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STIMULATORY EFFECTS OF EGG-LAYING HORMONE AND GONADOTROPIN-RELEASING HORMONE ON REPRODUCTION OF THE TROPICAL ABALONE, *HALIOTIS ASININA* LINNAEUS

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ABSTRACT Egg-laying hormone (ELH) is a neuropeptide hormone that stimulates ovulation of gastropods, including *Aplysia californica* and *Lymnaea stagnalis*. Other neuropeptides, gonadotropin releasing hormones (GnRHs), also play important roles in controlling reproduction in both vertebrates and invertebrates. In the current study, the effects of abalone ELH (aELH) and several GnRHs on somatic growth, sex differentiation, gonad maturation, and spawning of *Haliotis asinina* were investigated in 3 experiments. In experiment 1, groups of 4-mo-old juveniles (11.8 ± 0.03 mm shell length (SL) and 0.33 ± 0.04 g body weight (BW)) were injected with aELH and GnRHs, including buserelin (mammalian GnRH analogue), octopus GnRH (octGnRH), and tunicate GnRH-I (tGnRH-I), at doses of 20 ng/g BW and 200 ng/g BW. The aELH induced early sex differentiation with a bias toward females, but with normal somatic growth, whereas the different isoforms of GnRH had no effect on sexual differentiation or somatic growth. In experiment 2, groups of 1-y-old-abalone (SL, 4.04 ± 0.02 cm; BW, 20.15 ± 0.25 g) were injected with aELH and the 3 isoforms of GnRH including buserelin, octGnRH, and lamprey GnRH (lGnRH-I) at doses of 500 ng/g BW and 1,000 ng/g BW, and all produced stimulatory effects. For each peptide treatment, the gonads reached full maturation within 5–6 wk and spawning occurred, whereas control groups took 8 wk to reach maturity. In experiment 3, injections of ripe abalone with aELH stimulated spawning of both sexes in a dose-dependent manner. Buserelin had a lesser effect on inducing spawning, and octGnRH had no apparent effect. The gametes released from induced spawnings by aELH and GnRH showed normal fertilization and development of larvae. Altogether, these findings provide further knowledge on manipulating abalone reproduction, which is important in improving abalone aquaculture.

KEY WORDS: abalone, egg laying hormone, gonadotropin releasing hormone, sexual differentiation, gonad maturation, spawning, *Haliotis asinina*

INTRODUCTION

Haliotis asinina Linnaeus, 1 of 3 abalone species in Thailand, is an economically important gastropod used widely in commercial aquaculture because of its high proportion of flesh, and rapid growth rate (Singhagraiwan & Doi 1993). There have been several studies on the reproductive biology of *H. asinina*, including the processes of gamete proliferation and maturation in males and females, and analyses of the reproduction cycle, involving spawning of mature adults, fertilization, larval development and metamorphosis, and juvenile development into adults (Jarayabhand & Paphavasit 1996, Apisawetakan et al. 1997, Capinpin et al. 1998, Counihan et al. 2001, Litaay & Silva 2003). Recent studies have determined the optimum ratio of sperm and eggs for artificial fertilization, egg changes occurring after fertilization, and early larval developmental (Suphamungmee et al. 2010a, Suphamungmee et al. 2010b). To enhance abalone aquaculture production, early research focused on the production of artificial diets for increasing growth rate and body mass of juveniles (Capinpin & Corre 1996, Fleming et al. 1996, Bautista-Teruel et al. 2003), and the use of hydrogen peroxide and ultraviolet irradiation to induce spawning (Morse et al. 1976, Morse et al. 1979, Moss et al. 1995). However, these techniques can be disadvantageous in giving asynchronous spawning. Our knowledge of the neuropeptides and their control of reproductive functions in abalone species is limited; with a better understanding of abalone neuropeptides, the problem of asynchronous spawning of males and females might be overcome.

Several studies have reported 2 neuropeptides involved in controlling the reproductive process in gastropods species: an egg-laying hormone (ELH; Geraerts & Bohliken 1976, Scheller et al. 1982, Nagle et al. 1989)) and a gonadotropin releasing hormone (GnRH; Young et al. 1999, Zhang et al. 2000)). In *Aplysia*, ELH is synthesized and cleaved from a larger preprohormone sequence (Nambu & Scheller 1986), and a homologous hormone, caudodorsal cell hormone, in *Lymnaea* is also synthesized from a preprohormone (Geraerts et al. 1988). The final ELH peptide, containing 36 amino acids, can stimulate egg-laying and ovulation in *Aplysia* via electrical discharge triggering of neurons (Geraerts et al. 1988). Studies on the temperate abalone species, *Haliotis rubra*, identified a gene encoding of an ELH (aELH; (Wang & Hanna 1998)). In *H. rubra*, aELH immunoreactivity was found in neurosecretory cells of cerebral and pluropedal ganglia, statocysts, and trabeculae of female gonads (Cummins et al. 2001). Similar aELH immunoreactivity was identified in *H. asinina*, in which the neurosecretory cells of cerebral, pluropedal, and visceral ganglia, and granulated cells within the trabeculae and capsules of gonads, were positively stained (Saitongdee et al. 2005). However, the function of this peptide in abalone has not yet been investigated.

Another neuropeptide, GnRH, has also been found to be important in controlling reproduction in both vertebrate and

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invertebrate species (Fernald & White 1999, Gorbman & Sower 2003). Currently, GnRH has been characterized into 25 molecular isoforms, 14 distinct and 11 variant isoforms in vertebrates and invertebrates, respectively. Several studies have reported the presence of GnRH immunoreactivity in the central nervous system and gonadal tissues of gastropods (Tsai et al. 2003, Iwakoshi et al. 2004, Zhang et al. 2008) and bivalves (Pazos & Mathieu 1999). Furthermore, numerous studies have demonstrated the function of various GnRH isoforms involved in reproductive processes. For example, a synthetic mammalian GnRH causes egg laying in the snail *Helisoma trivolvis* (Young et al. 1997), and lamprey GnRH-I (lGnRH-I) and tunicate GnRH-I (tGnRH-I) stimulate spawning and gamete release in the chitons, *Mopalia* sp. (Gorbman et al. 2003) and *Cion intestinalis* (Terakado 2001). In 2 recent reports, a marked shortening of ovarian maturation cycles in the penaeid shrimp *Penaeus monodon* resulted through treatments with luteinizing hormone releasing hormone, salmon GnRH, lGnRH-I, and aELH (Ngernsoungnern et al. 2008, Ngernsoungnern et al. 2009). Therefore, we investigated the effects of aELH and GnRHs on somatic growth and sex differentiation of juvenile *H. asinina*, as well as their effects on gonad maturation and spawning of adults.

