Aquaculture Reports 37 (2024) 102257

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Serotonin plus spiperone induce ovarian maturation, spawning and potential release of ovarian maturation inducing pheromones in the Pacific white shrimp *Litopenaeus vannamei* (Penaeidae)

Isabel Quesada-Ávila [a], Jorge Alfaro-Montoya [a,*], Rodolfo Umaña-Castro [b], André Braga [c], Marvin Ramírez-Alvarado [a], Tomer Ventura [d]

[a] *Universidad Nacional, Escuela de Ciencias Biológicas, Estación de Biología Marina, Puntarenas, Costa Rica*
[b] *Universidad Nacional, Escuela de Ciencias Biológicas, Laboratorio de Análisis Genómico (LAGen), Costa Rica*
[c] *Universidad Autónoma de Baja California, Instituto de Investigaciones Oceanológicas, Ensenada, Mexico*
[d] *University of the Sunshine Coast, Centre for BioInnovation, School of Science, Technology and Engineering, Queensland, Australia*

ARTICLE INFO

Keywords:
Litopenaeus vannamei
shrimp reproduction
serotonin
spiperone
ovarian maturation

ABSTRACT

In this study, we further investigate the hypothesis that serotonin plus spiperone (Ser+Spi) injection induces ovarian maturation and releases ovarian maturation inducing pheromones into the water, thereby stimulating neighboring untreated females. *Litopenaeus vannamei* females (b.w. = 40.40 ± 5.84 g; n = 120) and males (b.w. = 34.25 ± 4.68 g; n= 90) were divided into three 18-m^2 maturation tanks. Each tank contained 40 non-ablated females and 30 males with two treatments per tank. In tank 1, 20 females received a low dose of Ser+Spi (Ser at 25 μg g-1 b.w. and Spi at 2.0 μg g-1 b.w.) along with a control vehicle-injection. In tank 2, 20 females received a high dose of Ser+Spi (Ser at 40 μg g-1 b.w. and Spi at 4.0 μg g-1 b.w.) with the same control vehicle-injection applied to tank 1. In tank 3, unilateral eyestalk ablation (ESA) was performed on 20 females, while 20 control females were maintained non-ablated/injected. The experiment spanned 18 weeks and was divided into three phases. Ovarian maturation was observed in both Ser+Spi-treated and control females, with stages III-IV of maturation occurring one week after injections at both tested doses. Statistical analysis revealed that the average ovarian maturation index (OMI) was significantly higher for the control group in tank 2 (OMI = 0.25), compared to the Ser40+Spi4.0 group. Additionally, control females in tank 1 matured similarly to Ser25+Spi2.0 group ($p \leq 0.05$). The control group of tank 3 displayed very low maturation activity (OMI = 0.1). Controls from tanks 1 and 2 exhibited a higher number of mated females and spawning events. These results support the potential of Ser+Spi to induce ovarian maturation and spawning and suggest that Ser+Spi-treated females may release a metabolite into the water that acts as an ovarian maturation inducer for neighboring females.

1. Introduction

Crustacean reproduction is regulated by several organs including the brain, thoracic ganglia, mandibular organ, X organ - sinus gland complex, among others (Swetha et al., 2011; Prasad et al., 2014). Two key hormones involved in the gonad development of these invertebrates are the gonad inhibiting hormone (GIH) and gonad stimulating hormone (GSH). GIH is produced and secreted by the X organ-sinus gland neuroendocrine complex located in the eyestalk and plays a role in regulating the gonadal maturation of shrimp (Zapata et al., 2003; Chen et al., 2014; Diggles, 2019). While the identity of the GIH is well-established, the GSH remains speculative, though it is known to be produced by the thoracic ganglia and the supraesophageal ganglia (brain) (Cahansky et al., 2011; Prasad et al., 2014; Rotllant et al., 2018).

