. until color development). Samples were washed with tap water, counter-stained with hematoxylin, dehydrated and mounted in Permount solution. For controls, preparations were processed in the same manner but with primary antibody excluded. Additionally, consecutive slides were stained with Periodic acid-Schiff solution (PAS) to identify the presence of carbohydrate.

Immunofluorescent detections of GABA were performed on tissue sections and mucus collected on slides. Preparations were initially blocked in 4% BSA for 30 min, then washed 3 times for 5 min each in PBS (pH 7.4), and incubated in buffer containing 0.1% glycine for 15 min. Preparations were then incubated overnight with mouse anti-GABA in PBS (1:100), washed with buffer, then incubated with FITC-conjugated goat anti-mouse IgG (1:200; Zymed Laboratories), for 1 h. They were then washed three times with PBS and mounted in buffered glycerol. Slides not incubated with anti-GABA were used as negative controls.

All preparations were photographed under a Nikon Eclipse E600 microscope with Nikon digital camera DXM1200F.

2.2.4. Dot blots of mucus extracts

Supernatant obtained from mucus extractions were spotted (20 μL) onto nitrocellulose membranes and dried at RT. Membranes were washed in PBS (pH 7.4) then incubated in blocking buffer (5% skim milk in PBS) for 15 min. Following a 15 min wash in PBS, membranes were incubated with mouse anti-GABA (1:200) in 5% skim milk for 1 h at RT. The membranes were washed with PBS and then incubated for 30 min at RT with HRP-conjugated goat anti-mouse IgG (Zymed Laboratories) at a dilution of 1:500 containing 5% skim milk. Finally, membranes were incubated with tetraethylbenzidine (TMB) membrane peroxidase substrate 1-C (KPL, Gaithersburg, ML) until color development, then washed in water and dried.

2.3. Chemical analysis of mucus

Determination of GABA within aqueous mucus extracts was performed with an Agilent Hewlett-Packard 1100 series HPLC system with solvent degasser system, quaternary pump, and autosampler fitted with a UV and fluorescence detector. The system was controlled by a Hewlett-Packard Vectra X_m series 4 data analysis work station. The column used was a Lichropher 100 Rp-18 column 150×4.6 mm I.D., 5 μm (Agilent). The separation conditions were performed as described by Henderson et al. (2006). Derivatisation reagents included 10 mg OPA reagent [*o*-pthaldialdehyde (Sigma-Aldrich) dissolved in 100 μL of methanol (Univar)], 900 μL 0.4 M sodium borate (pH 10.4) (BDH AnalaR), and 20 μL 3-mercaptopropionic acid, which was added to FMOC reagent [2.5 mg 9-fluorenylmethyl chloroformate (Sigma-Aldrich) dissolved in 1 mL of dry acetone (Univar)]. Calibration standards of GABA (Sigma-Aldrich) at concentrations of 1×10^{-2}, 5×10^{-3}, 1×10^{-3}, 5×10^{-4}, 1×10^{-4}, 5×10^{-5}, and 1×10^{-5} M were prepared as standard references. Valine (Sigma-Aldrich), was used at a concentration of 1×10^{-3} M as an internal standard.

Time-of-flight mass spectrometry was performed on an Agilent 6210 MSDTOF instrument using acetonitrile as the mobile phase. The following ESI conditions were selected: drying gas, nitrogen (7 mL/min, 350 °C), nebulizer gas, nitrogen (16psi), capillary voltage 4.0 kV, vaporizer temperature 350 °C and cone voltage 60 V.

2.4. Mucus settlement induction assays with statistical analysis

Mucus samples from *H. rubra*, *H. asinina* and *H. diversicolor* were collected on plastic sheets following a 15 min attachment and removal of adults. The animals were either immobilized "fixed" at the same area or allowed to walk "trail" on the sheet to test settlement of larvae on the mucus from fixed or trailed abalone. The mucus collected on sheets was used immediately as fresh mucus, or used following rehydration of stored dried samples. Bioassays were performed by placing three sheets in plastic cylindrical containers containing 1.2 L UV filter-treated sea water supplemented with streptomycin and neomycin (Sigma) at 7.5 mg/L. Larvae which were competent to settle at 24 h post fertilization ($n=1300$), were each placed into nine containers (3 replicates of 3) to assess settlement after 6, 24 and 72 h. Counts were then made of the larvae settling on mucus covered areas, compared with the equivalent mucus-free areas.

2.4.1. Statistics

The settlement data was analyzed using SPSS11.5 software (SigmaStat; SPSS), for testing normality, and homogeneity of variance, of each data set (mucus types). Group comparisons were then made using multiple group analyses with one-way ANOVA, and post hoc Tukey's HSD test. Interspecies differences, as well as settlement with time were also analyzed.

3. Results

3.1. Immunolocalization of GABA in foot muscle and mucus of gastropods

Histologically, the foot of most gastropods is comprised of muscle cells, mixed fibers, and is covered by an epithelium which has numerous ciliated columnar cells (see control

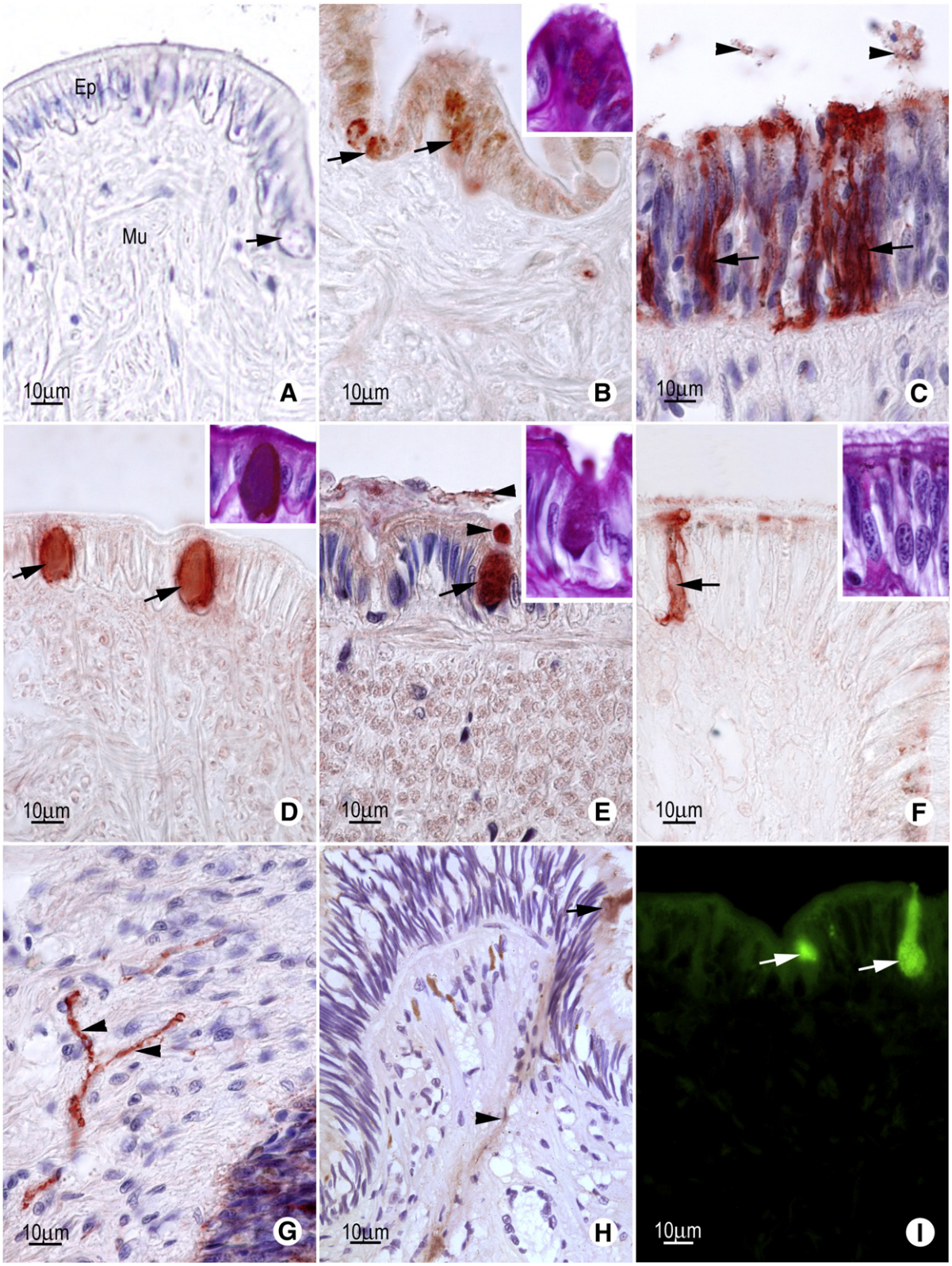

Fig. 1. Paraffin sections showing GABA-immunostaining in the foot region of gastropods. A, control (no anti-GABA) cross section of the foot of *H. asinina*, Ep-epithelium, Mu-muscle. B is an adjacent section to A, exhibiting GABA immunoreactivity in some mucosal cells (arrows) which was PAS positive (inset). C-I, GABA immunoreactivity in foot and mucus C, in mucosal cells of the foot epithelium of *H. diversicolor* (arrows), and mucus (arrow heads); D-F, in mucosal cells (arrows), as well as released mucus (arrow heads), in *Babylonia areolata*, *Strombus canarium*, and *Chicoreus ramosus*, respectively. Inset: cells were positive with PAS staining; G, within the foot of *H. diversicolor* (arrow heads); H and I, in mucosal cells (arrows), as well as nerve fibers in the foot (arrow head) of *H. rubra,* using immunoenzyme and immunofluorescence techniques, respectively.

Fig. 1A). The mucus secreting cells showed no staining in control sections, probed with pre-immune serum (Fig. 1A). When tissue sections were probed with anti-GABA, GABA was identified inside mucus secreting cells of the foot epithelia in *H. asinina*, *H. diversicolor*, *H. rubra, B. areolata*, *S. canarium*, and *C. ramosus* (Fig. 1B–F). These positive cells were typically goblet-shaped with distinctive oval nuclei. Moreover, their cytoplasm contained numerous GABA immunoreactive granules, which when stained with PAS contained high proportions of carbohydrate (glycogen, glycoprotein, proteoglycans), typically observed in mucus (Smith and Morin, 2002) secreting cells (Fig. 1B, D–F insets). Nerve fibers beneath the epithelium, and others located throughout the muscle tissue, displayed GABA reactivity (Fig. 1G, H), as did secreted mucus, which also contained GABA (Fig. 1C, E). Immunofluorescence also confirmed GABA immunoreactivity in the goblet cells, distributed throughout the epithelium of *H. rubra* (Fig. 1I).

GABA immunoreactivity was not observed in negative controls in which there was no anti-GABA applied (Fig. 2A). GABA immunoreactivity was observed in mucus wiped from the foot muscle of *H. rubra* (Fig. 2B), and in the mucus of the other gastropods investigated (data not shown). In particular, GABA was highly concentrated in some cells shed into the mucus (Fig. 2B), which are likely to be remnant goblet cells, based on appearance.

3.2. Chemical determination of GABA in mucus

Aqueous extracts of rehydrated mucus taken from *H. rubra* were tested for the presence of GABA by dot blots. GABA was detected at all dilutions between 1 µg and 0.01 µg (Fig. 3A). Controls were negative.

GABA was clearly present in *H. rubra* mucus as illustrated by HPLC chromatographs (Fig. 3B). The GABA standard (1.0×10^{-5} M) showed a peak with a retention time of 9.3 min, and a peak at 11.6 min corresponding to valine (internal standard). When the GABA standard was spiked with abalone mucus extract a significant additive increase in the height of the peak assigned to GABA was observed, whereas the peak height for the internal standard remained constant. Using time-of-flight mass spectrometry (TOF-MS), GABA was shown to be present in rehydrated mucus. This was achieved by identifying several characteristic ions that were present in both the rehydrated mucus and a standard GABA sample. The most significant of these peaks were m/z 104.1 which corresponds to the $[M+H]^+$ of GABA ($C_4H_9NO_2$) (Fig. 3C).

Although GABA was detected by HPLC in all abalone mucus extracts, the concentration varied. To investigate whether this variation was dependent on gender, the mucus of males and females ($n=20$; 100±10 mm) were separately analyzed. No significant difference could be detected (Fig. 4). The concentration of GABA in the sample group ranged from 2.8 to 0.13 mM, with a mean of 0.68 mM.

3.3. Settlement induction of larvae by mucus

H. asinina larval settlement was tested on the mucus of three abalone species, *H. asinina*, *H. rubra*, and *H. diversicolor* (Table 1). Six hours after exposure of competent larvae to *Haliotis* mucus, the total settlement was 11.7±1.2%. This value was significantly greater than that on control areas devoid of mucus, 1.4±0.4% ($p<0.05$). Settlement of larvae was 9.7±1.3% on *H. asinina* mucus, 12.1±0.9% on *H. rubra* mucus, and 13.7±1.7% on *H. diversicolor* mucus ($p<0.05$).

After 24 h, total larvae settlement on mucus increased to 41.4±3.6%, nearly four-fold of that at 6 h (11.7±1.2%, $p<0.05$). Moreover, this value was still greater than 2.5±0.9%; being the total settlement on controls ($p<0.05$). Larvae settlement at 24 h was 36.1±3% on *H. asinina* mucus, 33.1±3.1% on *H. rubra* mucus, and 48.9±4.4% on *H. diversicolor* mucus. The value of settled larvae to *H. diversicolor* mucus was significantly higher in proportion to the other two values ($p<0.001$).