MATERIALS AND METHODS

Preparation of aELH and GnRHs

Recombinant aELH peptide was generated and purified as described previously (Wang & Hanna 1998). Four isoforms of GnRH—namely, buserelin, lGnRH-I, octopus (octGnRH) and tGnRH-I—were used in experiments. Buserelin (Suprefact), a mammalian GnRH analogue, was purchased from Aventis Pharma Ltd. (Bangkok, Thailand), and the octGnRH and tGnRH-I peptides were purchased from GenScript Corporation (Piscataway, NJ). The lGnRH-I was kindly provided by Dr. Stacia A. Sower (University of New Hampshire, Durham, NH). All hormone preparations were made up in normal saline solution (NS).

Experiment 1: Effects of Hormones on Somatic Growth and Sex Differentiation of Juvenile Abalone

Four-month-old juvenile abalone ($n = 1{,}500$), of shell length (SL) 11.8 ± 0.03 mm ($\bar{X}$ ± SEM) and body weight (BW) 0.33 ± 0.04 g, were obtained from a land-based culture system located at the Coastal Fisheries Research and Development Center, Department of Fisheries, Prachaubkhirikhan, Thailand. They were maintained in cement tanks with plastic shelters, running seawater, and a natural photoperiod, and were fed with seaweed *ad libitum*.

Animals were randomly divided into 10 groups with 150 in each. Groups 1 and 2 (controls) consisted of 1 noninjected group and the other injected with 20 µL NS. Groups 3 and 4 were injected with 20 µL aELH at concentrations of 20 ng/g BW and 200 ng/g BW, respectively. Groups 5–7 were injected with 20 µL buserelin, octGnRH, and tGnRH-I at a dose of 20 ng/g BW; groups 8–10 received 200 ng/g BW. Each injected group received a total of 14 injections at 7-day intervals. At 14-day intervals, SL and BW ($n = 50$) were measured in each group. Daily growth rates in terms of SL and BW were also calculated according to Capinpin and Corre (1996). Ten of these abalone were sacrificed and immediately fixed in Bouin's solution for histological studies of gonad development and sex differentiation, using light microscopic observations of hematoxylin-eosin-stained sections. The experiment was performed in replicate.

Experiment 2: Effect of Hormones on Gonad Maturation in Adult Abalone

One-year-old adult *H. asinina* females were reared in concrete tanks (1.2 × 1.8 × 1 m) containing running seawater with 22.5–32.5 ppt salinity, at 22–26°C. Prior to the start of the experiment, 350 animals were tagged and measured (SL, 4.04 ± 0.02 cm; BW, 20.15 ± 0.25 g). Visual gonad index (VGI) was determined on each abalone, as defined by Grubert and Ritar (2004). The index consists of 4 visually assessed categories: 0, sex indistinguishable; 1, sex distinguishable, thin gonad with pointed tip; 2, gonad partially enlarged with pointed tip; and 3, gonad swollen with rounded tip. Animals with a VGI of 1 were used in the experiment, which was performed May through July.

Animals were randomly divided into 10 groups, each containing 35 animals. Groups 1 and 2 were control groups; one without injections and the other injected with NS. Groups 3 and 4 were injected with aELH at doses of 500 ng/g BW and 1,000 ng/g BW, respectively. Groups 5–7 were injected with 500 ng/g BW buserelin, lGnRH-I, and octGnRH; groups 8–10 were injected with 1,000 ng/g BW. Animals received a total of 7 injections at 7-day intervals; concomitantly, the VGI was observed in each animal. Furthermore, 4 abalone from each group were sacrificed and the gonads immediately fixed in Bouin's solution for histological examination to determine the developmental stage as defined by Sobhon et al. (1999), and to evaluate the gonad index (GI). GI indicates gonad development based on measurements of tissue slices, and is the percent of gonad area versus the total area of the conical apex, based on the equation of Fukazawa et al. (2007), with some modification:

$$I_G = 100 \times (L - L')/L$$

where I_G is GI, L is the diameter of the cross-section at 0.5 cm from the tip of the conical apex, and L′ is the diameter of the hepatopancreas in the same cross-section.

Experiment 3: Effects of Hormones on Spawning of Adult Abalone

Adult male and female *H. asinina* (SL, 4.25 ± 0.21 cm; BW, 21.09 ± 2.44 g), with a VGI of 3, were held separately in rectangular fiberglass tanks (500 L) until the experiment began. All animals were fed daily with *Gracilaria* spp. The experiment was performed 4 days before full moon and dark (new) moon periods to avoid natural spawning, which generally occurs twice a month. During the experimental period, male and female abalone were maintained in separate rectangular tanks (16 × 20 × 20 cm) containing 2 L ultraviolet filter-treated seawater with aeration.

Animals were randomly divided into 9 groups; each group contained 10 animals of each sex. Group 1 (control group) was injected with NS. Groups 2–4 were injected with aELH at doses of 250, 500, and 1,000 ng/g BW, respectively. Groups 5–7 were injected with buserelin at doses of 250, 500, and 1,000 ng/g BW, respectively. Groups 8 and 9 were injected with octGnRH at doses of 500 ng/g BW and 1,000 ng/g BW, respectively. Animals were injected at 9:00 PM and then observed from 12:00 midnight to 6:00 AM for spawning activity, including the duration of spawning and the number spawned. After spawning, the number

and quality of gametes were recorded before initiating fertilizations in which the ratio of sperm and egg was calculated according to Poomtong et al. (1997). The percent fertilization and viability of veliger larvae were also recorded.

Statistical Analyses

The data were analyzed using SigmaStat software (SPSS Science Inc., Cary, NC). The relevant data are presented as mean and SEM ($\bar{X} \pm$ SEM). Statistical significance was then evaluated by 1-way analysis of variance with Tukey-HSD multiple comparison tests. A difference was considered significant if $P < 0.05$.

RESULTS

Effects of aELH and GnRHs on Somatic Growth and Sex Differentiation of Juvenile Abalone

The effects of aELH and GnRHs on somatic growth of juvenile abalone, following weekly intramuscular injections for 14 wk, are given in Figure 1. Doses of 20 ng/g BW and 200 ng/g BW aELH had no apparent effect on SL or BW. Small fluctuations during the experimental period were not consistent. These fluctuations were more pronounced during weeks 4–10, in which 200 ng/g BW of octGnRH and tGnRH-I appeared to have had an adverse effect on BW growth. However, by week 12 all groups were similar to the controls. At 14 wk, the groups given doses of 20 ng/g BW of octGnRH and tGnRH-I were significantly larger in SL and BW ($P < 0.05$). However, the growth rates of the aELH and GnRHs groups during the 14-wk experiment were not significantly different to the control groups. However, the 20 ng/g BW tGnRH-I-treated juveniles showed significant increases in growth rates of SL (in millimeters per day) and BW (in milligrams per day) by the end of the experiment (Fig. 2). It was noted that the groups given octGnRH and tGnRH-I did not show the rapid sexual differentiation observed in the aELH- and buserelin-treated groups (see histology data presented next).