Unilateral eyestalk ablation (ESA) has been demonstrated to accelerate ovarian maturation processes in female shrimp, thus boosting larval production in controlled systems (Benzie, 1998; Okumura, 2007; Swetha et al., 2011; Uawisetwathana et al., 2011; Kannan et al., 2015; Uengwetwanit et al., 2018; Diggles, 2019). However, this practice has detrimental effects on the organism compromising the quantity and

* Corresponding author.
E-mail addresses: isa.8quesada@gmail.com (I. Quesada-Ávila), jalfarom05@mail.com (J. Alfaro-Montoya), rodolfo.umana.castro@una.cr (R. Umaña-Castro), andre.braga@uabc.edu.mx (A. Braga), marvin.ramirez.alvarado@una.cr (M. Ramírez-Alvarado), tventura@usc.edu.au (T. Ventura).

https://doi.org/10.1016/j.aqrep.2024.102257
Received 27 February 2024; Received in revised form 6 June 2024; Accepted 12 July 2024
Available online 19 July 2024

quality of spawns reducing the number of hatchings and negatively impacting other health parameters of shrimp (Swetha et al., 2011; Kannan et al., 2015). Bray et al. (1990) reported that in *Litopenaeus stylirostris* less than 25 % of ESA females were producing 70 % of the larvae. Ren et al. (2020) found that 20–30 % of *Litopenaeus vannamei* ablated females did not spawn after 30 days of culture. Selecting *L. vannamei* females with multiple spawning capacities has been proposed as an alternative to compensate for the low proportion of ablated females responsible of the nauplii production (Palacios et al., 2000).

The first evidence of serotonin (Ser) stimulatory effect on decapod crustaceans was reported by Sarojini et al. (1995) in the red swamp crayfish, *Procambarus clarkii*. Further research reported varying degrees of ovarian maturation induced by Ser in penaeid species including *L. vannamei*, *Penaeus monodon*, and *Fenneropenaeus indicus* (Vaca and Alfaro, 2000; Wongprasert et al., 2006; Santhoshi et al., 2009). Additionally, the dopamine antagonist, spiperone (Spi) has been shown to trigger in vitro ovarian growth in the crab, *Chasmagnathus granulate* (Zapata et al., 2003).

The combined use of serotonin and spiperone (Ser+Spi) was first reported by Alfaro et al. (2004) in two shrimp species, *L. stylirostris* and *L. vannamei*. This study demonstrated the high rates of ovarian maturation and spawning, suggesting that the release of maturation-inducing pheromones into the water contributed to the high maturation rates observed in control females. Complementarily, Tinikul et al. (2009) evaluated the effects of Ser+Spi, separately and in-combination of the giant freshwater prawn, *Macrobrachium rosenbergii*. The findings indicated that the two compounds acted synergistically, shortening the periods of ovarian maturation and embryonic development, likely by inhibiting the release of GIH and/or stimulating the release of GSH. However, Ryan (2018) evaluated the injection of Ser+Spi (25 μg and 5 μg g-1 b.w., respectively) in *P. monodon*, observing very low spawning activity after four injections.

Recently, Dineshan and Sudha Devi (2022) reported Ser accelerated ovarian maturation in the freshwater crab, *Travancoriana schirnerae*. Moreover, Harlıoğlu et al. (2020) emphasized the importance of conducting further research to evaluate the commercial benefits of this approach. In additional to Alfaro et al. (2004), the existence of ovarian maturation inducing pheromones in crustaceans has been proposed in other studies. Chandrakant-Sitaram (2001) suggested that a male pheromone is required for normal ovarian growth and maturation in *Macrobrachium idella*. Tropea et al. (2018) reported that adult males of *Neocaridina davidi* release cues that stimulate ovarian maturation, while adult females release cues that delay maturation.

In this study, we statistically evaluate the hypothesis proposed by Alfaro et al. (2004) that Ser+Spi injection induces direct ovarian maturation and the release of ovarian maturation-inducing pheromones into the water, thereby stimulating neighboring non-treated females. Additionally, the overall aim is to evaluate the effect of two doses of Ser+Spi on the direct and indirect induction of ovarian maturation and spawning in *L. vannamei*.