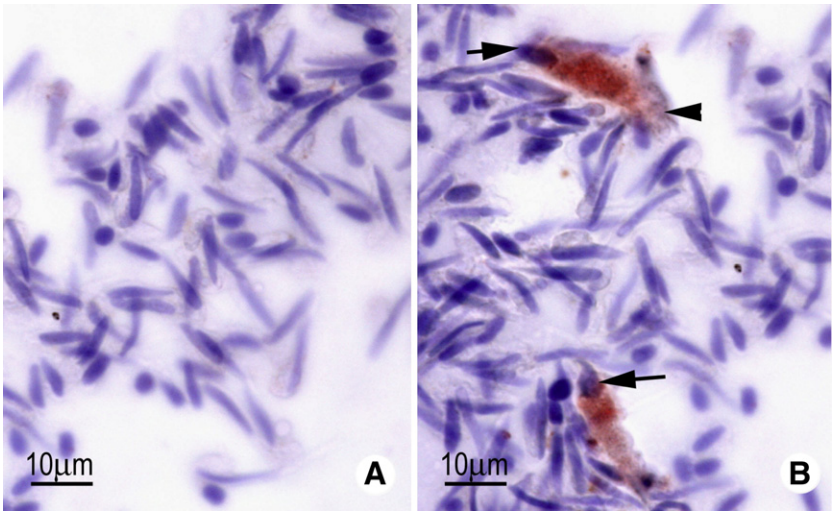

Fig. 2. Immunolocalization of GABA in mucus taken from the foot of *H. asinina*. A, micrograph of mucus used as a negative control (immunostaining without anti-GABA). B, mucus showing GABA immunoreactivity located in "shed cells" (arrows), as well as some reactivity in the mucus (arrowhead).

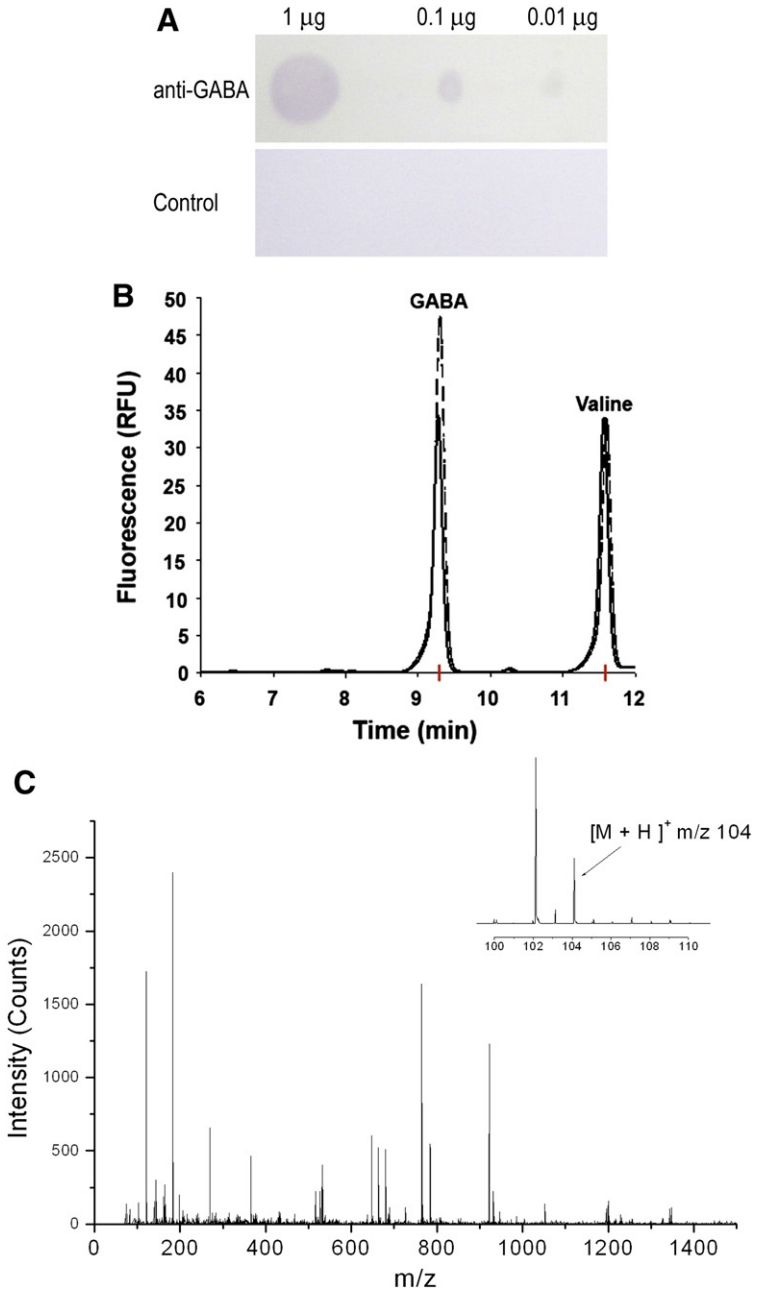

Fig. 3. Chemical determination of GABA in mucus. A, Dot blot analysis of an aqueous extract of rehydrated mucus from *H. rubra* stained with anti-GABA, and without anti-GABA as a control. B, HPLC chromatograph of a GABA standard (_____), plus a GABA standard spiked with abalone mucus (— — —), GABA retention time of 9.3 min. Valine was used as an internal standard with retention time of approximately 11.6 min. C, TOF-MS chromatograph of rehydrated mucus showing the peak of m/z 104.1 which corresponds to the $[M+H]^+$of GABA.

After 72 h, total larval settlement on mucus was 29.7±2.1%, compared to control (3.8±0.6%), $p<0.05$. Settlement numbers were still higher than that observed at 6 h, but reduced when compared with that at 24 h. Larval settlement on mucus at 72 h was significantly different on *H. asinina* mucus 29.7±2%, compared to *H. rubra* mucus 8.3±1.3%, and *H. diversicolor* mucus 35±2.4% ($p<0.05$).

Mucus obtained from abalone trails induced significantly greater larval settlement when compared to mucus collected from stationary (fixed) abalone (Fig. 5). As well, it was noted that fresh mucus resulted in better settlement than dried mucus. After 6 h (Fig. 5A), settlement on trail mucus was 14.4±1.3%; compared to that on fixed mucus, at 9.6±1.1% ($p<0.05$). Moreover, at 24 h (Fig. 5B) and 72 h (Fig. 5C), settlement increased on trail mucus to 44.6±3.8% and 35.5±1.9% respectively, and at values significantly higher than that on fixed mucus, viz, 38.9±3.5% and 25±2.2%, respectively ($p<0.05$).

In comparing fresh and dried mucus for induction of larvae, it was found that larval settlement on fresh mucus was 12.1±1.6%, and on dried mucus 11.4±0.9%. These were not significantly different at 6 h, but at 24 h and 72 h, fresh

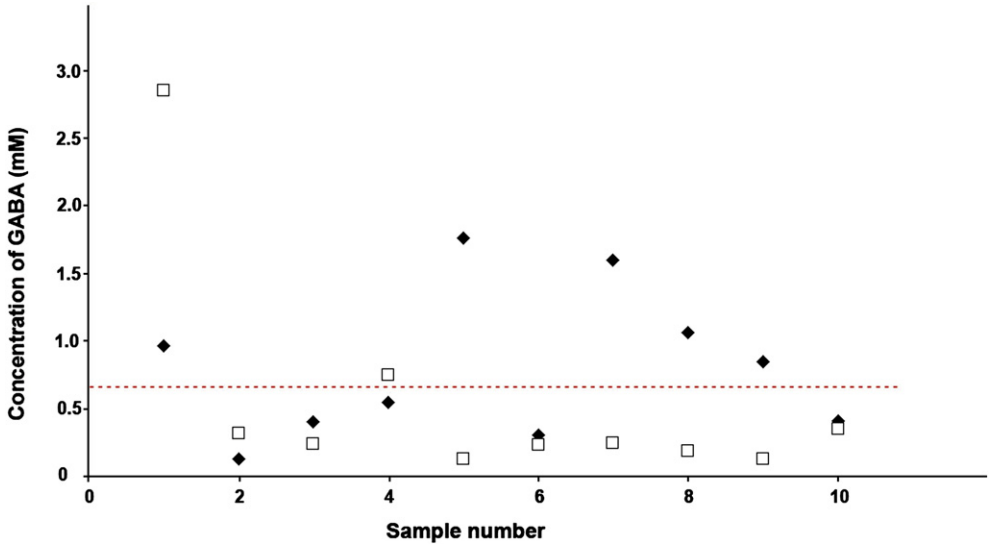

Fig. 4. Concentrations of GABA in aqueous extracts of mucus samples of *H. rubra* (◆, males; □, females). Mean (- - -) of all samples (n=20) was 0.68 mM.

mucus had significantly higher settlement values, i.e. 49.6± 3.5% and 34.9±3.7% respectively, than dried mucus i.e. 40.9± 2.8% and 20.7±1.6%, respectively (p<0.05).

4. Discussion

There have been numerous reports of GABAergic neurons in the CNS of different gastropod species (Cooke and Gelperin, 1988; Vehovszky et al., 1989; Richmond et al., 1991; Arshavky et al., 1993; Hernadi, 1994). However, similar studies in abalone species have been limited to a recent investigation in which evidence for GABA reactivity in the nerve bundles and radiating nerve fibers of *H. asinina* was shown (Wanichanon et al., 2004). They found that GABA was highly concentrated in neuroepithelial cells, which were distributed widely among the epithelial columnar cells and goblet-like cells of the tentacles. This current study has now shown that in *H. rubra*, GABAergic nerve fibers are distributed throughout the foot muscle and beneath the epithelium.

Our most important finding has been that haliotids and other gastropods possess GABA producing goblet-like cells in foot epithelia. When these cells are shed with mucus, they provide high concentrations of GABA which appeared to be slowly released into the surrounding seawater. We infer that the function of these cells is to secrete GABA in complex with mucus, thereby creating an inducer for gastropod larval settlement. It is possible that GABA is a general inducer whereas another component of mucus may provide species or genus specificity.

The supernatant obtained from rehydrated dried mucus samples were assayed for the presence of GABA by preliminary dot blot assays, followed by more detailed HPLC and TOF-MS analysis. The concentration of GABA (mean 0.68 mM), as determined by HPLC, was considerably higher than the 0.4–10 μM GABA concentrations that have been used in aquaculture trials (Roberts, 2001; Gapasin and Polohan, 2004). It should be noted that the concentrations determined in these experiments were entirely dependent on the volume of water used for rehydration, and the concentration of GABA in any secreted mucus sample might be higher than the current values. Variables that could attribute to, or minimize calculated GABA concentrations include (1) solubility of GABA from dried mucus into solution upon rehydration, (2) numbers of GABAergic cells shed-shed cells containing high levels of GABA were, however, identified by immunocytochemistry, (3) unknown dynamics of GABA degradation and or leaching from mucus, and (4) bacterial degradation or production of GABA. The latter notion is unlikely considering mucus samples were immediately incubated for 1 h at 60 °C, thereby minimizing any subsequent contribution by endogenous marine bacteria during extractions.

Abalone larvae have been shown to have highly specific cues (GABA and analogs) for initiating settlement and metamorphosis. Morse et al. (1979a) demonstrated that GABA induced planktonic larvae of the red abalone, *H. rufescens*, to settle and commence behavioral and developmental metamorphosis. Additional findings generally have shown that to stimulate settlement and metamorphosis of competent larvae, it is better to have a substratum covered with encrusting diatoms, and mucus secretions from grazing juveniles (Seki and Kan-no, 1981; Searcy-Bernal et al., 1992). In the present study, abalone larvae settled in greater numbers when exposed to either freshly

Table 1
Settlement of *H. asinina* larvae at 6, 24 and 72 h, when given a choice of mucus from three species of *Haliotis* (pooled data, n=9 replicates, means of percentages±s.e.m.)

Species	Percent settlement at 6 h	Percent settlement at 24 h	Percent settlement at 72 h
H. asinina	9.7±1.3 [a]	36.1±3.0	29.7±2.0 [a]
H. rubra	12.0±0.9	33.0±3.1	8.3±1.3 [a]
H. diversicolor	13.7±1.7	48.9±4.4 [b]	35.0±2.4 [a]
Mean (total)	11.7±1.2	41.4±3.6	29.7±2.1
Control	1.4±0.4 [a]	2.5±0.9 [b]	3.8±0.6 [a]

[a] Significant differences between species (Tukey's post hoc test), p<0.05.
[b] p<0.001.