Histological analyses of sex differentiation of developing gonads during hormone treatments are summarized in Table 1. The juvenile gonads at the start of the experiments showed undifferentiated gonads (Fig. 3A, B). This was accompanied by a gonad cavity and connective tissue covering the hepatopancreas. At week 6, abalone in the aELH- and buserelin-injected groups showed rapid increases in the number (40–50%) of differentiated male and female germ cells (Fig. 3D, E). In contrast, the control groups and groups given octGnRH and tGnRH-I showed little differentiation (0–20%) and basically contained early germ cells (Fig. 3C, F). At week 10, the gonads of animals within the control groups, and the octGnRH- and tGnRH-I-treated groups, had begun to differentiate (20–40%) into male or female characteristics (Figs 3G–I), but most animals in the aELH- and buserelin-treated groups had completed sex differentiation (40–90%). However, by 14 wk most groups showed a high number of sexually differentiated animals (Fig. 3J–L). Notably, the sex ratio in the aELH-treated groups was significantly biased toward females (1 male:4 females), during the 14 wk of treatment and sampling. This bias in sex ratio was not observed in the control groups or the GnRH-treated groups, including buserelin.

Effects of aELH and GnRHs on Gonad Maturation of Adult Abalone

The results of the VGI and GI studies were similar, and are shown in Figure 4A and B, respectively. Initial values were VGIs of 1 and GIs of approximately 10, and sections of gonads within all groups showed a proliferative stage in maturation (Fig. 5A). Except for the 1,000-ng/g BW aELH group at weeks 3 and 4, the others groups showed increases over the 4 wk. The

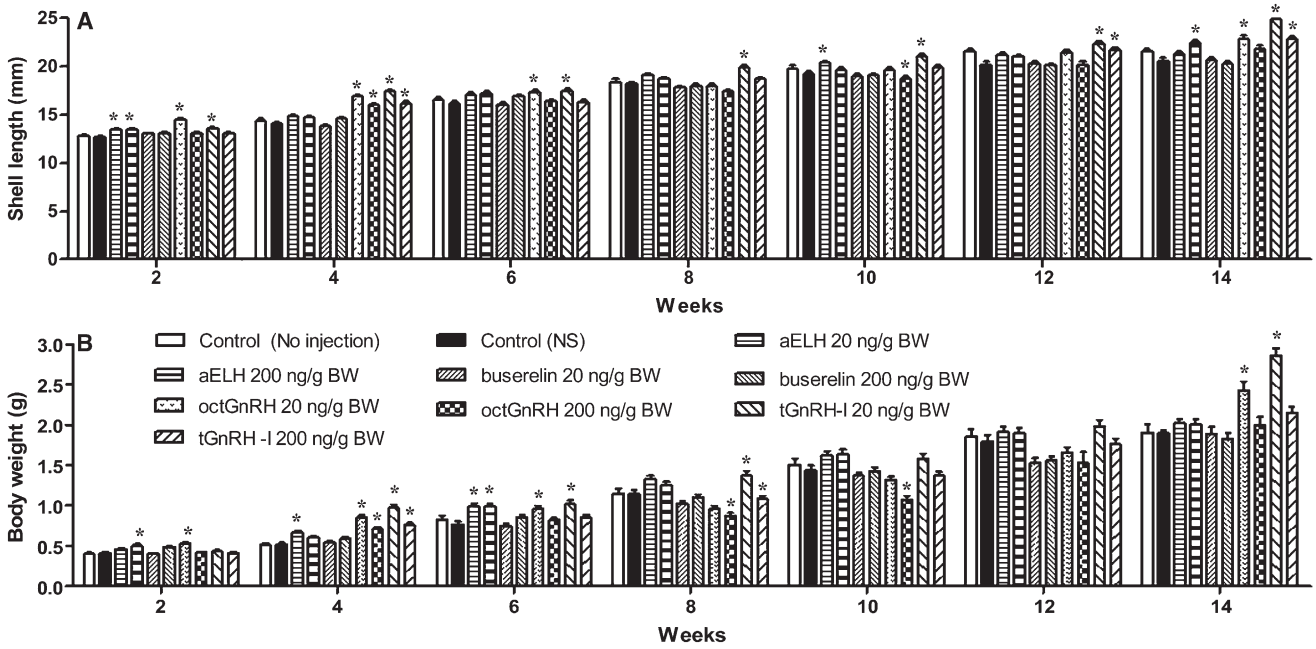

Figure 1. (A, B) Graphs showing shell length (A) and body weight (B) of juvenile abalone during 14 wk of treatments with aELH and GnRHs at 0 (control), 20, and 200 ng/g BW. Vertical bars indicate $\bar{X} \pm$ SEM; $n = 50$. Asterisks indicate significant differences ($P < 0.05$).

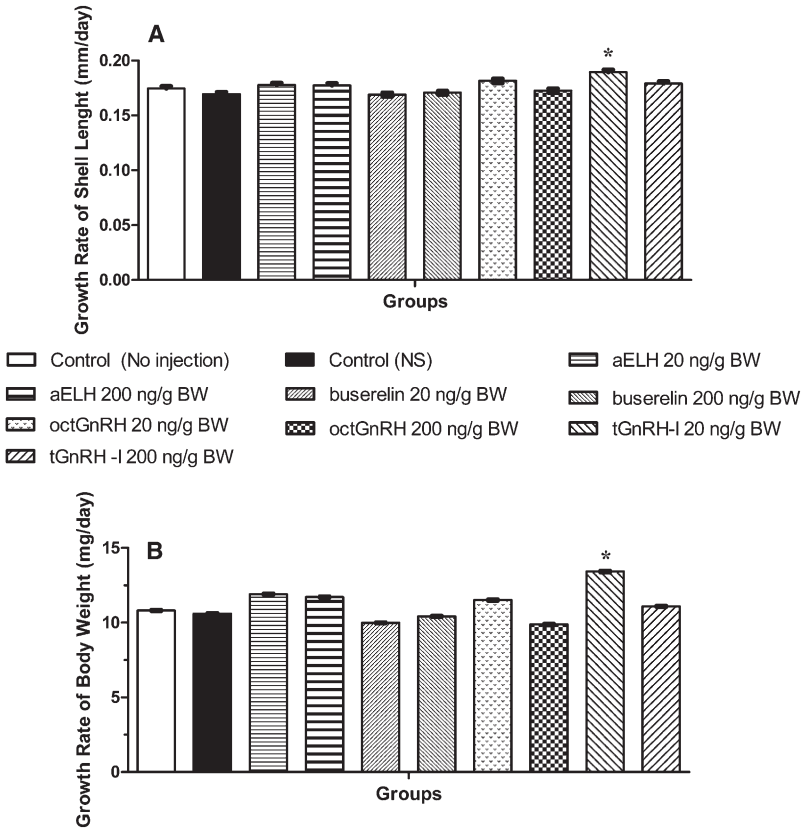

Figure 2. (A, B) Graphs showing the growth rate of shell length (A) and body weight (B) of juvenile abalone after 14 weekly treatments with aELH and GnRHs at 0 (control), 20, and 200 ng/g BW. Vertical bars indicate $\bar{X} \pm$ SEM. Asterisks indicate significant differences ($P < 0.05$).

controls continued this pattern through to 8 wk, and the stage of gonad maturation was similar. When the VGI and GI reached the highest values, the female gonads had become dark green, indicating a fully mature stage in readiness to spawn (Fig. 5B).