2. Materials and methods

2.1. Pond-grown brooders

Subadult *L. vannamei* (body weight = 24.9 ± 3.0 g) were selected before harvesting from the commercial semi-intensive farm ISLAMAR, located in Canjelito, Nandayure, Guanacaste, Costa Rica (10°01'44.3"N 85°13'36.5"O) in November 2021. Growth was continued at Estación de Biología Marina (EBM) in three external 20-m^3 tanks with natural photoperiod (13 h light: 11 h dark), seeded at a density of 13 shrimp m^{-2}. The tanks were covered with saran wrap and used a flow-through system (10 % daily exchange) with new marine water (26–28 °C; 26–35 ppt of salinity) pretreated by high-pressure silica sand filtration and sedimentation. Shrimp were fed daily at 3 % body weight (b.w.) in two rations of a dry commercial feed (BIOMAR, 35 % protein). After 7 months, females (b.w. = 40.40 ± 5.84 g) and males (b.w. = 34.25 ± 4.68 g) were selected based on general healthy appearance and transferred to the Controlled Reproduction Laboratory at EBM. This research was conducted under resolution CPI-SINAC-PNI-ACT-004–2021, which grants accesses to genetic and biochemical resources for the project titled "Neurotransmitters and pheromones of ovarian maturation and spawning in reproduction of the world-farmed shrimp, *Litopenaeus vannamei*" (PPAA 413–20, UNA- MICITT).

2.2. Experimental design

Three 18-m^2 maturation tanks accommodated two groups of females per tank: a treatment group and control vehicle group for tanks 1 and 2. Additionally, tank 3 housed an ESA group and a non-treated control group. Due to constraints in laboratory resources and animal availability, no repetitions of tanks were feasible in the experimental design. However, this design incorporates three control groups, adequate number of replicates of experimental females (n = 20) and facilitates a long-term study conducive to robust statistical comparisons.

Water depth was maintained at 0.50 m, with a total daily water replacement of 30 %, using new water treated by high pressure silica sand filtration and sedimentation. Salinity levels ranged between 26 and 35 ppt, while temperatures fluctuated between 26 and 28 °C. The photoperiod followed a natural cycle of 13 h light and 11 h dark, with reduced light intensity. *L. vannamei* were fed at 15 % b.w. day^{-1}, with a mixture of fresh frozen squids, bloodworms, and sardine at equal ratios, once daily. The experiment commenced following one week of shrimp acclimation in experimental tanks. Each maturation tank was seeded with 40 non-ablated females and 30 males, with two treatments per tank. Treatment was identified by marking females with different eyestalk color-bands; within treatments no individual identification was implemented. In tank 1, females were injected with Ser+Spi at a low dose (n = 20), while control females received vehicle-injections (n = 20). Tank 2 had females injected with Ser+Spi injected females at high dose (n = 20), as well as control vehicle-injected females (n = 20). Tank 3 contained unilateral ESA females (n = 20) and control non-ablated/injected females (n = 20). This experimental setup was designed to facilitate direct comparisons of maturation and spawning induction between the two doses of Ser+Spi and ESA females, as well as to investigate the hypothesis of maturation-inducing pheromones affecting control groups of females.

The doses administered were determined based on previous studies (Vaca and Alfaro, 2000; Alfaro et al., 2004). The high dose of Ser was set at 40 μg g^{-1} b.w. (4,0 $\times 10^{-6}$ moles $shrimp^{-1}$), administered in 0.01 ml injection, and Spi at 4.0 μg g^{-1} b.w. (4,0 $\times 10^{-7}$ moles $shrimp^{-1}$) in 0.05 ml injection. The low dose of Ser was set at 25 μg g^{-1} b.w. (2,5 $\times 10^{-6}$ moles $shrimp^{-1}$) and Spi at 2.0 μg g^{-1} b.w. (2,0 $\times 10^{-7}$ moles $shrimp^{-1}$). Ser (5-HT creatinine sulfate, Sigma, St. Louis, MO, USA) solution was prepared in a sterile saline solution (0.85 % NaCl), and Spi (Sigma) solution was prepared in 95 % ethanol. A water bath at 50 °C was used to aid dissolution without altering the chemical composition of the molecules. Control groups in Tanks 1 and 2 received 0.10 ml of sterile saline solution and 0.05 ml of ethanol, while the control group of Tank 3 remained untreated.