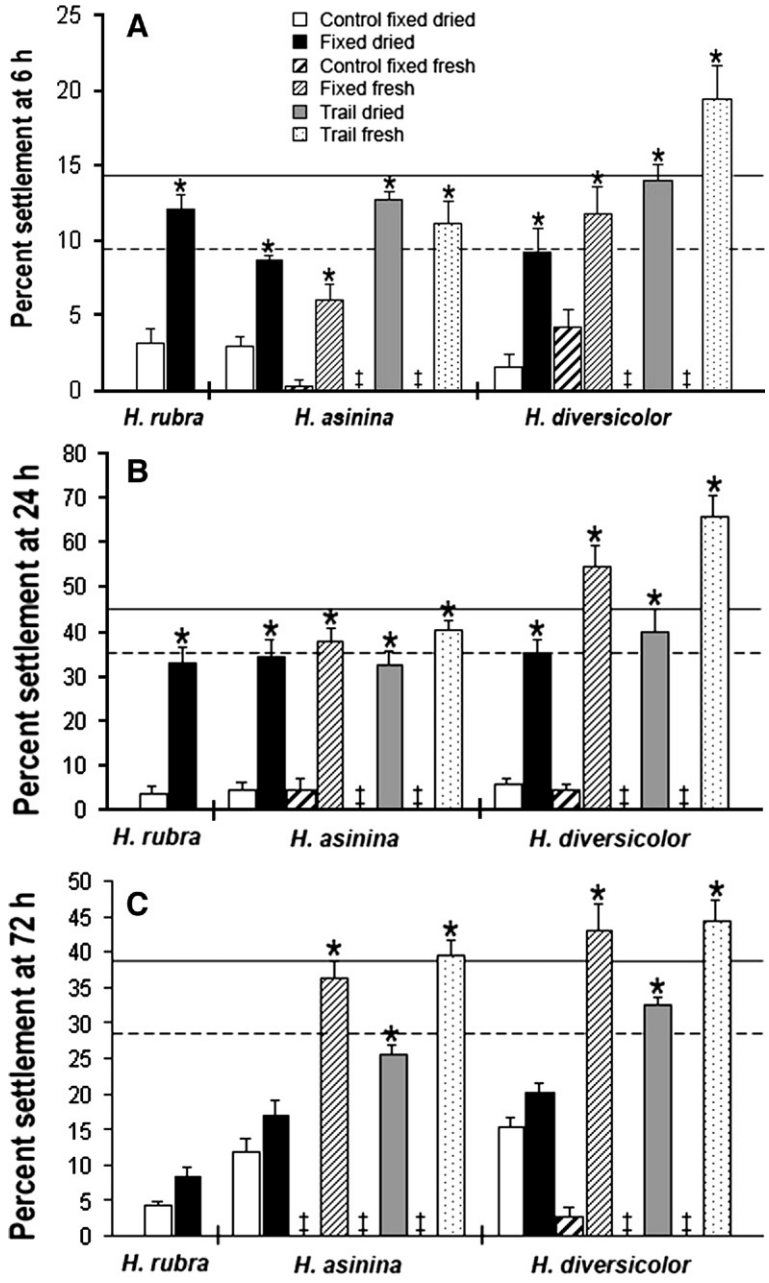

Fig. 5. Settlement of competent *H. asinina* larvae on mucus obtained from *H. asinina*, *H. rubra* and *H. diversicolor*, at 6 h (A), 24 h (B), and 72 h (C), following the addition of larvae. Columns indicate settlement on mucus (mean ± s.e.m.; $n = 9$ replicates) collected from abalone immobilized (fixed), or allowed to crawl freely (trail), on plastic sheets for 15 min. Treatments included: control for fixed dried (no mucus), fixed dried mucus, control for fixed fresh mucus (no mucus), fixed fresh mucus, trail dried mucus, and trail fresh mucus. Settlement in controls for treatments which have zero values, have no columns and are shown as fixed fresh, trail dried and trail fresh (‡).

obtained mucus, or rehydrated dried mucus samples. However, when mucus was absent, settlement was much lower. We observed no differences between the numbers of larvae settling on the mucus of three *Haliotis* species. However, when trail and fixed mucus preparations were compared, variations became apparent. Differences also emerged when settlement over time was compared. In particular, the reduction at 72 h was possibly attributed to natural mortality and/or waste build-up. These data support the findings of Seki and Kan-no (1981), who found that larvae of *H. discus hannai* settled on conspecific mucus trails, as well as the mucus trails of three other haliotids. Furthermore, they found that larvae did not settle as well on mucus trails of non-haliotid gastropods. Bryan and Qian (1998) found that the addition of mucus from animals with free movement increased larval settlement. Likewise, our tests showed that significantly more larvae settled on conspecific mucus trails from free moving animals, rather than on mucus from stationary individuals.

We have found that gastropod mucus contains GABA in sufficient quantities to induce larval settlement. It was already known that GABA-like molecules on the substrate surface of CCA also stimulate efficient settlement of gastropods (Morse

et al., 1979a, 1984), however, there appear to be unknown cues that distinguish one mucus form from another, particularly in attracting larvae. We believe the unknown cues could either be the mucus itself or released into the seawater as pheromones. Water-borne pheromones, as well as contact pheromones have recently been characterized in a number of marine invertebrates, including *Aplysia vaccaria, A. californica*, *Loligo pealeii*, and *Telmessus cheiragonus* (Buresch et al., 2003; Susswein and Nagle, 2004; Kamio et al., 2005). In *A. californica*, egg capsule secreting glands are responsible for the release of pheromones for sexual attraction (Cummins et al., 2004; Painter et al., 2004). In marine arthropods, copulatory behavior occurs in response to chemical cues released in urine (Kamio et al., 2005). We are now investigating the possible presence of pheromones in gastropod mucus, and such findings would strengthen our understanding of gastropod recruitment in the wild, which could be applied in aquaculture.

Acknowledgements

We thank Tony Smith at Bay City Seafarm, Geelong, Australia, and Tanes Poomtong at the Coastal Aquaculture Research and Development Center Prachuabkirikun, Thailand, for the use of animals. Critical comments about the statistical outcomes by Assoc. Prof. Montip Tiensuwan were appreciated. Assistance by Dr Xavier Conlon, from the Institute of Biotechnology (BioDeakin) Deakin University, in the TOF-MS instrument set up, and subsequent analysis of results was also greatly appreciated. This project was supported by grants from the Thailand Research Fund (Senior Research Scholar Fellowship to Prof. Prasert Sobhon), the Commission on Higher Education Thailand (Ph.D. scholarship to Praphaporn Laimek), and Mahidol University. **[RH]**

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Aquaculture 279 (2008) 142–149

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Existence of APGWamide in the testis and its induction of spermiation in *Haliotis asinina* Linnaeus

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ARTICLE INFO

Article history:
Received 7 September 2007
Received in revised form 10 March 2008
Accepted 21 March 2008

Keywords:
Haliotis asinina
Spawning
APGWamide
Immunolocalization

ABSTRACT

Immunolocalization by using rabbit polyclonal antibody against APGWamide demonstrated that there was APGWamide immunoreactivity (APGW-ir) in the varicosed nerve fibers in the capsule, connective tissue and trabeculae of the testis whereas there was no APGW-ir in the ovary of sexually mature broodstocks of *Haliotis asinina*. *In vivo* bioassay, by injecting APGWamide into foot muscle of mature males and females (age over 24 months), showed that most males (87.50%) injected with 200 μl of 10^{-3} M APGWamide (170 μg APGWamide/animal) could be induced to spermiate whereas fewer males (43.75%) injected with 200 μl of 10^{-6} M APGWamide (0.17 μg APGWamide/animal) could spermiate. Following injection with 10^{-3} M, 53% of males spawned within 3 to 4 h, while the remaining males spawned within 2 to 3 h. The former tended to release more sperm at the range of 1.04 to 2×10^9, and the latter at the range 3.62×10^7 to 2.07×10^8 cells, respectively, and that the sperm of the former group appeared to be more motile. Following the injection of 10^{-6} M, all inducible males spawned within 2–3 h, and 43% of spawned males have sperm around 2×10^9, while 57% have fewer sperm number ranging from 2.75×10^7 to 9.94×10^8 cells. In contrast to males, none of the mature females with ripen ovary could be induced to ovulate. These data suggest that APGWamide is an important signaling molecule that plays a role in the male reproductive process that could be used to induce spermiation.

1. Introduction

APGWamide, an amidated tetrapeptide Ala–Pro–Gly–Trp–NH_2, was first isolated from neural ganglia of a prosobranch, *Fusinus ferrugineus* (Kuroki et al., 1990), and African giant snail *Achatina fulica* (Liu et al., 1991). It acts as a neurotransmitter and neuromodulator in many invertebrate species, such as *Lymnaea* (Croll and Van Minnen, 1992; De Boer et al., 1997; McCrohan and Croll, 1997), *Aplysia* (Fan et al., 1997), bivalve mollusks (Henry et al., 2000), sea mussel (Ohtania et al., 2000), and cuttlefish (Henry and Zatylny, 2002). APGWamide is thought to play a key role in the regulation of reproduction (Croll and Van Minnen, 1992; De Boer et al., 1997) and feeding behaviors in these animals (De Boer et al., 1997). Evidence for these functions comes from injection of APGWamide and *in vivo* electrical stimulation of the neurons in the right anterior lobe of cerebral ganglia, both of which cause eversion of the penial complex in the snail *Lymnaea stagnalis* (De Boer et al., 1997). Studies in *Helix aspersa* using anterograde and retrograde tracing methods infer the axon projection from the mesocerebral neurons to the penis, via penile nerves containing APGWamide (Li and Chase, 1995). In *Aplysia*, the movement of radula during feeding is controlled by APGWamide-containing neurons (Jing and Weiss, 2001; Morgan et al., 2002). Moreover, immunohistochemical analysis demonstrated APGWamide-containing axonal projections in the central nervous system as well as in peripheral tissues including the adductor muscle, foot, gill, labial pulps, lips, tentacles of a wide range of gastropods and bivalves (Croll and Van Minnen, 1992; De Lange and Van Minnen, 1998). Additionally, the gonads of juvenile and adult sea scallop of both sexes also contain this neuropeptide (Smith et al., 1997). In the oyster *Crassostrea gigas*, APGWamide is directly involved in the oocyte transport by modulating the contraction of adductor muscle that causes repetitive shell closure (Bernay et al., 2006).

Like other snails *Haliotis asinina*, a tropical abalone, is expected to use this amide as a neuromodulator for ovulation and spermiation. In this study we reported on the existence of APGWamide in the gonad and its function in the induction of ovulation and spermiation. Sexually mature male and female abalones raised in aquaculture

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doi:10.1016/j.aquaculture.2008.03.058

system usually have asynchronous spawning, so there may not be sufficient numbers of eggs and sperm available for fertilization at the same time. The priming of either fully ripe males or females, or both, by this amide could help inducing simultaneous spawning of eggs and sperm, and thus increase fertilization success.

2. Materials and methods

2.1. Preparation of antibody

To produce polyclonal antibody against APGWamide, two 6-week-old female New Zealand white rabbits, obtained from the Animal Care Unit, Mahidol University, with the approval of the Animal Ethics Committee, were used. Before immunization the preimmune sera were collected from both rabbits. Then the immunogen was prepared by coupling 2 mg of APGWamide to 10 mg of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louise, MO, USA) as carrier protein by using 20 mg of linking reagent, 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) (Sigma-Aldrich), in a 0.5 ml of 50 mM NaH_2PO_4, at pH 7.2. The mixture was incubated overnight at 4 °C, and then concentrated using Centricon-10 microconcentrator (Millipore, Bedford, MA, USA). After centrifugation at 10,000 rpm, 10 min, 4 °C, the precipitated component was washed four times with 2 ml of 50 mM NaH_2PO_4 (pH 7.2), then resuspended in 0.5 ml of the same buffer and transferred to a new tube. The antigen (~200 μg) in 0.25 ml phosphate-buffered saline (PBS) was mixed into emulsion with 0.25 ml of Freund's complete adjuvant, and then this antigen mixture was used to immunize the rabbits by subcutaneous injections. At 14, 28 and 42 days following the first injections, the rabbits were further boosted by subcutaneous injection with the same amount of protein mixed with Freund's incomplete adjuvant. The animals were killed at 56 days after the first injection, and the antisera were collected and kept at −20 °C until use.

2.2. Specificity of polyclonal antibody against APGWamide

BSA and BSA-linked to APGWamide were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A five microliter aliquot containing 5 μg of protein of the respective sample (BSA and BSA-linked to APGWamide) was applied to the gel, and the broad range molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA) were run in parallel as the molecular weight markers. The separated proteins were blotted onto nitrocellulose membranes, and non-specific bindings were blocked with 5% skim milk in 10 mM Tris buffer saline, 0.1% Tween-20, pH 7.4. Rabbit antiserum against BSA-APGWamide was preabsorbed with BSA in 0.1 M PBS (250 mg/ml) at the ratios 1:20, 1:40, and 1:80 of antiserum: BSA (v/v). The nitrocellulose strips were incubated in the non-absorbed as well as the BSA-preabsorbed antisera, and bound antigen–antibody complexes were detected by using goat anti-rabbit IgG labeled with HRP (Zymed Laboratory Inc. San Francisco, CA, USA). Reactions were detected using the ECL detection kit (Amersham Biosciences, Buckinghamshire, England), according to the manufacturer's instructions. For negative control, the membrane was incubated with rabbit preimmune serum instead of non-absorbed and preabsorbed antisera. In addition BSA-preabsorbed antiserum was also preabsorbed again by APGWamide in 0.1 M PBS (250 mg/ml) at the ratio of 1:80 of antiserum:APGWamide (v/v). This BSA-APGWamide preabsorbed antiserum was then used to detect the bands on the nitrocellulose membrane as another negative control.