The time for gonad maturation was considerably shorter for the aELH- and GnRH-treated groups. Groups treated with 1,000 ng/g BW aELH showed partial spawning at weeks 3–5 (Fig. 5C), and spawning by week 6 (Fig. 4B), which was significantly different from controls ($P < 0.05$). The group given 500 ng/g BW and 1,000 ng/g BW octGnRH showed fully matured gonad stages with a partial spawning at week 5 (Fig. 4B), which was significantly different from controls ($P < 0.05$). Moreover, all other groups injected with aELH and GnRHs showed premature to mature stages of gonad development by

TABLE 1.

Effects of aELH and GnRHs on the development of juvenile abalone gonads ($n = 10$/group/week).

	6 Wk				10 Wk				14 Wk			
Groups (ng/g BW)	UD	M	F	% Sex Differentiation	UD	M	F	% Sex Differentiation	UD	M	F	% Sex Differentiation
Control*	10	0	0	0	8	1	1	20	4	4	2	60
Control†	8	1	1	20	6	2	2	40	2	6	2	80
aELH (20)	5	1	4	50	3	1	6	70	3	1	6	70
aELH (200)	6	1	3	40	1	2	7	90	1	2	7	90
Buserelin (20)	6	2	2	40	6	2	2	40	2	4	4	80
Buserelin (200)	5	3	2	50	3	4	3	70	3	2	5	70
octGnRH (20)	10	0	0	0	7	1	2	30	6	3	1	40
octGnRH (200)	10	0	0	0	8	1	1	20	4	2	4	60
tGnRH-I (20)	8	0	2	20	8	0	2	20	3	6	1	70
tGnRH-I (200)	10	0	0	0	7	0	3	30	5	2	3	50

* No injections. † NS injections

F, female; M, male; UD, undifferentiated sex.

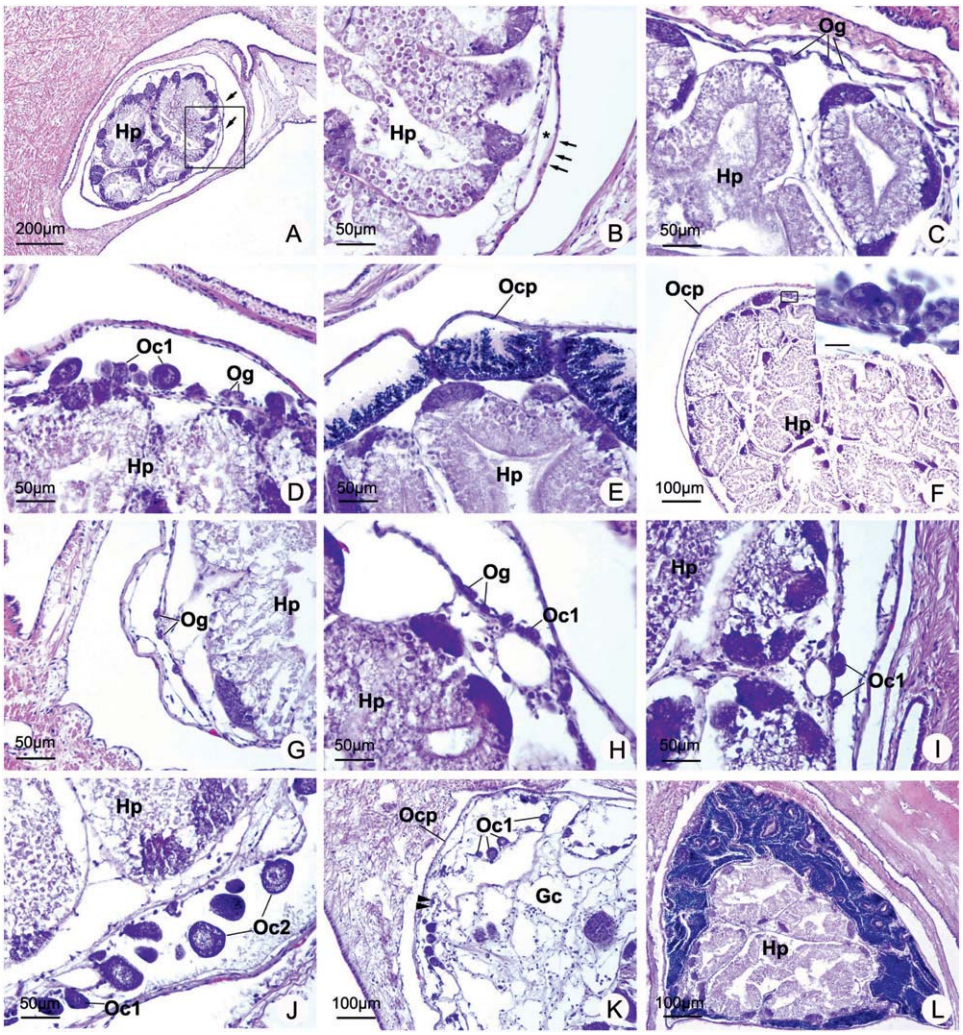

Figure 3. (A–L) Light micrographs showing the effects of hormones on juvenile abalone gonad development (hematoxylin-eosin staining). (A, B) Low (A) and medium (B) power of an undifferentiated gonad of a control juvenile at the start of the experiment showing the formation of the gonad cavity (asterisk) and a thin layer of connective tissue (arrows) covering the hepatopancreas (Hp). (C) Medium power of a gonad at 6 wk of the control group (normal saline solution injection) showing the appearance of oogonia (Og). (D) Medium power of a gonad at 6 wk after weekly injections with 200 ng/g BW aELH showing female characteristics with the appearance of Og and stage I oocytes (Oc1). (E) Medium power of a gonad with an outer capsule (Ocp) at 6 wk after weekly injections with 20 ng/g BW buserelin showing male characteristics with numerous germ cells (white asterisk). (F) A gonad at 6 wk after weekly injections with 20 ng/g BW tGnRH-I showing only undifferentiated early germ cells (inset, bar = 10 μm). (G) Medium power of a control group (no treatment) gonad at 10 wk showing female characteristics with the appearance of Og. (H, I) Female gonad at 10 wk after weekly injections with 20 ng/g BW octGnRH and tGnRH-I, respectively, showing increased numbers of Og and Oc1. (J) Female gonad at 14 wk of a vehicle control group showing an appearance of Oc1 and stage II oocytes (Oc2). (K) Female gonad at 14 wk after weekly injections with 20 ng/g BW aELH showing a large gonad cavity (Gc) and increased numbers of Og (arrowheads) and Oc1. (L) Male gonad at 14 wk after weekly injections of 200 ng/g BW buserelin showing a large gonad size and numerous male germ cells at various stages of development.

week 5 (Figs. 4 and 5D, E). Notably, most hormone-treated groups had spawned by week 6, with the exception of the 500-ng/g BW buserelin-treated groups, which required a further week to spawn (Fig. 4B), which was significantly different from controls ($P < 0.05$). After spawning, the values of VGI and GI decreased markedly and the gonads exhibited a typical spent stage (Fig. 5F).