The experiment spanned 18 weeks, divided into three phases based on the reported spawning activity reductions after 6 weeks (Palacios et al., 1999). Phase 1 extended from week 0–8, during which injections were applied at the lateral section of the second abdominal segment every two weeks (0, 2, 4 and 6) and ablation was performed by cutting the right eyestalk with red-heated forceps at week 0. Phase 2 encompassed week 8–12, devoid of injections, while phase 3 from week 12–18, reintroducing injections every two weeks (Fig. 1).

2.3. Parameters

Maturation stages were assessed thrice weekly across all three tanks,

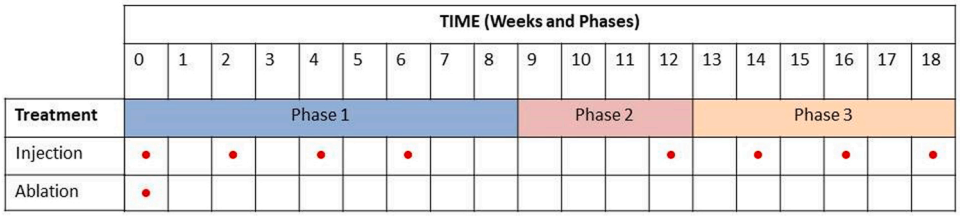

Fig. 1. Schematic representation of the three phases followed for serotonin plus spiperone injection and eyestalk ablation in *Litopenaeus vannamei* during an 18-week study. Red dots indicate weeks where treatments were applied.

employing the method previously outlined in Alfaro et al. (2004). The evaluation criteria were categorized into four stages.

Stage I. Ovaries appear transparent without a distinct outline.

Stage II. A thin opaque line along the dorsal central axis becomes visible.

Stage III. The ovary exhibits a thick and yellow band.

Stage IV. Ovaries are characterized by a turgid, broad, and dark red or orange hue.

Upon reaching full maturity, females were allowed to mate, and impregnated females were relocated to individual spawning tanks (200 l) in the evening. These tanks contained 100 l of 1-μm filtered and UV-irradiated treated seawater. The following morning, females were reintroduced to their respective tanks, and the quality of spawns was evaluated by sampling eggs and nauplii. For sampling, three homogenized 50 ml water samples were collected at the morning (eggs) and in the afternoon (nauplii), followed by stereo microscope counting.

At the end of the trial, sections of ovaries representing various stages of maturation were surgically removed excised from females under ice anesthetized ranging from 10 to 12 °C. The excised ovaries were preserved in Davidson's fixative for 24 h and stored in 50 % ethanol, according to Bell and Lightner (1988). Tissue samples were paraffin embedded, sectioned, and stained with hematoxylin-eosin by the Del Cruz Laboratories Company, San José, Costa Rica.

2.4. Statistical analysis

Weekly maturation status (n = 3 replicates) for each treatment was quantified using an ovarian maturation index (OMI), based on the following equation:

OMI = (females in stages III and IV) / (total number of females)

The means and standard deviations of the OMI were calculated, and a time series plot was generated illustrating the values across the 18 experimental weeks for the six treatments, for all three experimental phases. The normality of the OMI data was assessed, and due to rejection of the normality hypothesis, the non-parametric test Kruskal-Wallis was used to determine the significant differences of this variable between the treatment and control within each tank with a significance level of $\alpha = 0.05$. Subsequently, a post-hoc Wilcoxon pair test was performed to compare the values between all treatments and controls. The mean and standard deviation of the OMI were calculated for the three experimental phases.

Spawning quality parameters, including the average number of eggs per spawning, the average number of nauplii per spawning, and the average hatching percentage, were calculated along with their respective standard deviations. Data visualization and statistical analyses were performed using R programming language version 4.1.0.

3. Results

Upon injection of Ser+Spi an immediate alteration in swimming activity was observed, characterized by flexion of walking legs and agitation of pleopods lasting for a few minutes. Mortality rates at the end of phase 2 (12 weeks) are as follows: T1- control = 50 %, T1- Ser25+Spi2.0 = 45 %, T2 – control = 40 %, T2 – Ser40+Spi4.0 = 44 %, T3 – control = 40 %, and T3 – ESA = 50 %.