2.3. Screening of APGWamide in various organs by dot blot assay

To demonstrate the presence of APGWamide in different organs, dot blot assay was carried out using specific anti-APGWamide antiserum that has been preabsorbed with BSA as mentioned in Section 2.2. Various organs including male and female gonads, hepatopancreas, cerebral ganglia and pleuropedal ganglia were dissected. The organs were homogenized in a buffer containing 10 mM Tris–HCl, 150 mM NaCl, 0.5% TritonX-100, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride was added during the procedure. Protein-containing supernatants were collected following centrifugation at 10,000 rpm, 20 min 4 °C. After measuring the protein concentrations by Lowry method (Lowry et al., 1951) the proteins were diluted to obtain the concentrations of 1 μg, 100 ng, 10 ng, to 1 ng per 1 μl. One microliter of each sample was applied onto a nitrocellulose membrane pre-soaked in PBS and air-dried for 15 min. The membrane was blocked with 3% skim milk and 1% BSA in PBS and then incubated in the BSA-preabsorbed rabbit anti-APGWamide serum. For a negative control, the membrane was incubated with rabbit preimmune serum and BSA-APGWamide-preabsorbed antiserum instead of BSA-preabsorbed antiserum. After washing, the membrane was then incubated with goat anti-rabbit IgG labeled with HRP and treated with ECL detection kit. Stained dots on a white background indicated positive results. APGWamide at the same serial concentrations as those in the homogenates of organs were also dot-blotted on the nitrocellulose strip, and probed with the same set of antibodies for detection as a positive control.

2.4. Immunolocalization of APGWamide

2.4.1. Specimen preparation

Adult male and female *H. asinina*, with age over 24 months and body weight ranging between 70 and 80 g were obtained from a land-based aquaculture system at the Coastal Aquaculture Research and Development Center, Department of Fisheries, Prachaubkirikun Province, Thailand. They were anesthetized by immersing in 5% $MgCl_2$ in sea water, and then their shells were removed. The gonads were quickly dissected and fixed in Bouin's solution in 0.14 M NaCl for 24 h. After washing with 70% ethanol, the specimens were dehydrated in ascending concentrations of ethanol, cleared in dioxane three times, infiltrated, and embedded in paraffin. Serial transverse sections of the gonads were cut at 5 μm thickness using a rotary microtome, and mounted on glass slides coated with 3-aminopropyl triethoxy silane solution for strong adhesion. For a positive control the cerebral ganglion of *Aplysia californica* embedded in a paraffin block (courtesy of Dr. Scott Cummins University of Queensland, Australia) was also cut at the same thickness and treated similarly.

2.4.2. Immunoperoxidase staining

The gonad sections, as well as sections of *Aplysia* cerebral ganglion, were deparaffinized in xylene and rehydrated in descending concentration of ethanol (in 100% three times, 95%, 90%, 80%, and 70% once each). The residual picric acid from Bouin's solution was neutralized in 70% ethanol containing 1% Li_2CO_3 for 15 min (Wener et al., 1997). Subsequently, the endogenous peroxidase and free aldehyde groups from the fixative were blocked by immersing the sections in 0.3% H_2O_2 in 30% methanol for 30 min, and 0.1% glycine in PBS for 5 min, and washed three times with 0.05 M PBS containing 0.4% Triton X-100 (PBST). Non-specific binding was blocked by incubating the sections in 2% normal goat serum and with 4% BSA in PBST for 2 h. The sections were incubated overnight in the primary antibody (BSA-preabsorbed anti-APGWamide at the absorption ratio of 1:80 v/v) at a dilution of 1:200 in blocking solution, at room temperature overnight. Afterwards the sections were washed three times in PBST, and incubated in the secondary antibody (biotinylated goat anti-rabbit IgG, Sigma-Aldrich) diluted 1:200 in blocking solution for 45 min. After four washes, the slides were incubated with HRP-conjugated streptavidin (diluted 1:200 in PBST), for 45 min before exposure to substrate solution, Nova Red (Vector, Burlingame CA, USA.) containing H_2O_2 for 2–5 min, to develop color reaction. Finally, sections were rinsed with tap water for 10 min, dehydrated, and then

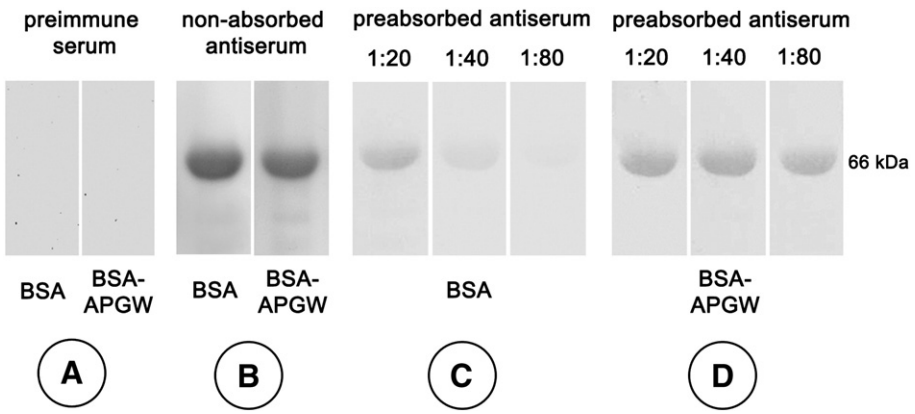

Fig. 1. Immunoblotting of BSA and BSA-APGWamide by non-absorbed and BSA-preabsorbed antiserum. (A) In controls, neither BSA nor BSA-APGW lanes show positive band with preimmune serum. (B) When using non-absorbed antiserum on BSA and BSA-APGW, both lanes show a similar pattern of positive banding. (C) Immunoblotting of BSA and BSA-APGW separated by SDS-PAGE and blotted onto nitrocellulose strips using rabbit antiserum preabsorbed with BSA in 0.1 M PBS (250 mg/ml) at the ratios of 1:20; 1:40; 1:80 (v/v) shows positive bands at 66 kDa in BSA lanes at the absorption ratios of 1: 20 and 1:40 but not at 1:80. (D) In BSA-APGW lanes, the positive bands appear equally intense, at all absorption ratios, i.e., 1:20; 1:40; and 1:80, respectively. Thus, BSA-preabsorbed antiserum at the ratio 1:80 is considered highly specific to APGWamide and was used for dot blot assays (Fig. 2) and immunolocalization (Figs. 3–5).

mounted with Permount medium (Sigma-Aldrich). However, to reveal histological details, some treated sections were counter-stained with Mayer's hematoxylin, washed in water, and processed similarly. For negative controls, the sections were processed in the same manner using preimmune rabbit serum or BSA-APGWamide-preabsorbed antiserum instead of the BSA-preabsorbed antiserum. All sections were examined and photographed using Nikon Eclipse E600 microscope fitted with Nikon digital camera DXM1200.

2.5. Spawning induction

Twenty-four mature males with fully ripen creamy yellow testis and twenty-four mature females with fully ripen dark-green ovary with and the body weight ranging between 70 and 80 g were each divided into 3 groups. The animals were each kept in separate 5-l tanks placed in a reversed day–night room for at least 3 days to allow the animals to acclimatize before beginning the experiment, which was carried out in the day time at 7 days prior to the full moon, which is the natural time that the animals usually spawn. In the two experimental groups, the animals were intramuscularly injected with APGWamide into the sole of foot muscle at two different doses, i.e., 200 µl of 10^{-3} M (170 µg APGWamide/animal) and 200 µl of 10^{-6} M (0.17 µg APGWamide/animal) respectively. Afterwards each animal was placed in a clear plastic tank filled with 2 l of filtered sea water and observed for their spawning behavior at half an hour intervals. Immediately following spawning a 20 µl of sea water containing spawned sperm was taken from the tank and placed in a glass slide with concave well and the sperm motility was assessed by observing under the light microscope, and another 10 µl aliquot was taken, fixed in equal volume of 4% paraformaldehyde in 0.1 M PBS and the sperm number counted under a light microscope using hemocytometer. The total number of sperm per spawn (i.e., in total volume of each tank) was then calculated. In the control groups the injections of mollusc physiological saline (Hepes 13 g, NaCl 25.66 g, KCl 0.82 g, $CaCl_2$ 1.69 g, $MgCl_2$ 10.17 g, Na_2SO_4 2.56 g, dH_2O 1 l, at pH 7.2) at the sxame volume per animal was performed instead of APGWamide. Each experiment was duplicated.

3. Results

3.1. Specificity of anti-APGWamide

The specificity of preabsorbed APGWamide antiserum was tested by western blot analysis (Fig. 1). No positive bands were detected in

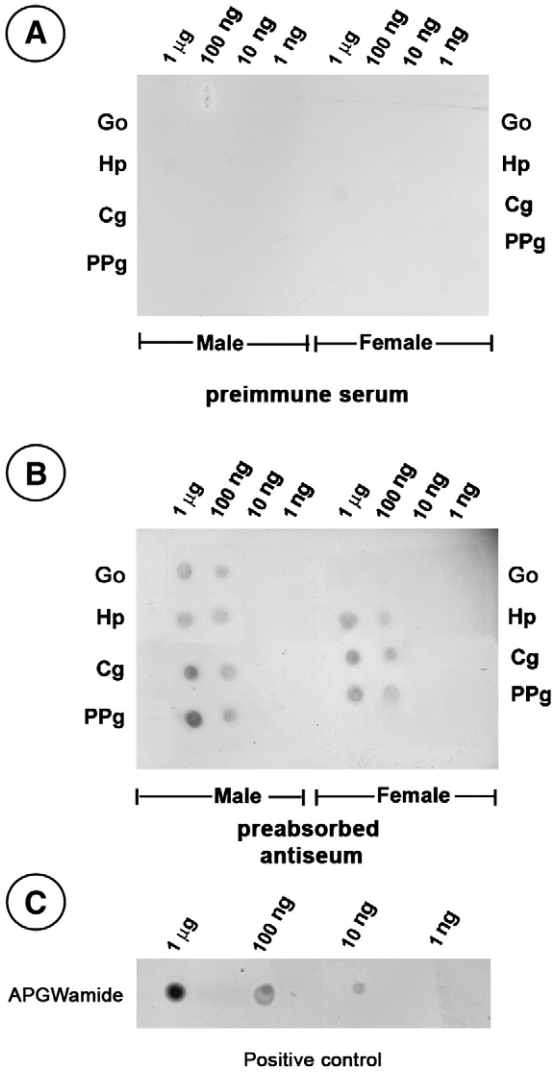

Fig. 2. (A, B) Dot blot assays to detect APGW-ir in homogenates of various organs of the male and female abalone. The amount of proteins of homogenates was serially diluted at 1 µg, 100, 10, and 1 ng/µl, and one microliter was the final aliquot dotted on nitrocellulose paper. APGW-ir could be observed in all organ samples except in the female gonad. (C) Positive control with similar concentrations of APGWamide at 1 µg, 100, 10, and 1 ng/µl showing detection levels of APGWamide in dot blot assays. Cg–cerebral ganglia, Hp–Hepatopancreas, Go–Gonads, PPg–pleuropedal ganglia.