Effects of aELH and GnRHs on Spawning of Adult Abalone

After injections with aELH or GnRHs, some male and female abalone (VGI, 3) spawned within 6–8 h, which was 2–3 days prior to natural spawning. The 250-ng/g BW aELH dose stimulated 20% of males and 30% of females to spawn. However, increasing the peptide concentration to 500 ng/g BW and 1,000 ng/g BW induced more abalone to spawn, with 70% males and 80% females spawning at the higher dose (Table 2). In the GnRH-treated groups, buserelin at 250 ng/g BW did not induce abalone spawning (data not shown), but with 500 ng/g BW buserelin, 60% of males and 50% of females spawned, but the number spawning was less with 1,000 ng/g BW. Only 2 females injected with octGnRH at 1,000 ng/g BW spawned, and there was the same percentage of abalone spawning as in the control group.

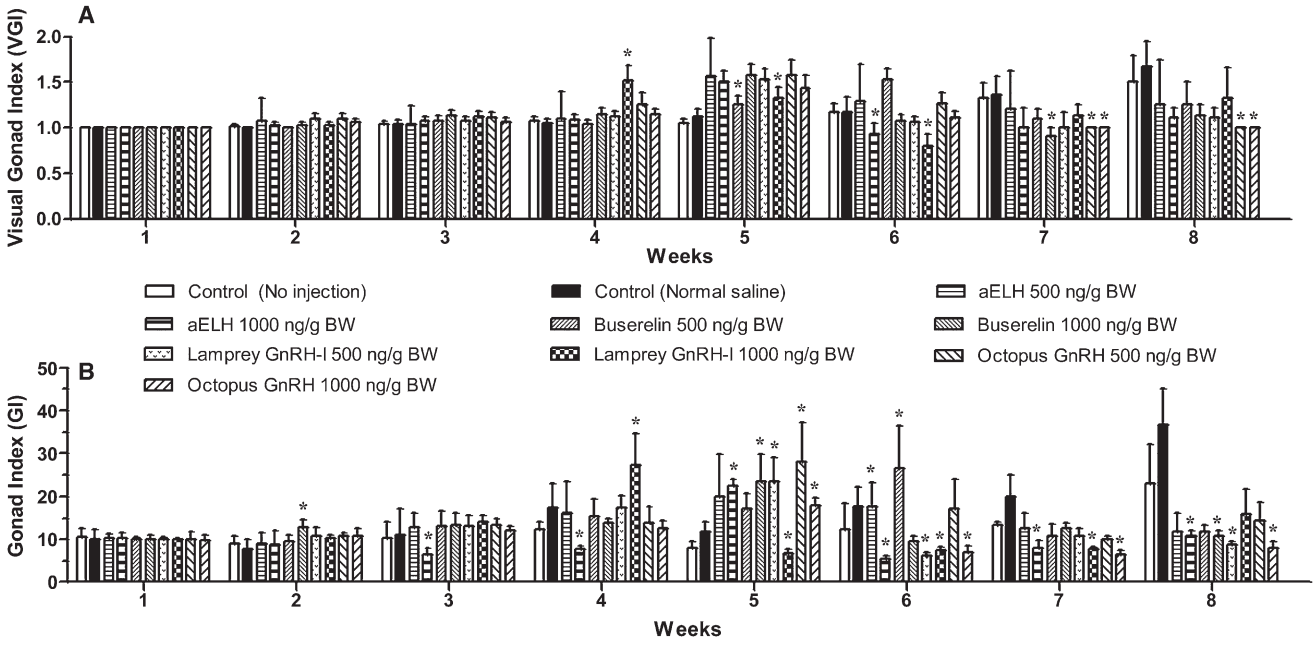

Figure 4. (A, B) Graphs showing visual gonad index (A) and gonad index (B) at each week after treatment with aELH and GnRHs. Vertical bars indicate $\bar{X} \pm$ SEM, $n = 4$. Asterisks indicate significant differences ($P < 0.05$).

Overall, the quantity and quality of sperm and eggs were not different in each group. Sperm released from all groups showed fast, continuous movement and the eggs were typically round and had jelly coats. The gametes released from the 500 ng/g and 1,000 ng/g BW aELH- and buserelin-treated groups showed a higher fertilizing ability than the control group, but this was not significantly different ($P > 0.05$). After fertilization, all groups showed normal development until becoming veliger larvae after 17 h (data not shown).

DISCUSSION

First, we have shown that there were no apparent effects of aELH and GnRHs injections on somatic growth parameters (SL and BW), except for small but significant increases in both SL and BW after 14 wk of injections with 20 ng/g BW octGnRH and tGnRH-I (Fig. 1). This needs further investigation. The growth rates of juvenile abalone in the controls, and all aELH- and GnRH-treated groups, were not different from normal abalone (data not shown), and were approximately 0.18 mm/day for SL and 11.10 mg/day for BW (Fig. 2). The *H. asinina* SL growth rate obtained in our study is greater than that of a Philippine study that reported a growth rate of 0.13 mm/day (Fermin & Buen 2002), and was considerably more than a study using artificial diets for 90 days that obtained a growth rate of 0.10 mm/day (Kruatrachue et al. 2004). Several studies have indicated that the growth rate of abalone depends on type of food, temperature, and density. For example, juvenile *H. asinina* fed the red alga *Gracilariopsis heteroclada* and an artificial diet grew faster than those fed the red alga *Kappaphycus alvarezii* (Capinpin & Corre 1996). In addition, growth rates of juvenile *H. asinina* decrease when stocking densities increase (Capinpin et al. 1999).

Sexual differentiation in juvenile abalone was approximately 6 wk shorter in aELH-treated juveniles and those treated with 200 ng/g BW buserelin than the controls and juveniles treated with the other GnRHs. In addition well, aELH induced sexual differentiation with a strong bias toward females (1 male:~4 females). The various isoforms of GnRH showed no bias toward differentiation of gonads with either male or female characteristics. It has been reported that neurotransmitter and neuroendrocrine hormones influence sexual differentiation, even under the normal genetic control of sex determination (Wilson & Davies 2007). For example, estrogen or androgen treatment can produce all female or male fish stocks (Piferrer & Lim 1997), estrogen treatments during sexual differentiation cause the formation of ovotestis in male quail embryos (Halldin et al. 2005), and phytoestrogen treatments of Sharptooth catfish juveniles increases the percentage of females to nearly 70% (Yılmaz et al. 2009). In regard to ELHs, De Lange et al. (1994) found the gene to be expressed during post-embryonic development of *Lymnaea stagnalis*. Likewise, *Aplysia californica* bag cell neurons produce a small amount of ELH during early-stage development (Strumwasser et al. 1969). These data suggest that a long-term exposure of ELH during the sex differentiation of developing juvenile abalone might upregulate ELH gene transcription and expression in neural ganglia. This could possibly accelerate and bias sex differentiation. However, further studies of abalone juvenile development are necessary to clarify this hypothesis. Nevertheless, our results indicate that aELH can be used for the induction of rapid sexual differentiation, and with a propensity of females.