Ovarian maturation was successfully induced under the experimental conditions outlined in this study. Fig. 2 depicts the various stages of ovarian maturation, ranging from the immature stage I displaying previtellogenic oocytes (photos A-C), to early development stage II with oocytes in primary vitellogenesis (photos B-D), and stages III-IV (photos E-F) with oocytes in secondary vitellogenesis and pre-maturation with cortical crypts (photos G-H).

Under culture conditions using pond-tank grown females, ovarian maturation was induced in Ser+Spi injected females, control females, and ESA females (Fig. 2). Stages III-IV of maturation were observed one week post-injections in both tested doses. However, ESA females exhibited a delayed progression to stages III-IV. This trend suggests that Ser+Spi injections induced maturation in treated females and simulated the maturation process in control females at comparable rates. Initially, spontaneous maturation was observed in every tank. However, following the first week, a significant increase in reproductive activity was recorded, particularly in tank 2 with the high dose of Ser40+Spi4.0.

This heightened reproductive activity was pronounced during phase 1 of the experiment (Fig. 3), coinciding with the periodic injections. In phase 2, with no injections, a notable reduction in ovarian maturation was observed. In phase 3, following reintroduction of injection, maturation activity improved compared to phase 2, but remained inferior to that observed in phase 1.

Statistical analysis of the data spanning the experimental period (Fig. 4) revealed that the average OMI was highest for the control group of tank 2 (OMI = 0.25). Significant differences were observed between Ser40+Spi4.0 and the control, as well as between ESA and the control ($p \leq 0.05$). Furthermore, this analysis suggests that control females in tank 1 exhibited maturation rates similar to Ser25+Spi2.0 ($p \leq 0.05$). Notably, control females in tank 3 (ESA) displayed a very low maturation activity (OMI = 0.1), statistically lower to the control of Ser40+Spi4.0, as determined by the complementary analysis of the Wilcoxon post-hoc test (Table 1, $p \leq 0.05$).

Regarding mating activity, this reproductive process was predominantly measured during phase 1 (Fig. 5), as 67 % of mating activity occurred. Tanks 1 and 2 exhibited superior mating responses, as mating behavior and transfer of spermatophores were primarily associated with the control of tank 1 (26.5 % mating rate), and both control (23.5 %) and Ser40+Spi4.0 treated females (20.6 %) from tank 2. Mating activity in tank 3 (ESA) was comparatively low (ESA = 11.8 %; control = 8.8 %). Additionally, complete spawning was more frequent in tanks 1 and 2, particularly among control females (control tank 1 = 9 spawns; control tank 2 = 11 spawns; control tank 3 = 3 spawns; Fig. 6).

The quality of spawning is presented in Table 2. The data indicate that the average values for eggs and nauplii production are comparable across treatments, with the exception for the untreated control in tank 3. However, it is worth noting that these values are low for *L. vannamei*. Hatching rates were approximately 60 % and 40–50 % for tanks 1 and 2, respectively, while the ESA yielded a hatching rate of 46 % and its control group had a very low hatching rate of 6 %.