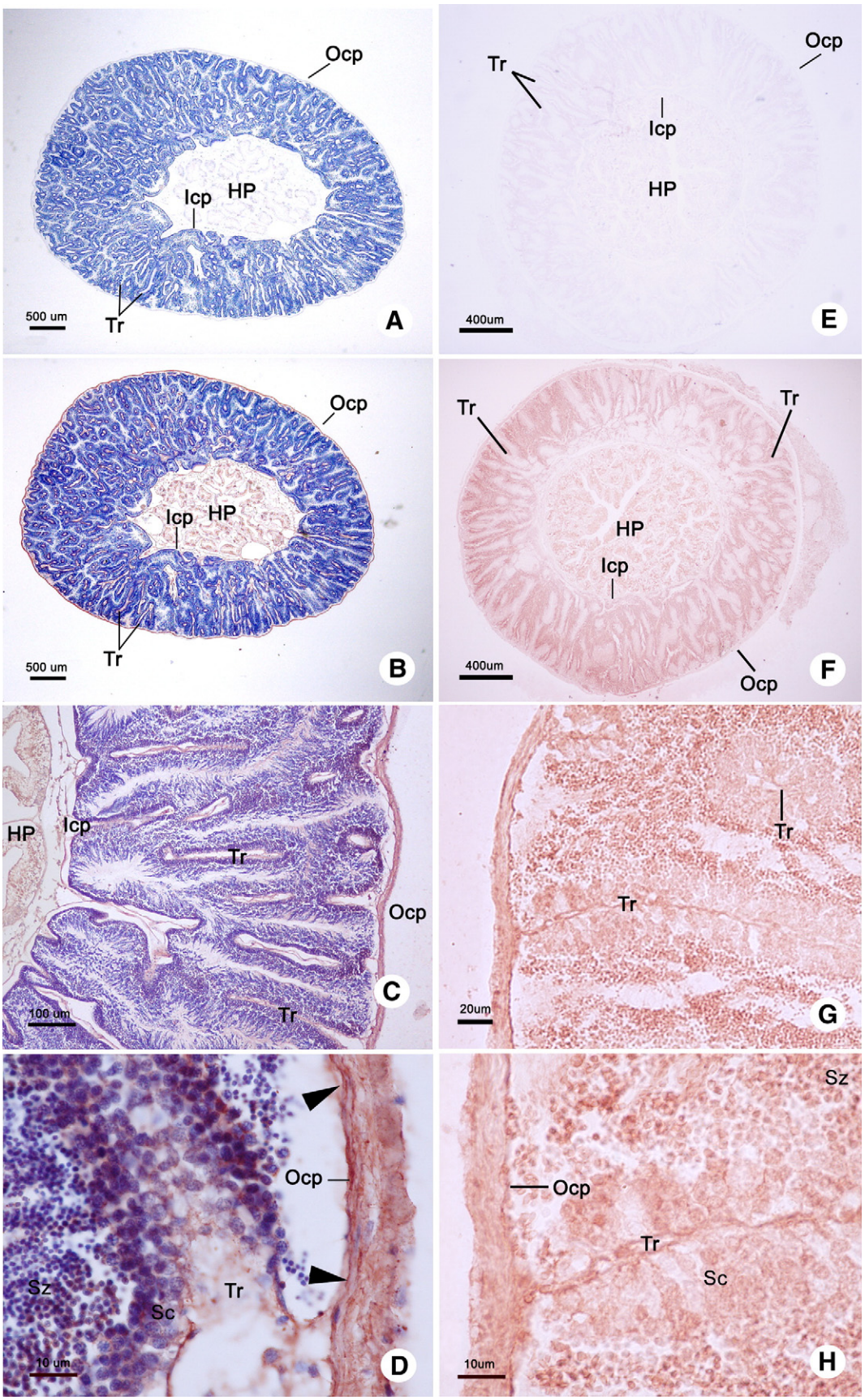

Fig. 3. Sections of fully mature testis stained with anti-APGWamide. (A) Low power micrographs of control sections of fully mature testis stained with preimmune serum and counter-stained with hematoxylin, and without counter stain (E). Low power micrographs of testicular sections showing APGW-ir in the capsule and trabeculae in counter-stained (B) and noncounter-stained sections (F). Medium power micrographs showing APGW-ir in the outer capsule (Ocp), hepatopancreas (HP) and trabeculae (Tr) of counter-stained(C) and noncounter-stained (G) sections. High powered micrographs showing APGW-ir in the nerve fibers (arrow head) in the outer capsule and trabeculae in counter-stained (D) and noncounter-stained(H) sections. Ocp–outer capsule, HP–hepatopancreas, Icp–inner capsule, Tr–trabeculae, Sz–spermatozoa, Sc–spermatocytes.

either lane of the negative controls, which were probed by rabbit preimmune serum (Fig. 1A), whereas lanes containing either BSA or BSA-APGW showed positive bands with non-absorbed antiserum (Fig. 1B). In BSA lanes, a less intense but still positive band was detected by the antiserum preabsorbed with BSA at the ratios 1:20 and 1:40 while no positive band was detected at the ratio 1:80 (Fig. 1C). However positively stained protein bands at a molecular weight 66 kDa was observed in all BSA-APGWamide lanes probed with antiserum preabsorbed with BSA at the ratio 1:20, 1:40 and 1:80 (v/v) (Fig. 1D). On the other hand no band was detected using antiserum preabsorbed with both BSA and APGWamide at the ratio 1:80 (v/v) (data not shown). Hence antiserum after preabsorption with BSA at the ratio 1:80 was quite specific to APGWamide, and this was used for the detection of APGW-ir in dot blot assay and in gonadal tissue sections.

3.2. Dot blot assay for APGWamide

The homogenates from different organs were tested by dot blot assay for the presence of APGW-ir (Fig. 2). No staining was detected in the dot blots of male or female organs probe with preimmune serum (Fig. 2A). In males, APGW-ir was detected in the homogenates of testis, hepatopancreas, cerebral ganglia, and pleuropedal ganglia, with the least intensity in hepatopancreas at the lowest concentration of 100 ng/1 µl. In females APGW-ir was found in the homogenates of cerebral ganglia, pleuropedal ganglia and hepatopancreas, but was absent in ovary (Fig. 2B). The dot blot of APGWamide at comparable concentrations by the same antibody showed positive staining at the lowest concentration of 10 ng/1 µl (Fig. 2C). This suggests that the amount of APGWamide detectable in homogenates could be approximately ten times lower than when using pure APGWamide in comparable dot blot assays.

3.3. Distribution of APGWamide in gonadal tissue using immunoperoxidase staining

There was no APGW-ir in the control gonadal sections both counter-stained and noncounter-stained (Figs. 3A, E, 4A). Intense APGW-ir was observed in the outer capsule of the testis which contained muscle, nerve fibers, and epithelial cells in both counter-stained (Fig. 3B, C, D) and noncounter-stained sections (Fig. 3F, G, H). Similar level of intense staining was found in the trabeculae which were the extending parts of the capsule separating the testis into many compartments (Fig. 3B–D, F–H). In both the capsule and trabeculae, APGW-ir was confined to connective tissue and possibly nerve fibers shown as wavy and varicosed lines (Fig. 3D, H). Hepatopancreas in males exhibited very light staining (Fig. 3B, C, F). In contrast both the control (Fig. 4A,B) and experimental sections (Fig. 4C,D) of the ovary displayed no APGW-ir although the hepatopancreas in females also exhibited very light staining (Fig. 4C). For positive control APGW-ir in the cerebral ganglion of *Aplysia* was distinct (Fig. 5), with fairly intense immunoreactivity present in the giant neurons located at the right and left sides of the ganglion, with slightly fewer positive cells in the latter (Fig. 5A). The strong immunostaining was noted in the neurons of both noncounter-stained (Fig. 5B) and counter-stained sections

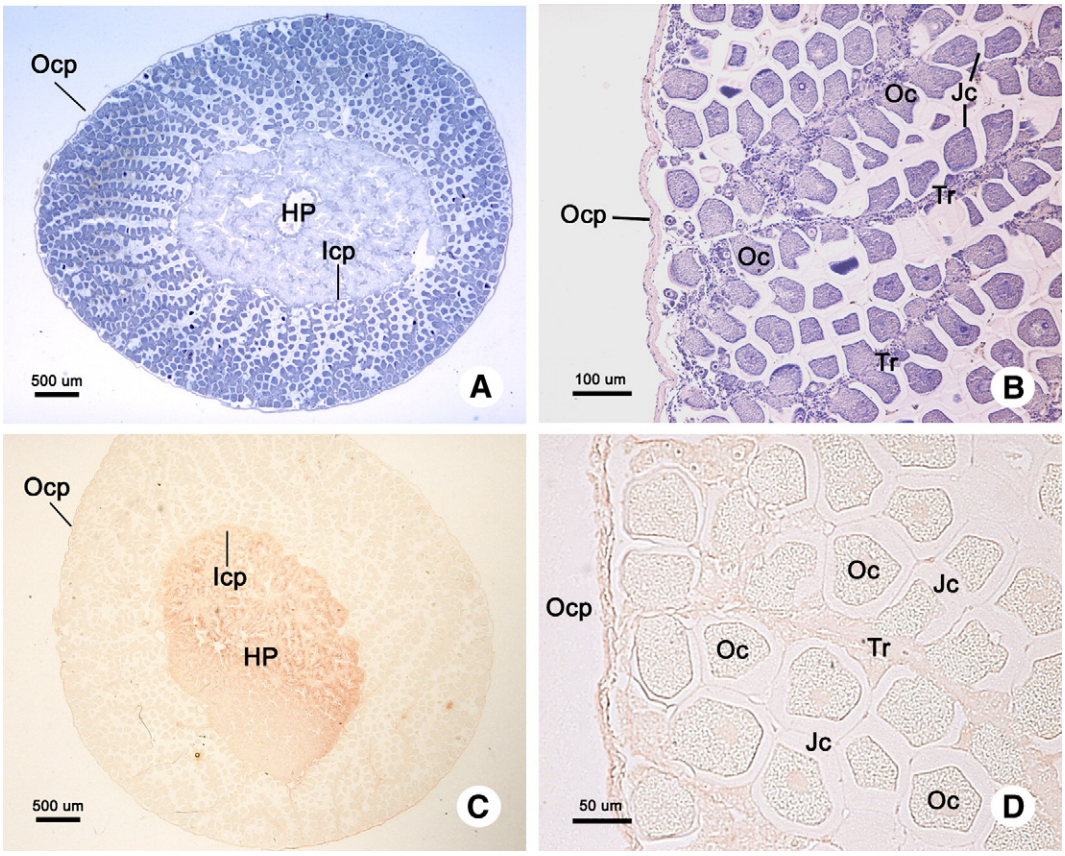

Fig. 4. Sections of fully mature ovary stained with anti-APGWamide. (A) A low power micrograph of a control ovarian section, counter-stained with hematoxylin showing no positive staining. (B) A medium power micrograph of counter-stained control ovarian section showing no positive staining for APGW-ir. (C) A low power micrograph of an ovarian section which is probed with anti-APGWamide and not counter-stained with hematoxylin, showing no positive signal for APGW-ir. Only hepatopancreas (HP) showed light staining. (D) A high power micrograph of C showing no staining for APGW-ir in the capsule or trabeculae of the mature ovary that contains fully developed oocytes. Oc–oocyte, Jc–jelly coated, Ocp–outer capsule, HP–hepatopancreas, Icp–inner capsule.

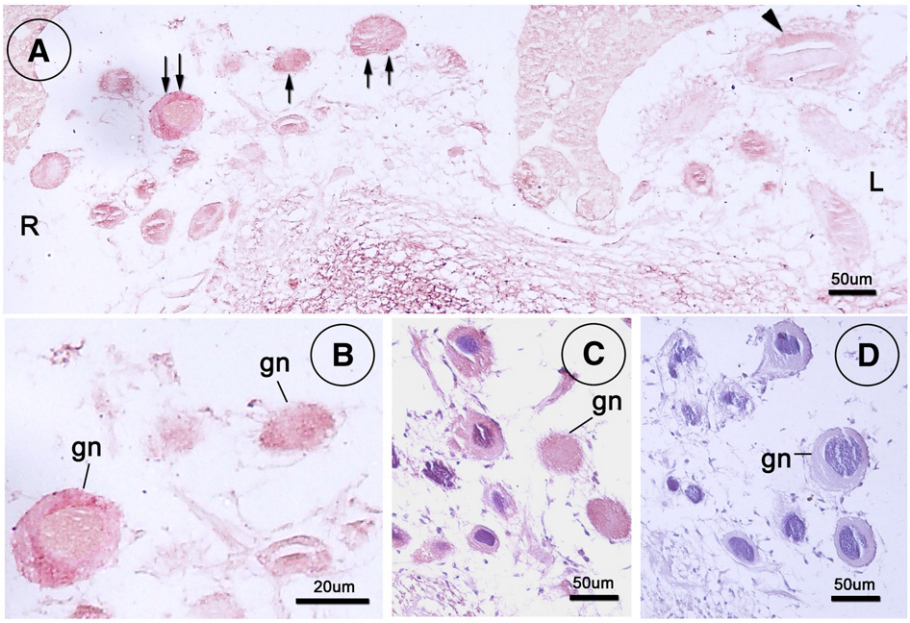

Fig. 5. Cerebral ganglia of *Aplysia californica* stained with anti-APGWamide. (A) A low power micrograph of the *Aplysia* cerebral ganglion showing APGW-ir in neurons,with more numbers and more staining intensity on the right side (arrow) than on the left side (arrow head). (B) A high power micrograph of (A) show intensely stained giant neurons (gn) in noncouter-stained (B) as well as in counter-stained (C) sections. (D) The control section using preimmune serum showing no positive immunoreactivity in the giant neurons (gn) where nuclei are counter-stained with hematoxylin. R–right side, L–left side.

(Fig. 5C). Immunoreactivity was not observed in control sections using preimmune serum (Fig. 5D) nor in sections that were stained with antiserum preabsorbed with BSA-APGW (data not shown).

3.4. Induction of spawning by APGWamide

The injection of APGWamide induced spawning (spermiation) only in the male abalones. Immediately after APGWamide injection, the foot muscle became spasmodic and all tentacles rose up for about 5 min, subsequently the treated males released their gametes within 2 to 4 h. After the injection of APGWamide at dosage of 200 μl of 10^{-3} M (170 μg/animal) most males (87.5%) could be induced to spawn (Fig. 6), whereas only 43.75% of males could be induced to spawn with the injection of 200 μl of 10^{-6} M (0.17 μg/animal) of APGWamide (Fig. 6). The sperm was immediately checked for motility by examination of the movement of their tails for half an hour in the light microscope. The motility was rated from + to +++. At +++ all sperm exhibited fast movement over the period of observation time, at ++ some sperm exhibited fast while the rest exhibited slow movement, and at + all sperm exhibited very slow movement to no movement (Table 1). It was noted that, following injection of 10^{-3} M APGWamide 53% of male abalones spawned within 3–4 h, with the lowest to highest sperm number ranging from 1.04 to 2×10^9 cells, and 47% of male abalones spawned within 2–3 h with the range of sperm number from 3.62×10^7 to 2.07×10^8 cells following injections. Hence, the males that spawned later (3–4 h) tended to release more sperm that were also more active than those that spawned earlier (2–3 h). In comparison, following the injection of 10^{-6} M APGWamide the spawned males released their sperm at 2–3 h and 43% of these spawning male exhibited around 2×10^9 sperm per spawn, whereas 57% exhibited sperm number that ranged from 2.75×10^7 to 9.94×10^8 cells. The numbers of sperm per spawn are within the range observed in natural spawning occurring at full moon, which is about 1.15–6.2×10^9 cells per spawn. In contrast, mature females could not be induced to spawn at either dose, nor control males that received mollusc physiological saline.