We expected that there would be a stimulatory effect on gonad maturation when mature abalone, with gonads with a VGI of 1 and a low GI were given a 7-wk course of synthetic hormones at high doses (500–1,000 ng/g BW). Mollusk aELH, and GnRHs, were already known to stimulate ovarian maturation and spawning in a decapod crustacean (Ngernsoungnern et al. 2008, Ngernsoungnern et al. 2009). The VGI, GI, and

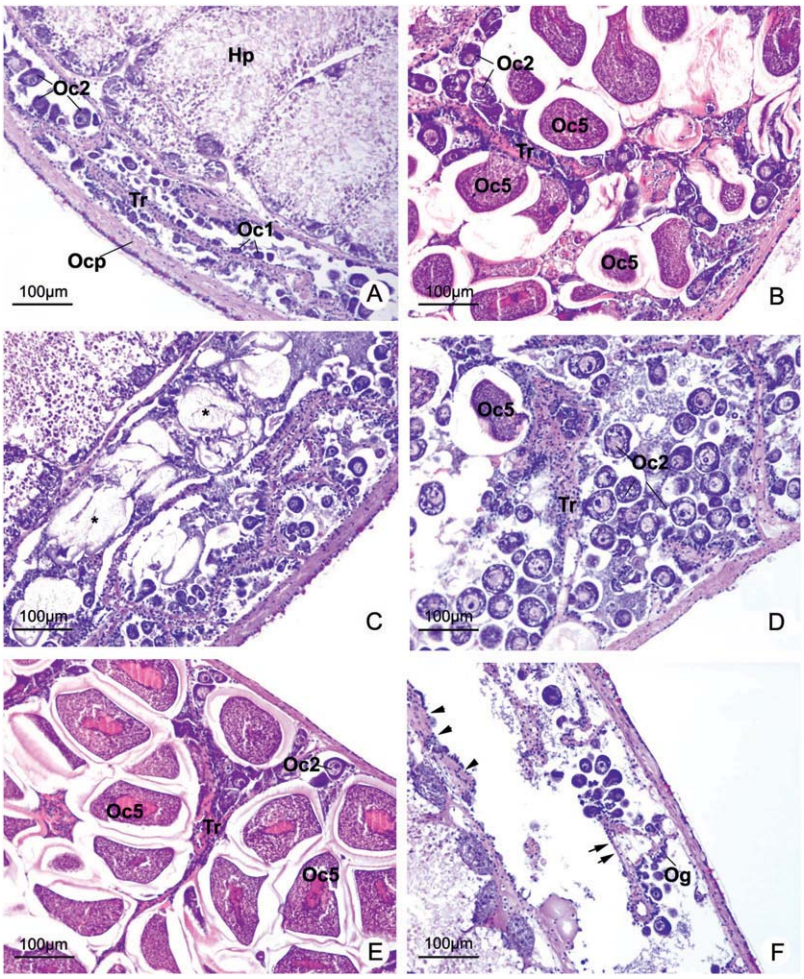

Figure 5. (A–F) Light micrographs of adult abalone gonads showing the effects of hormones on gonad maturation (hematoxylin-eosin staining). (A) First-week control group showing a proliferative stage with numerous stage I and stage II oocytes (Oc1, Oc2). (B) Control group show a fully mature stage of gonad at 8 wk, filled with abundant stage V oocytes (Oc5). (C) Gonad after 5 weekly injections of 1,000 ng/g BW aELH showing a partial spawning. Asterisks indicate the remnant of released oocytes. (D) Gonad after 5 weekly injections with 500 ng/g BW lGnRH-I showing a premature stage with abundant Oc2 containing lipid droplets and some Oc5. (E) Gonad after 5 weekly injections with 1,000 ng/g BW buserelin showing a fully mature stage, large size, and with abundant numbers of Oc5. (F) Gonad after 6 weekly injections with 1,000 ng/g BW buserelin showing a spent stage with disintegration of old trabeculae (Tr; arrows) and the renewal of trabeculae (arrowheads). Some clusters of early germ cells are still attached to the Tr. Hp, hepatopancreas; Icp, inner capsule; Ocp, outer capsule; Og, oogonia.

histological results did show gonad maturation within 4–6 wk, at which time spawnings occurred. These results were significantly different from controls ($P < 0.05$), and the gonads then commenced redevelopment. Controls with no stimulating hormones were still developing at 8 wk. The variation of hormone effects can be explained by differential affinity of each hormone with its receptor (Ngernsoungnern et al. 2008), but it should be noted that the hormone isoforms used in this study were also synthetic peptides, and were based on sequence data obtained from neural cells of other taxa. The characterizations of both ELH and GnRH in *H. asinina* are yet to be done and are part of our current research. In addition, when mature adults (VGI of 3) were injected with aELH and buserelin at high doses, both showed spawning and gave the normal fertilization of gametes. This is the first report showing that aELH and GnRHs had a stimulatory effect on spawning in *H. asinina*. Previous studies have reported ELH and GnRH involvement in regulating reproduction in other species. For example, injection of ovulation hormone (caudodorsal cell hormone) causes synchronous maturation of the female reproductive system and spawning in *L. stagnalis* (Dogterom et al. 1983a, Dogterom et al. 1983b), whereas GnRH stimulates ovarian maturation in the reeves shad (Wang et al. 1998), and the black tiger shrimp, *Penaeus monodon* (Ngernsoungnern et al. 2008). Our results imply that synchronous maturation and spawning may be caused by stimulatory effects of ELH on the nervous system, which acts directly on the gonad and stimulates spawning, and supports the report of ELH-induced spawning in *Aplysia* (Wayne et al. 2004), and *Lymnaea* (Geraerts and Bohlken 1976). Conversely, GnRHs tend to have a regulatory effect on the modulation of ovarian maturation or suppression activity of neural cells involved in spawning. What is now needed is molecular knowledge of the expression of 2 hormones in *H. asinina*, and studies to determine the relationship between ELH and GnRH in controlling reproduction. The current data on the existence of these hormones in a wide range of invertebrate taxa are

TABLE 2.

Effects of aELH and GnRHs on the spawning of mature male and female abalone.

Group (ng/g BW)	No. Spawning		Percent Spawning	No. Sperm/mL ($\times 10^6$) ($\bar{X}$ ± SEM)	No. Eggs ($\times 10^5$) ($\bar{X}$ ± SEM)	Percent Fertilization ($\bar{X}$ ± SEM)
	Male ($n = 10$)	Female ($n = 10$)				
Control	2	2	20	1.40 ± 0.02	4.70 ± 1.99	69.00 ± 9.03
aELH (250)	2	3	25	6.83 ± 5.57	1.48 ± 0.09	62.99 ± 12.83
aELH (500)	6	6	60	3.66 ± 0.86	5.24 ± 1.48	86.94 ± 5.10
aELH (1,000)	7	8	75	2.43 ± 0.71	9.43 ± 6.37	84.14 ± 7.14
Buserelin (500)	6	5	55	16.80 ± 9.70	3.42 ± 2.05	88.12 ± 3.19
Buserelin (1,000)	5	2	35	3.97 ± 3.47	3.19 ± 1.56	85.42 ± 1.51
octGnRH (1,000)	0	2	20	0	1.08 ± 0.33	—

incomplete, and are also questionable, so we are now doing a molecular survey of various phyla. However, in practical terms, our results have indicated that induction of gonad maturation and spawning with optimal doses of 500 ng/g BW aELH and buserelin may be a useful technique in the culture of abalone.

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LITERATURE CITED

Apisawetakan, S., A. Thongkukiatkul, C. Wanichanon, M. Kruatrachue, E. S. Upatham & P. Sobhon. 1997. The gametogenic processes in a tropical abalone, *Haliotis asinina* Linnaeus. *J. Sci. Soc. Thailand* 23: 225–240.

Bautista-Teruel, M. N., A. C. Fermin & S. S. Koshio. 2003. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: animal and plant protein sources. *Aquaculture* 219:645–653.

Capinpin, E. C. & K. G. Corre. 1996. Growth rate of the Philippine abalone, *Haliotis asinina,* fed an artificial diet and macroalgae. *Aquaculture* 144:81–89.

Capinpin, E. C., V. Encena & N. C. Bayona. 1998. Studies on the reproductive biology of the donkey's ear abalone, *Haliotis asinina* Linnaeus. *Aquaculture* 166:141–150.

Capinpin, E. C., J. D. Toledo, V. C. Encena, II & M. Doi. 1999. Density dependent growth of the tropical abalone *Haliotis asinina* in cage culture. *Aquaculture* 171:227–235.

Counihan, R. T., D. C. McNamara, D. C. Souter, E. J. Jebreen, N. P. Preston, C. R. Johnson & B. M. Degnan. 2001. Pattern, synchrony and predictability of spawning of the tropical abalone, *Haliotis asinina,* from Heron Reef, Australia. *Mar. Ecol. Prog. Ser.* 213:193–202.

Cummins, S., A. Thongkukiatkul & P. J. Hanna. 2001. Location of egg-laying hormone in reproductive structures and neurons of *Haliotis rubra* (Leach) using antibodies raised against recombinant fusion proteins. *J. Shellfish Res.* 20:705–710.

De Lange, R. P. J., J. V. Minnen & H. H. Boer. 1994. Expression and translation of the egg-laying neuropeptide hormone genes during post-embryonic development of the pond snail *Lymnaea stagnalis*. *Cell Tissue Res.* 275:369–375.

Dogterom, G. E., S. Bohlken & W. P. M. Geraerts. 1983a. A rapid *in vivo* bioassay of the ovulation hormone of *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.* 50:476–482.

Dogterom, G. E., H. V. Loenhout & W. P. M. Geraerts. 1983b. Synchronous maturation of the female reproductive system and of the ovulation hormone-producing system in *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.* 52:242–246.

Fermin, A. C. & S. M. Buen. 2002. Grow-out culture of tropical abalone, *Haliotis asinina* (Linnaeus), in suspended mesh cages with different shelter surface areas. *Aquacult. Int.* 9:499–508.

Fernald, R. D. & R. B. White. 1999. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. *Front. Neuroendocrinol.* 20:224–240.

Fleming, A. E., R. J. V. Barneveld & P. W. Hone. 1996. The development of artificial diets for abalone: a review and future directions. *Aquaculture* 140:5–53.

Fukazawa, H., T. Kawamura, H. Takami & Y. Watanabe. 2007. Oogenesis and relevant changes in egg quality of abalone *Haliotis discus hannai* during a single spawning season. *Aquaculture* 270:265–275.

Geraerts, W. P. M. & S. Bohlken. 1976. The control of ovulation in the hermaphrodite freshwater snail, *Lymnaea stagnalis*, by the neurohormone of the caudodorsal cells. *Gen. Comp. Endocrinol.* 28:350–357.

Geraerts, W. P. M., A. T. Maat & E. Vreugdenhil. 1988. The peptidergic neuroendocrine control of egg-laying behavior in *Aplysia* and *Lymnaea*. In: H. Laufer and R. G. H. Downer, editors. Endocrinology of selected invertebrate types. New York: Alan R. Liss. pp. 141–231.

Gorbman, A. & S. A. Sower. 2003. Evolution of the role of GnRH in animal (metazoan) biology. *Gen. Comp. Endocrinol.* 134:207–213.

Gorbman, A., A. Whiteley & S. Kavanaugh. 2003. Pheromonal stimulation of spawning release of gametes by gonadotropin releasing hormone in the chiton, *Mopalia* sp. *Gen. Comp. Endocrinol.* 131: 62–65.

Grubert, M. A. & A. J. Ritar. 2004. Temperature effects on the dynamics of gonad and oocyte development in captive wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. *Invertebr. Reprod. Dev.* 45:185–196.

Halldin, K., J. Axelsson & B. Brunstrom. 2005. Effects of endocrine modulators on sexual differentiation and reproductive function in male Japanese quail. *Brain Res. Bull.* 65:211–218.

Iwakoshi, E., K. Ukena, K. Takuwa-Kuroda, A. Kanda, K. Tsutsui & H. Minakata. 2004. Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (*Octopus vulgaris*) by in situ hybridization and immunohistochemistry. *J. Comp. Neurol.* 477: 310–323.

Jarayabhand, P. & N. Paphavasit. 1996. A review of the culture of tropical abalone with special reference to Thailand. *Aquaculture* 140: 159–168.

Kruatrachue, M., S. Sawatpeera, Y. Chitramvong, P. Sonchaeng, E. S. Upatham & S. Sangpradub. 2004. Comparative growth performance of early juvenile *Haliotis asinina* fed various artificial diets. *J. Shellfish Res.* 23:197–203.

Litaay, M. & S. S. Silva. 2003. Spawning season, fecundity and proximate composition of the gonads of wild-caught blacklip abalone (*Haliotis rubra*) from Port Fairy waters, south eastern Australia. *Aquat. Living Resour.* 16:353–361.

Morse, D. E., H. Duncan, N. Hooker & A. Morse. 1976. Hydrogen peroxide induces spawning in mollusks, with activation of prostaglandin endoperoxide synthetase. *Science* 196:298–300.