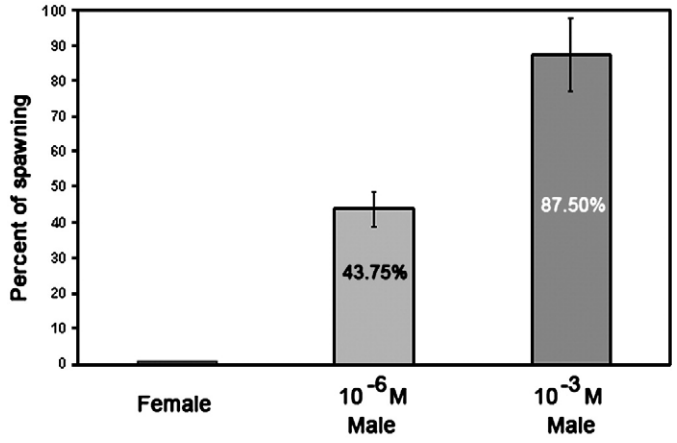

Fig. 6. Effect of APGWamide on spawning induction in male and female abalones. APGWamide injected at the dose of 200 μl of 10^{-3} M could induce sperm release in 87.50% of the treated male animals, whereas APGWamide injected at the dose of 200 μl of 10^{-6} M could induce spermiation in only about 43.75% of treated male, whereas female could not be induced to spawn at either dose. Bars represent standard deviation.

Table 1
The motility and quantity of the released sperm after injecting male abalones with APGWamide at 200 μl per animal of 10^{-3} and 10^{-6} M

APGW Conc.	Ratio% of male that spermiate	Time of spawning (h)	Motility	Range of sperm number per spawn
10^{-3}	53	3–4	+++	$1.04–2\times10^9$
	47	2–3	++	$3.62\times10^7–2.07\times10^8$
10^{-6}	43	2–3	+++	2×10^9
	57	2–3	++	$2.75\times10^7–9.94\times10^8$
Natural spawning	N/A	N/A	+++	$1.15–6.2\times10^9$

The sperm motility is qualitatively estimated as follows: +++, all sperm exhibit fast movement over the period of observation time; ++, some sperm exhibited fast while the rest exhibited slow movement; + all sperm exhibited very slow movement to no movement. Time of spawning is the hours that spawning occurs post injection.

4. Discussion

APGWamide is a well known regulatory neuropeptide that controls the reproductive and feeding behaviors in many mollusks (Li and Chase, 1995; De Boer et al., 1996; De Lange and Van Minnen, 1998). Immunohistochemical localization of APGWamide in several species of gastropod mollusks suggests that APGWamide is a major neuromodulator present in the neural tissue. In a comparative study by De Lange and Van Minnen (1998), it was found that APGW-ir was present in clusters of neurons of cerebral ganglia in the basommatophoran gastropod, *Bulinus truncates*, the stylommatophoran gastropods, *Arion ater* and *Limax maximus*, and in a prosobranch, *Littorina littorea*. Additionally, there are cluster of APGWamide-containing neurons located in the neural ganglia of *A. californica* (Fan et al., 1997), right pedal ganglion of *H. aspersa* (Li and Chase, 1995), and neural ganglia of *L. stagnalis* (Croll and Van Minnen, 1992; De Boer et al., 1997). Most of these cells have projections into the penial nerve which control muscles of the penile complex, or into a pedal nerve which has a pathway into the penial complex (Koene et al., 2000). In this study we chiefly aimed to investigate the distribution of APGWamide in both gonad and neural tissues of male and female *H. asinina*. In dot blot assays, it was observed that the male gonad exhibited more APGW-ir than the female gonad, and APGW-ir was also detected substantially in the neural ganglia including cerebral and pleuropedal ganglia of *H. asinina*, however grading from the intensity the level of APGWamide in the neural ganglia of both sexes, indicates no gender specific differences. Our initial immunohistochemical staining of these neural ganglia also showed APGW-ir in neurons lying in clusters in cerebral and pleuropedal ganglia of both male and female (data not shown), which also supports the dot blot assays. It has been suggested that APGWamide in the neural ganglia may play other neuromodulatory roles, like those observed in *L. stagnalis*, where the isolated light green cells in the cerebral ganglia may be induced to release insulin-like hormone by the action of APGWamide (Geraerts, 1976; Tol-Steye et al., 1999). However, the different levels of gonadal APGWamide in males and females of *H. asinina* may have a more direct bearing on the different pattern of gamete release in both sexes. Our immunohistochemical staining supports this latter notion as APGW-ir is more intense in the connective tissue scaffold, viz., the capsule and trabeculae of the testis. Earlier our group investigated the detailed organization of these two structures and found that they are composed of varicosed nerve fibers and muscle cells arranged as layers in the connective tissue scaffold (Apisawetakan et al., 2001). The pattern of APGW-ir distribution in this structure matches the pattern of varicosed nerve fibers observed in our earlier study. Furthermore the anti-APGWamide that was used as a probe and the existence of APGWamide in these locations are quite specific as it has been proven that anti-APGWamide also specifically stained the APGW-ir containing-giant neurons of *Aplysia* cerebral ganglion as reported earlier (Fan et al., 1997; De Lange and Van Minnen, 1998). Hence, we believe that APGWamide stored in the nerve fibers may be released to stimulate the contraction of muscle cells in the capsule and trabeculae, so that this contraction initiates the release of sperm from the testis.

In functional studies, electrical stimulation of clusters of the APGWamide neurons in the right cerebral ganglion of *A. califonica* and *H. aspersa* causes contraction of the penial complex (Koene et al., 2000), whereas injection of APGWamide can evoke penial eversion (Yu and Blankenship, 1997). In this study, the injection of two concentrations of APGWamide 10^{-3} M and 10^{-6} M could induce sperm release in males *H. asinina*, with 87.50% positive response in the group treated with the higher concentration. In contrast, ovulation could not be induced in females by APGWamide given at the same concentrations and doses. Altogether, it is suggestive that in *H. asinina* this peptide plays a major role in spermiation. This observation does however appear to be different from an earlier report, which demonstrated that SepOvatropin, APGWamide-liked peptide is involved in the oocyte transport in cuttlefish, *Sepia officinalis* (Zatylny et al., 2000). Furthermore, in a more recent investigation in the oyster, *C. gigas*, APGWamide in association with the hexapeptide PIESVD, triggers a contraction of the adductor muscle to cause the release of oocytes to the external medium (Bernay et al., 2006). These two findings suggest that APGWamide can induce germ cell release in both males and females of these species. In contrast, in our study it was demonstrated that APGWamide has a predominantly more male-associated function, perhaps, due to the presence of different amounts of APGWamide in the gonads of the two sexes. It is also possible that the difference in observations from those of Zatylny et al. (2000) is due to the varied peptides structures of both APGWamide and SepOvatropin, as well as species specifications, leading to tangibly different outcomes (Cummins and Hanna, 2004). In the latter investigation it appears that APGWamide might operate in conjunction with PIESVD in females. Therefore stimulation by both peptides may be needed to induce female spawning, whereas one peptide is insufficient. This assumption is possible, as it was recently suggested that mollusc APGWamide family members act through the gonadotrophin releasing hormone (GnRH) along with it's receptor (GnRH-R) (Filipe et al., 2007) to induce imposex, namely, development of the male accessory sex organs in females (Oberdörster and McClellan-Green, 2002). Therefore it is possible that APGWamide directly triggers the onset of spawning in male abalone, whereas in females, it may require other cofactors to have any significant physiological effects on gamete release.

The numbers of sperm received per spawn from animals after treatment with different doses of APGWamide were not significantly different from one another, and fell within the range of numbers of sperm per spawn released during natural spermiation during the full moon period. Furthermore, the sperm released by APGWamide induction exhibited similar level of motility as those released under natural conditions. This implies that the APGWamide-induced released sperm should comprise mostly of mature sperm, although their ability to fertilize eggs needs to be tested further. Additionally, we have lower percentages of spermiation when using FMRFamide (unpublished observation), suggesting that the APGWamide effect is specific, although given the high dose used we cannot yet totally exclude an effect though an unspecific pathway.

Acknowledgements

This research was supported by the Thailand Research Fund (Senior Research Scholar Fellowship to Prasert Sobhon) and the Mahidol University. We would also like to thank the Coastal Aquaculture Research and Development Center, Department of Fisheries, Prachaubkirikun Province, Thailand, for providing abalone specimens, and Dr. Scott Cummins from the University of Queensland, Australia for providing the block of cerebral ganglion of *A. californica*.

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Aquaculture Research

Aquaculture Research, 2008, 1–9 doi: 10.1111/j.1365-2109.2008.01982.x

Larval settlement of the tropical abalone, *Haliotis asinina* Linnaeus, using natural and artificial chemical inducers

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Abstract

Many kinds of chemical and biological materials have been used as inducers of settlement of abalone larvae, as well as other species of marine gastropods, with responses being highly variable, even to the same chemical cue. The present study tested chemical inducers, γ-aminobutyric acid (GABA), δ-aminovaleric acid (5-AVA) and L-glutamic acid (GA) and the effects they have on larval settlement of *Haliotis asinina*. Additionally, a relatively inexpensive commercial substance, monosodium glutamate (MSG), was trialed. The datum provided shows all chemicals to be active inducers of settlement in this study, in order of effectiveness of 5-AVA, GABA, MSG to GA. Induction as adjudged from larval numbers settled was best at 6 h 62%, with 10^{-1} mM 5-AVA. At 24 h, induction was the highest at 78% when exposed to 10^{-2} mM 5-AVA. Larvae that were allowed to settle up to 72 h showed the highest numbers of settled larvae, and declined back to 60% when exposed to 10^{-2} 5-AVA and 10^{-1} mM GABA respectively. Monosodium glutamate, although third in settlement standings would bypass the other chemicals, with regard to cost versus yield. The assessment of settlement surface, rough or smooth proved to be irrelevant, which had no significant impact on larval settlement.

Keywords: *Haliotis asinina*, larval settlement, 5-AVA, GABA, L-glutamic acid, MSG

Introduction

Abalone cultivation in both wild and commercial aquaculture requires reliable production of large quantities of seeds. Although production is often compounded by disease, the numbers of larvae that complete settlement and metamorphose are important (Anguiano, Seary, Lizarraga & Marcial 1998; Huang, Liu & Lee 2001; Nicolas, Basuyaux, Mazurie & Thebault 2002). As such, attempts to improve settlement by researchers have yielded variable results, and the numbers of artificial cues known to influence the settlement of larvae are broad (Morse 1990; Roberts & Nicholson 1997; Roberts 2001).

Morse, Hooker, Duncan and Jensen (1979) first reported that 10^{-3} mM γ-aminobutyric acid (GABA) could significantly promote settlement and metamorphosis of larvae of *Haliotis rufescens*. Using larvae of *Haliotis iris*, Roberts and Nicholson (1997) showed that 10^{-3} mM GABA could induce 90–100% attachment and 20–60% metamorphosis within 2 days, and with *H. virginea* larvae, 65–100% settlement over the same time period. γ-aminobutyric acid in seawater stimulated attachment, and to a minor extent metamorphosis in *Haliotis diversicolor*, at 10^{-6}–10^{-7} mM (Bryan & Qian 1998). Larvae of *Haliotis asinina* exposed to GABA over 72 h showed settlement and metamorphosis, but the results varied according to the dose, with the optimal concentration being 0.45 μM (Gapasin & Polohan 2005). Overall, GABA

treatment has been found to induce successfully settlement of competent *Haliotis* larvae (Searcy-Bernal, Salas-Garza, Flores-Aguilar & Hinojosa-Rivera 1992; Moss 1999; Roberts 2001; Gapasin & Polohan 2005).

The settlement-inducing action of GABA has been shown to occur through the inhibition of veliger cilia movement (Morse Hooker, Duncan *et al.* 1979). γ-aminobutyric acid analogues, in particular, the isoforms of GABA, viz. γ-hydroxybutyric acid, γ-aminovaleric acid (5-AVA) and ε-aminocaproic acids, could stimulate synchronous settlement induction of planktonic larvae and metamorphosis into juveniles of several *Haliotis* spp. (Morse Hooker, Duncan *et al.* 1979; Morse, Hooker & Duncan 1980; Morse, Froyd & Morse 1984; Morse & Morse 1984a, b).

Recently, 5-AVA was isolated from the coralline alga *Hydrolithon samoense* (Suenaga, Hori, Ishida, Nukaya, Roberts & Tsuji 2004). In the examination of algal crusts, 5-AVA was found to be a naturally occurring compound in dry weight extracts (0.72 mg g^{-1}), whereas the presence for GABA could not be established. Using *H. rufescens* larvae, commercially available 5-AVA at 1 mM has produced a settlement efficacy similar to GABA, and attachment without metamorphosis, in *H. virginea* at the same concentration (Suenaga *et al.* 2004).

Other compounds have also been used to induce settlement of Haliotid larvae. For example, KCl at 10, 20 and 30 mM has also stimulated attachment behaviour in *H. diversicolor* (Bryan & Qian 1998). However, GABA has generally promoted better attachment, metamorphic responses and survival than KCl (Gapasin & Polohan 2004).

L-glutamic acid and L-aspartic acid have induced *Haliotis discus hannai* larvae to settle on substrates with benthic diatoms present at 82 ± 6.9% and 78.7 ± 5.1%, respectively, after 24 h (Kang, Kim, Kim & Kim 2003). However, without benthic diatoms present, settlement induction was significantly reduced with 33.3 ± 8.8% for GA and 16.7 ± 3.3 for L-aspartic acid (Kang *et al.* 2003).