Morse, D. E., N. Hooker, H. Duncan & L. Jensen. 1979. γ-Aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. *Science* 204:407–410.

Moss, G. A., J. Illingworth & L. J. Tong. 1995. Comparing two simple methods to induce spawning in the New Zealand abalone (paua), *Haliotis iris*. *N. Z. J. Mar. Freshw. Res.* 29:329–333.

Nagle, G. T., S. D. Painter & J. E. Blankenship. 1989. The egg-laying hormone family: precursors, products and functions. *Biol. Bull.* 177: 210–217.

Nambu, J. R. & R. H. Scheller. 1986. Egg-laying hormone genes of *Aplysia*: evolution of the ELH gene family. *J. Neurosci.* 6:2026–2036.

Ngernsoungnern, A., P. Ngernsoungnern, W. Weerachatyanukul, J. Chavadej, P. Sobhon & P. Sretarugsa. 2008. The existence of gonadotropin-releasing hormone (GnRH) immunoreactivity in the ovary and the effect of GnRHs on the ovarian maturation in the black tiger shrimp *Penaeus monodon*. *Aquaculture* 279:197–203.

Ngernsoungnern, P., A. Ngernsoungnern, W. Weerachatyanukul, P. Meeratana, P. J. Hanna, P. Sobhon & P. Sretarugsa. 2009. Abalone egg-laying hormone induces rapid ovarian maturation and early spawning of the giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture* 296:143–149.

Pazos, A. J. & M. Mathieu. 1999. Effects of five natural gonadotropin-releasing hormones on cell suspensions of marine bivalve gonad: stimulation of gonial DNA synthesis. *Gen. Comp. Endocrinol.* 113: 112–120.

Piferrer, F. & L. C. Lim. 1997. Application of sex reversal technology in ornamental fish culture. *Aquar. Sci. Conserv.* 1:113–118.

Poomtong, T., S. Sahawatcharin & K. Sanguanngam. 1997. Induced spawning, seed production, and juvenile growth of the donkey's ear abalone *Haliotis asinina* Linnaeus. *Phuket Mar. Biol. Center Spec. Publ.* 17:229–235.

Saitongdee, P., S. Apisawetakan, N. Anunruang, T. Poomthong, P. J. Hanna & P. Sobhon. 2005. Egg-laying-hormone immunoreactivity in the neural ganglia and ovary of *Haliotis asinina* Linnaeus. *Invert. Neurosci.* 5:165–172.

Scheller, R. H., J. F. Jackson, L. B. McAllister, J. H. Schwartz, E. R. Kandel & R. Axel. 1982. A family of genes that codes for ELH, a neuropeptide eliciting a stereotyped pattern of behaviour in *Aplysia*. *Cell* 28:707–719.

Singhagraiwan, T. & M. Doi. 1993. Seed production and culture of a tropical abalone, *Haliotis asinina* Linne. Rayong, Thailand: The Eastern Marine Fisheries Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives. 33 pp.

Sobhon, P., S. Apisawetakan, M. Chanpoo, C. Wanichanon, V. Linthong, A. Thongkukiatkul, P. Jarayabhand, M. Kruatrachue, E. S. Upatham & T. Poomtong. 1999. Classification of germ cells, reproductive cycle and maturation of gonad in *Haliotis asinina* Linnaeus. *Sci. Asia* 25:3–21.

Strumwasser, F., J. W. Jacklet & R. B. Alvarez. 1969. A seasonal rhythm in the neural extract induction of behavioral egg-laying in *Aplysia*. *Comp. Biochem. Physiol.* 29:197–206.

Suphamungmee, W., P. Chansela, W. Weerachatyanukul, T. Poomtong, R. Vanichviriyakit & P. Sobhon. 2010a. Ultrastructure, composition and putative function of egg coats in *Haliotis asinina*. *J. Shellfish Res.* 29:687–697.

Suphamungmee, W., A. Engsusophon, R. Vanichviriyakit, P. Sretarugsa, J. Chavadej, T. Poomtong, V. Linthong & P. Sobhon. 2010b. Proportion of sperm and egg numbers for maximal *in vitro* fertilization in *Haliotis asinina* and the chronology of early development. *J. Shellfish Res.* 29:757–763.

Terakado, K. 2001. Induction of gamete release by gonadotropin releasing hormone in a protochordate, *Ciona intestinalis*. *Gen. Comp. Endocrinol.* 124:277–284.

Tsai, P. S., T. A. Maldonado & J. B. Lunden. 2003. Localization of gonadotropin-releasing hormone in the central nervous system and a peripheral chemosensory organ of *Aplysia californica*. *Gen. Comp. Endocrinol.* 130:20–28.

Wang, L. & P. J. Hanna. 1998. Isolation, cloning and expression of a DNA sequence encoding an egg-laying hormone of the blacklip abalone (*Haliotis rubra* Leach). *J. Shellfish Res.* 17:789–793.

Wang, H. P., K. J. Wei, H. Yao, J. J. Lin & J. B. Mai. 1998. Induced ovarian development, maturation and ovulation of domestic reeves shad by hormone implantation and injection. *Asian Fish. Sci.* 11: 43–50.

Wayne, N. L., W. Lee, S. Michel, J. Dyer & W. S. Sossin. 2004. Activity-dependent regulation of neurohormone synthesis and its impact on reproductive behavior in *Aplysia*. *Biol. Reprod.* 70:277–281.

Wilson, C. A. & D. C. Davies. 2007. The control of sexual differentiation of the reproductive system and brain. *Soc. Reprod. Fert.* 133: 331–359.

Yılmaz, E., S. Çek & Y. Mazlum. 2009. The effects of combined phytoestrogen administration on growth performance, sex differentiation and body composition of Sharptooth catfish *Clarias gariepinus* (Burchell, 1822). *Turk. J. Fish. Aquatic Sci.* 9:33–37.

Young, K. G., J. P. Chang & J. I. Goldberg. 1999. Gonadotropin-releasing hormone neuronal system of the freshwater snails *Helisoma trivolvis* and *Lymnaea stagnalis*: possible involvement in reproduction. *J. Comp. Neurol.* 404:427–437.

Young, K. G., R. Zalitach, J. P. Chang & J. I. Goldberg. 1997. Distribution and possible reproductive role of a gonadotropin-releasing hormone-like peptide in the pond snail, *Helisoma trivolvis*. *Soc. Neurosci.* 23:696.

Zhang, L., J. A. Tello, W. Zhang & S. Tsai. 2008. Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, *Aplysia californica*. *Gen. Comp. Endocrinol.* 156:201–209.

Zhang, L., N. L. Wayne, N. M. Sherwood, H. R. Postigo & P. S. Tsai. 2000. Biological and immunological characterization of multiple GnRH in an opisthobranch mollusk, *Aplysia californica*. *Gen. Comp. Endocrinol.* 188:77–89.