Another cue also known to be effective in settlement induction of *Haliotis* spp. is mucus (Seki & Kan-no 1981; Takahashi & Koganezawa 1988; Yang & Wu 1995; Gallardo & Buen 2003). The role of mucus in the induction of settlement has been investigated recently in *H. asinina* (Laimek, Clark, Stewart, Pfeffer, Wanichanon, Hanna & Sobhon 2008), where the presence of GABA in mucus was determined and effective in inducing larval settlement, provided GABA was present in sufficient quantities.

In Thailand, the main cultivated species for commercial purpose is the tropical abalone *H. asinina*, commonly called donkey's ear abalone. It is considered to be suitable for artificial propagation in view of its relatively large size and high proportion of edible foot muscle (Singhagraiwan & Sasaki 1991). In order to increase *H. asinina* stocks and yields, research output in this species has grown extensively. One limiting factor, however, has been lack of knowledge on the settlement of this species. In this paper, we have focused on the effects of chemical inducers GABA, 5-AVA and GA, and have also explored the potential usefulness of monosodium glutamate (MSG) as a cheap alternative for settlement induction of this species. Monosodium glutamate has not been used in any previous settlement assay, but because its chemical properties are analogous to GA, its potential for settlement induction was investigated. Positive outcomes of the tests could provide better strategies to be implemented in settlement, and thereby aid in developing a sustainable aquaculture for *H. asinina* and other abalone species.

Materials and methods

Larval culture

Larvae of *H. asinina* were obtained from the Coastal Aquaculture Research and Development Center, Department of Fisheries, Klongwan, Prachaubkirikhun Province, Thailand. Sperm and eggs were obtained from a mixed mass spawning for a period of 20 min, and the eggs were washed to remove excess sperm. The fertilized eggs were transferred and allowed to develop to the late veliger stage in round plastic tanks housed in the shade, with circulated sand-filtered seawater and continuous aeration. The level of salinity used was 2.5–3.2 × 10^{-4} g L^{-1}, and the temperature was maintained between 25 and 26 °C (Singhagraiwan & Doi 1993). When competent to settle, late veligers that were suspended at the top of the water surface began displaying creeping and alternative suspending behaviour at 26 h. The proportion of these veliger that made the transition to creeping stage larvae were approximately 80%, as observed by the naked eye, and were used in bioassays.

Preparation of test chemicals

Before the assay, solutions of δ-aminovaleric acid (5-AVA), GABA, GA (Sigma, St Louise, MO, USA) and

MSG (Ayinomoto, Bangkok, Thailand) were freshly prepared as stock solutions and then 5-AVA, GABA and GA were assayed at concentrations of 10^{-1}, 10^{-2} and 10^{-3} mM. Monosodium glutamate was used at 5, 10, 15, 20 and 25 mM. Each solution was prepared using 0.45 μm filtered ultraviolet irradiated (500 mW h^{-1}) natural seawater, with salinity between 2.5 and 3.2 × 10^{-4} g L^{-1}.

Settlement bioassays

Assays were conducted in plastic containers with 1200 mL of filtered, ultraviolet-treated seawater, containing streptomycin and neomycin (Sigma) at concentrations of 0.0075 g L^{-1}. All cultures were maintained at 25–26 °C (approximate ambient water temperature), and under diffuse light. Competent veligers were used at a designated density of 600 $tank^{-1}$.

Assays were designed to test two kinds of conditioned plastic sheets (i.e. rough by crumpling the sheet or smooth) and three types of methods for treatment. Three separate assays were performed: (1) transparent plastic sheets, made of the same material, 10 × 15 cm in size, pre-coated with *Nitzschia* spp. for 5 days ($n = 3$ replicates $condition^{-1}$ $tank^{-1}$), were immersed in chemical solutions (5-AVA, GABA, GA) at concentrations of 10^{-1}, 10^{-2} and 10^{-3} mM, and MSG at 5, 10, 15, 20 and 25 mM, for 1 h, before being placed in tanks with filtered seawater, followed by 600 competent veligers; (2) 600 veligers were pre-immersed for 1 h in filtered seawater containing the chemical solutions at the same concentrations, after which the veligers were transferred, using an 80 μm sieve, into tanks that contained plastic sheets and filtered seawater; and (3) both the 600 veligers and the plastic sheets were exposed continuously in tanks with filtered seawater containing the chemical solutions at the concentrations as described in assay 1.

Data analysis

The numbers of larval settled on each plastic sheet were counted at 6, 24 and 72 h after the commencement of the experiment. Three replicates were performed. Data were expressed as a percentage of larval settlement. Statistical analyses were performed using statistical software SPSS 11.5 (SIGMASTAT, SPSS). One-way ANOVA with *post-hoc* Tukey's HSD tests were used to compare variance among the solutions, types of plastic sheets and experimental treatments. The results were considered to be significantly different at $P < 0.05$.

Results

The types of substrates (smooth or rough plastic sheets) had no influence on larval settlement numbers at each sampling time ($P > 0.05$). However, larvae preferred to settle on smooth sheets rather than on rough sheets, with an approximately 5–10% difference in assays with GA solution ($P < 0.05$). The exposure times of both larvae and plastic sheet were not related to the settlement of larvae.

In order of effectiveness, 5-AVA (greatest), GABA, MSG and GA (least) were identified as active inducers of settlement ($P < 0.05$). Details of the chemical assays are described below.

Effects of GABA concentration, type of plastic sheet and different modes of exposure to GABA on larval settlement

The values of the percentage settlement of *H. asinina* obtained under the different chemical treatments after 6, 24 and 72 h are shown in Figs 1–3 respectively.

Overall, GABA induced larval settlement with over 35% on plastic sheets at 6 h after commencement (Fig. 1) and around 45% after 24 h (Fig. 2). However, the numbers decreased to 43% at 72 h (Fig. 3). At a concentration of 10^{-1} mM, GABA induced 40–45% of larvae to settle at 6 h in experiments 1 and 2 (Fig. 1a–d). But the highest induction at 59% was observed with 10^{-3} mM GABA ($P < 0.05$) on the rough sheet on continuously exposing both larvae and the plastic sheet to GABA solution (Fig. 1e). Settlement increased at 24 h, and reached 65% when larvae were pre-exposed to 10^{-1} mM GABA for 1 h ($P < 0.05$; Fig. 2b) and 63% with continuous exposure of both the animal and the substrate in 10^{-3} mM GABA (Fig. 2e). However, these concentrations appeared to be possibly toxic as the numbers decreased when exposed to GABA for 72 h (Fig. 3).

Effects of 5-AVA concentration, type of plastic sheet and different modes of exposure to 5-AVA on larval settlement

Overall exposure of larvae to 5-AVA at 6 h after the commencement of the experiment demonstrated

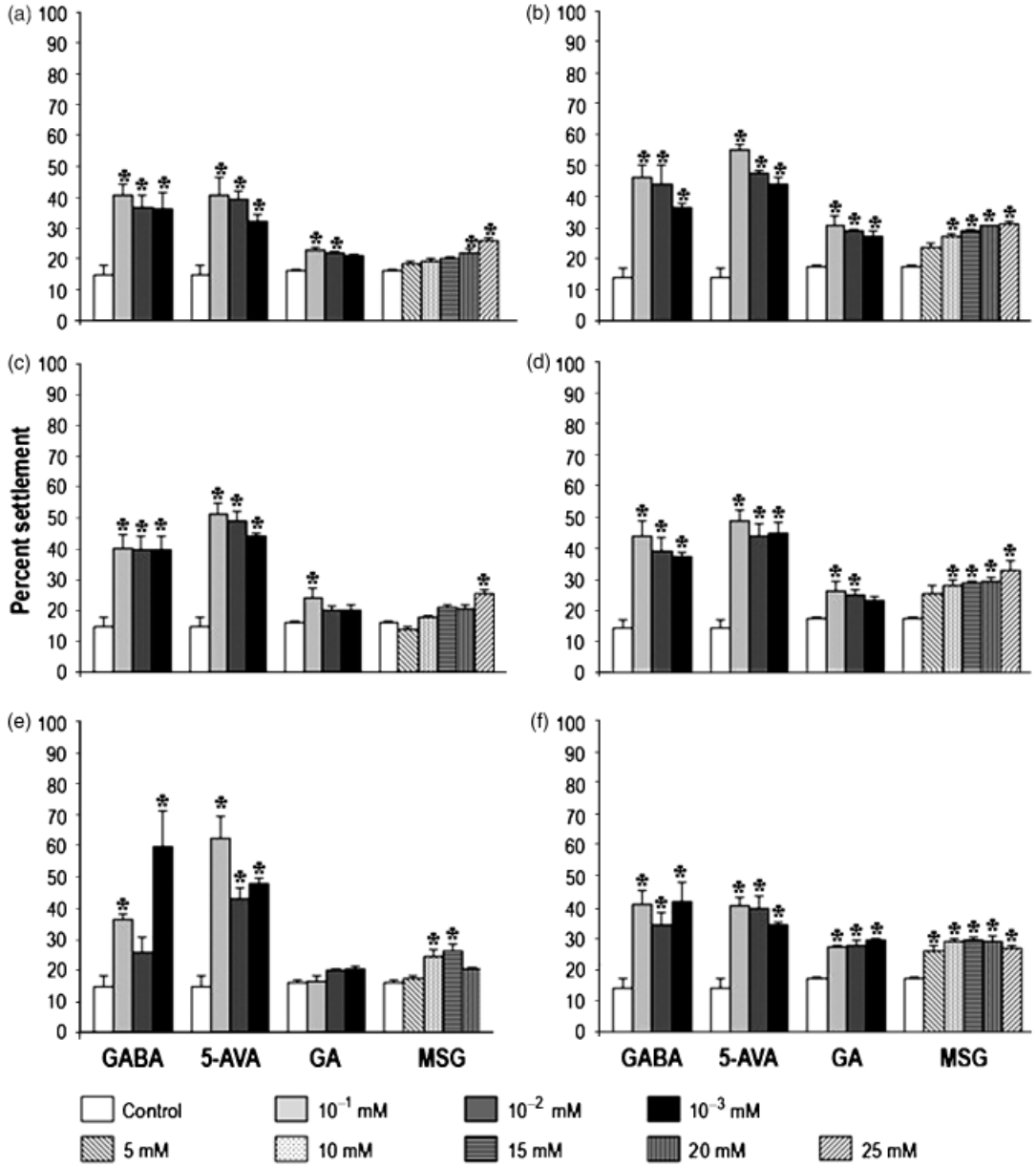

Figure 1 Effect of different chemicals, GABA, 5-AVA, GA and MSG, when compared with seawater (Control) on the settlement of *H. asinina* larvae at 6 h, using two types of substrates, i.e. rough plastic sheets (a, c, e) and smooth plastic sheets (b, d, f). Three experiments of chemical exposure were compared: either plastic sheets (a, b) or larvae (c, d) were pre-exposed for 1 h in a chemical solution or both were continuously exposed (e, f). All points represent the mean ± SEM. of triplicate determinations for each concentration. Asterisks indicate significant differences (*$P < 0.05$) from controls. GABA, γ-aminobutyric acid; 5-AVA, δ-aminovaleric acid; GA, L-glutamic acid; MSG, monosodium glutamate.

settlement of over 30% (Fig. 1). Settlement at 24 h increased to 55% (Fig. 2), but then decreased to 40% after 72 h (Fig. 3). Maximum numbers of larvae were induced to settle on rough sheets after continuous exposure for 6 h in 10^{-1} mM 5-AVA ($P < 0.05$; Fig. 1e). After 24 h, 10^{-2} mM 5-AVA induced the highest larval settlement, at 78%, on smooth sheets when the larvae were pre-exposed in 5-AVA solution for 1 h ($P < 0.05$; Fig. 2d). However, approximately 60% of larvae were settled on the smooth sheet, using the same exposure method with 10^{-2} mM 5-AVA at 72 h ($P < 0.05$; Fig. 3d).

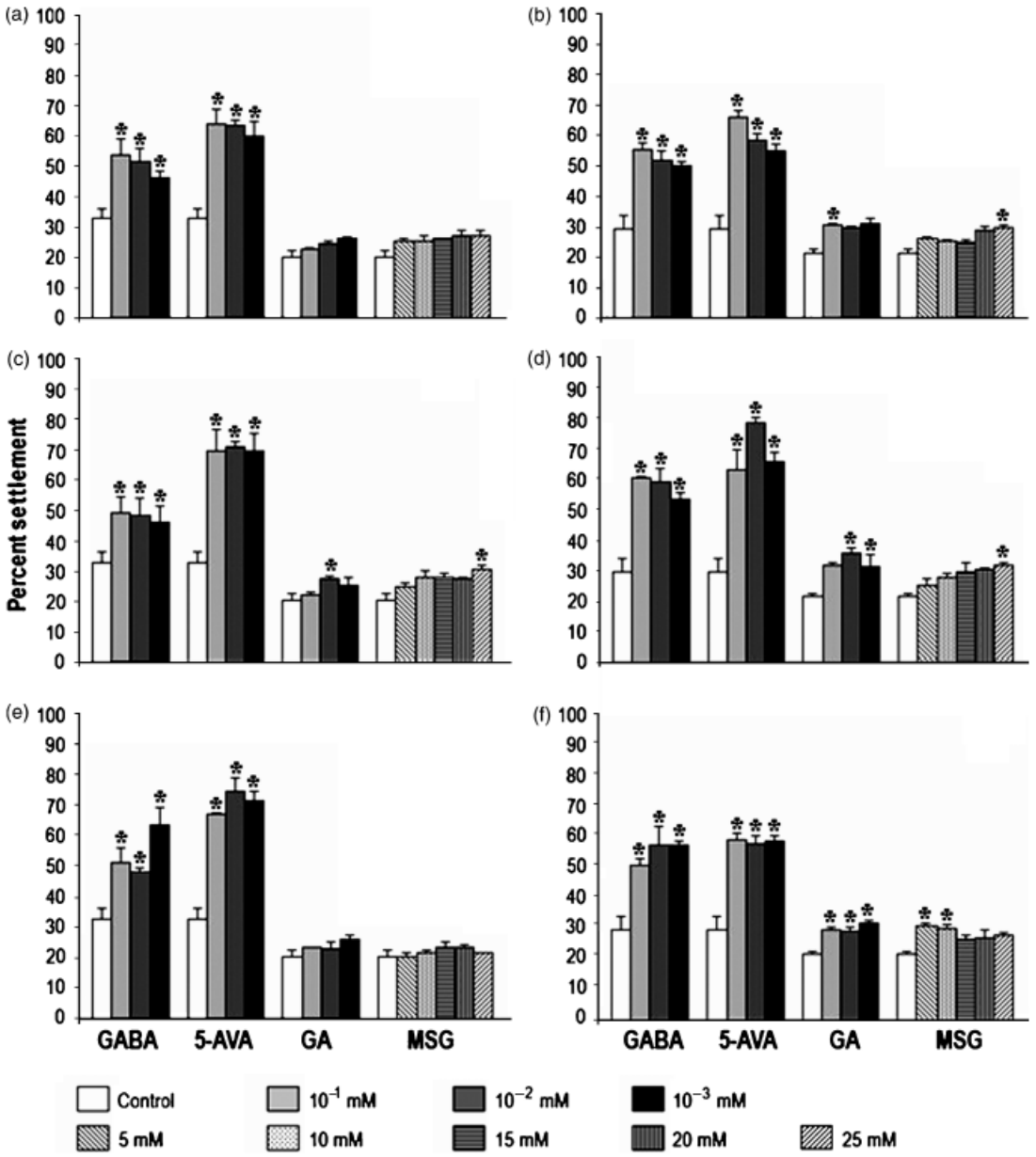

Figure 2 Effect of different chemical solutions, GABA, 5-AVA, GA and MSG, when compared with seawater (Control) on the settlement of *H. asinina* larvae at 24 h, using two types of substrates, i.e. rough plastic sheets (a, c, e) and smooth plastic sheets (b, d, f). Three experiments of chemical exposure were compared: either plastic sheets (a, b) or larvae (c, d) were pre-exposed for 1 h in a chemical solution or both were continuously exposed (e, f). All points represent the mean ± SEM of triplicate determinations for each concentration. Asterisks indicate significant differences ($^{*}P < 0.05$) from controls. GABA, γ-aminobutyric acid; 5-AVA, δ-aminovaleric acid; GA, L-glutamic acid; MSG, monosodium glutamate.

Similar percentages were obtained when both the plastic sheet and the larvae were continuously exposed to 10^{-2} mM 5-AVA (Fig. 3e). Therefore, doses of 10^{-1} and 10^{-2} mM 5-AVA induced higher percentages of settlement ($P < 0.05$) than the other doses.

Effects of GA concentration, type of plastic sheet and different modes of exposure to GA on larval settlement

Settlement of larvae was over 15% on plastic sheets at 6 h (Fig. 1), over 20% after 24 h (Fig. 2) and had

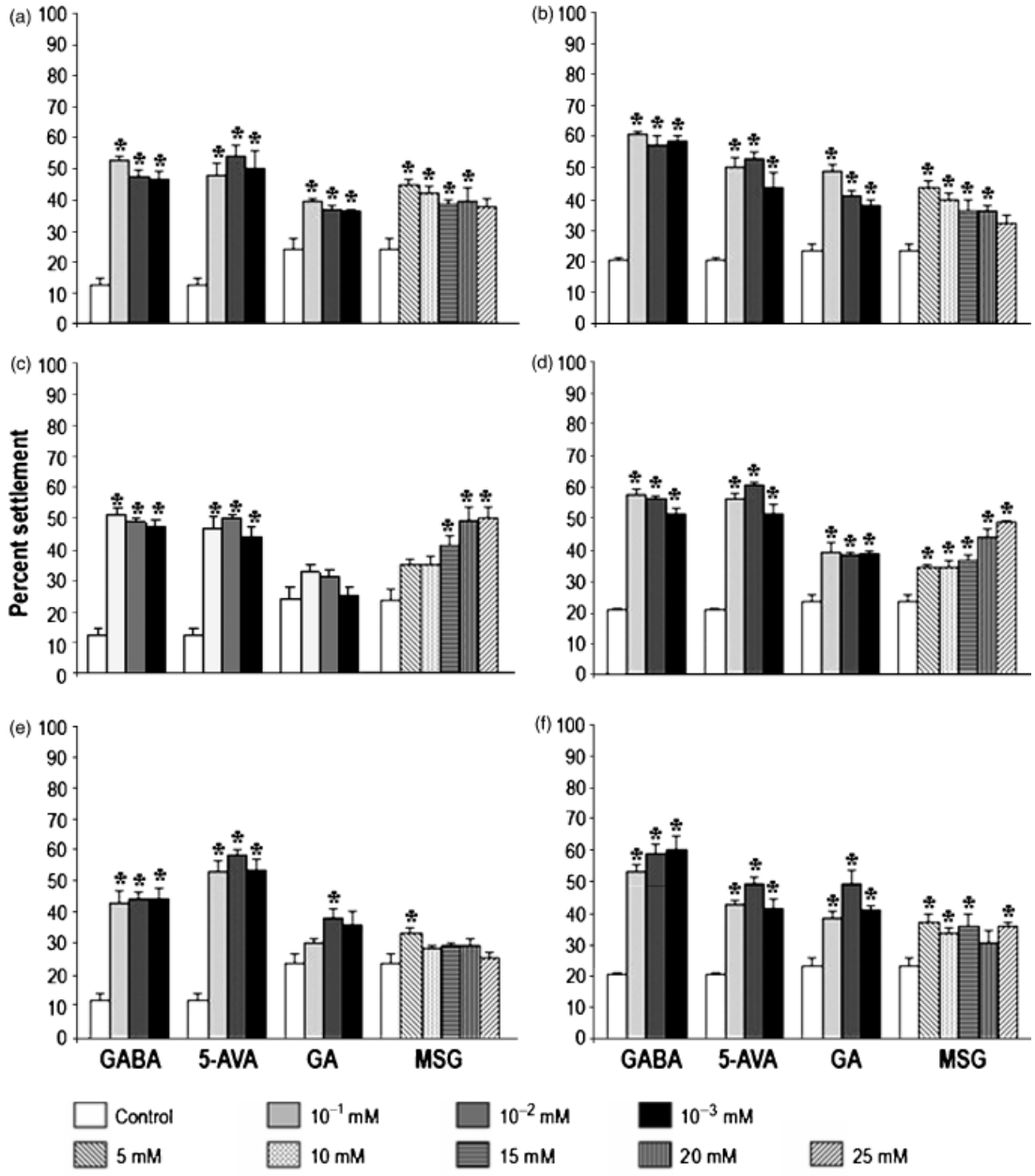

Figure 3 Effect of different chemical solutions, GABA, 5-AVA, GA and MSG, when compared with seawater (Control) on the settlement of *H. asinina* larvae at 72 h, using two types of substrates, i.e. rough plastic sheets (a, c, e) and smooth plastic sheets (b, d, f). Three experiments of chemical exposure were compared: either plastic sheets (a, b) or larvae (c, d) were pre-exposed for 1 h in a chemical solution or both were continuously exposed (e, f). All points represent the mean ± SEM of triplicate determinations for each concentration. Asterisks indicate significant differences ($^*P < 0.05$) from controls. GABA, γ-aminobutyric acid; 5-AVA, δ-aminovaleric acid; GA, L-glutamic acid; MSG, monosodium glutamate.

increased to 25% at 72 h (Fig. 3). At a concentration of 10^{-1} mM, GA induced significantly higher settlement behaviour at 6 h than at the other concentrations tested ($P < 0.05$) on both rough and smooth sheets. This was different when compared with exposure of larvae and plastic sheets in GA solution for 1 h (Fig. 1a–d), where higher responses occurred at a concentration of 10^{-3} mM ($P < 0.05$). At 24 h, more larvae settled on plastic sheets than at 6 h. An increase in the settlement percentage was also observed using

10^{-1} mM GA at 72 h ($P < 0.05$). Approximately 38–48% of the larvae were induced to settle on rough and smooth sheets when both larvae and plastic sheets were continuously exposed to 10^{-3} mM GA ($P < 0.05$).

Effects of MSG concentration with type of plastic sheet and different modes of exposure to MSG on larval settlement

Exposure to MSG resulted in settlements of over 12% for all treatment methods at 6 h. However, the settlement percentage at 24 h had increased to 20% (Fig. 2) and increased to 30% after 72 h (Fig. 3). Concentrations of 15 and 25 mM MSG significantly induced larvae to settle (30%) on both types of plastic sheets by 6 h with continuous exposure, and 33% at 6 h when exposing either larvae or the plastic sheet only for 1 h in MSG solution ($P < 0.05$; Fig. 1). The numbers settled remained about 30% for the same treatment at 24 h ($P < 0.05$; Fig. 2) and increased to 37–44% with continuous exposure to MSG for 1 h at a 5 mM concentration after 72 h ($P < 0.05$; Fig. 3a, b, e and f). Settlement increased up to 50% after larvae were immersed in 25 mM for 1 h, and then allowed to settle in seawater for 72 h (Fig. 3c and d).

Discussion

Most pelagic marine invertebrate larvae must pass through a period of settlement, thereby becoming benthic and then growing into adults. During the lifespan, this settlement stage is the bottle neck to reproductive capacity due to high mortality (Roberts 2001). In abalone aquaculture, this can be an obstacle in obtaining high production.

Settlement behaviour in larvae of *H. asinina* has been recorded previously (Singhagraiwan & Doi 1993). They found that at 26 h post fertilization competent larvae of *H. asinina* begin to swim downward and towards the substratum, periodically attaching their foot to the substratum, before settling and crawling.

In the present study, we have shown that competent larvae (26 h post fertilization) of *H. asinina* could be induced to settle, when exposed to 5-AVA, GABA, MSG or GA. Our data confirm earlier reports of the ability of the classic settlement inducer, GABA, to be effective in *H. asinina* larval settlement. γ-aminobutyric acid has previously been found to be effective in settlement in other abalone species, viz. *H. rufescens* (Morse Hooker, Duncan *et al.* 1979; Searcy-Bernal *et al.* 1992), *H. discus hannai* (Yang & Wu 1995), *H. iris* and *H. virginea* (Roberts & Nicholson 1997), *H. diversicolor* (Bryan & Qian 1998), *H. asinina* (Gapasin & Polohan 2004), as well as other marine species such as the mussel *Mytilus edulis* (Dobretsov & Qian 2003), *M. galloprovincialis*, the clam, *Venerupis pullastra*, the oyster, *Ostrea edulis* (Gacía-Lavandeira, Silva, Abad, Pazos, Sánchez & Parallé 2005), and the black chiton, *Katharina tunicata* (Rumrill & Cameron 1983). In our study, we found that GABA induced the best settlement of larvae at a concentration of 10^{-3} mM, which agrees with the report on *H. rufescens* (Morse 1992). It has also been shown that GABA can be ineffective at very low concentrations, while high doses can cause abnormal development (Searcy-Bernal & Anguiano-Beltran 1998), toxicity (as our results have indicated) or death of postlarvae (Morse, Hooker, Jensen & Duncan 1979). Akashige, Seki, Kan-no and Nomura (1981) reported that when *H. discus hannai* larvae were overexposed to GABA, velum ciliation movement was suspended, and after a temporary falling off, rotation occurred and they proceeded to die. We found that GABA at a temporary high concentration, such as 10^{-1} mM, was effective for inducing settlement, but not so if the larvae were exposed constantly to GABA. In contrast, when larvae were immersed in 10^{-3} mM (a lower dose) GABA or continuously exposed over 72 h, settlement numbers were good.

Previously reported differences in the efficacy of different chemicals in inducing settlement indicated that settlement may be due to a number of factors, including species differences, age and the differences in the methodologies used (Searcy-Bernal *et al.* 1992; Gapasin & Polohan 2004). This also extends to qualitative genetic differences as well, among batches of cultures of the same species, resulting in varying degrees of larval competence and responsiveness to chemical inducers. For example, in a previous study, 0.45–0.50 μM GABA elicited the best settlement (38–52%) and metamorphosis (42–55%) response in *H. asinina* after 72 h post commencement (Gapasin & Polohan 2004). However, we found that at 72 h, 10^{-3} mM GABA induced larvae to settle at 58%, and up to 60% at 10^{-1} mM in all the experimental methods used.

Neurotransmitter-related amino acid-derived compounds have proved effective for the reliable induction of settlement in a number of other economically valuable molluscan species. However, levels of settlement induction by GABA were significantly