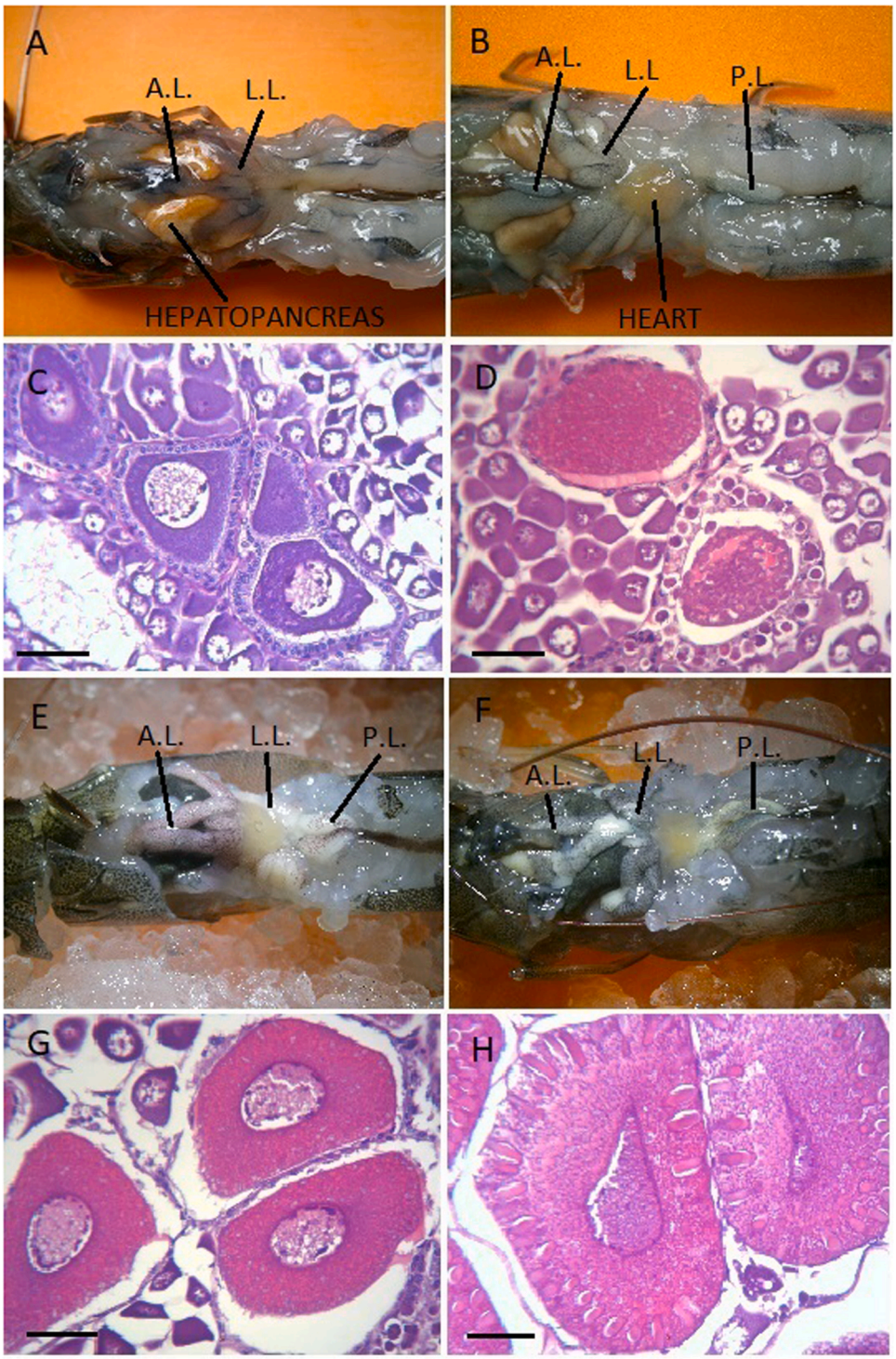

Fig. 2. Ovarian maturation stages of *Litopenaeus vannamei* at macroscopic and microscopic levels. A) Immature stage I, B) Early developmental stage II, C) Stage I, Pre-vitellogenesis, D) Stage II, Primary Vitellogenesis, E) Maturing state III, F) Advanced mature state IV, G) Stage III: Secondary Vitellogenesis, H) Stage IV: Pre-maturation. Scale bar = 50 μm. Ovary anatomy: A.L. = anterior lobe, L.L.= lateral lobe, P.L.= posterior or abdominal lobe.

4. Discussion

This contribution complements the findings of Alfaro et al. (2004) and Tinikul et al. (2009) regarding the reproductive impacts of Ser+Spi injections in decapod crustaceans. Methodologically, these studies followed a similar protocol, utilizing repetitive and separate injections of these molecules, with Ser being administered in 0.01 ml saline solution and Spi in 0.05 ml in 95 % ethanol. Tinikul et al. (2009) injected *M. rosenbergii* females every 4 days, while Alfaro et al. (2004) employed longer injection intervals of 2–3 weeks. In the present report, females were injected every 2 weeks. The altered posture observed post-injection is likely attributed to the role of Ser in activating a central motor program for postural flexion, a phenomenon documented in other crustaceans (Fingerman, et al., 1994).

The data presented demonstrate that ovarian maturation in *L. vannamei* is significantly induced by the combined Ser+Spi injection

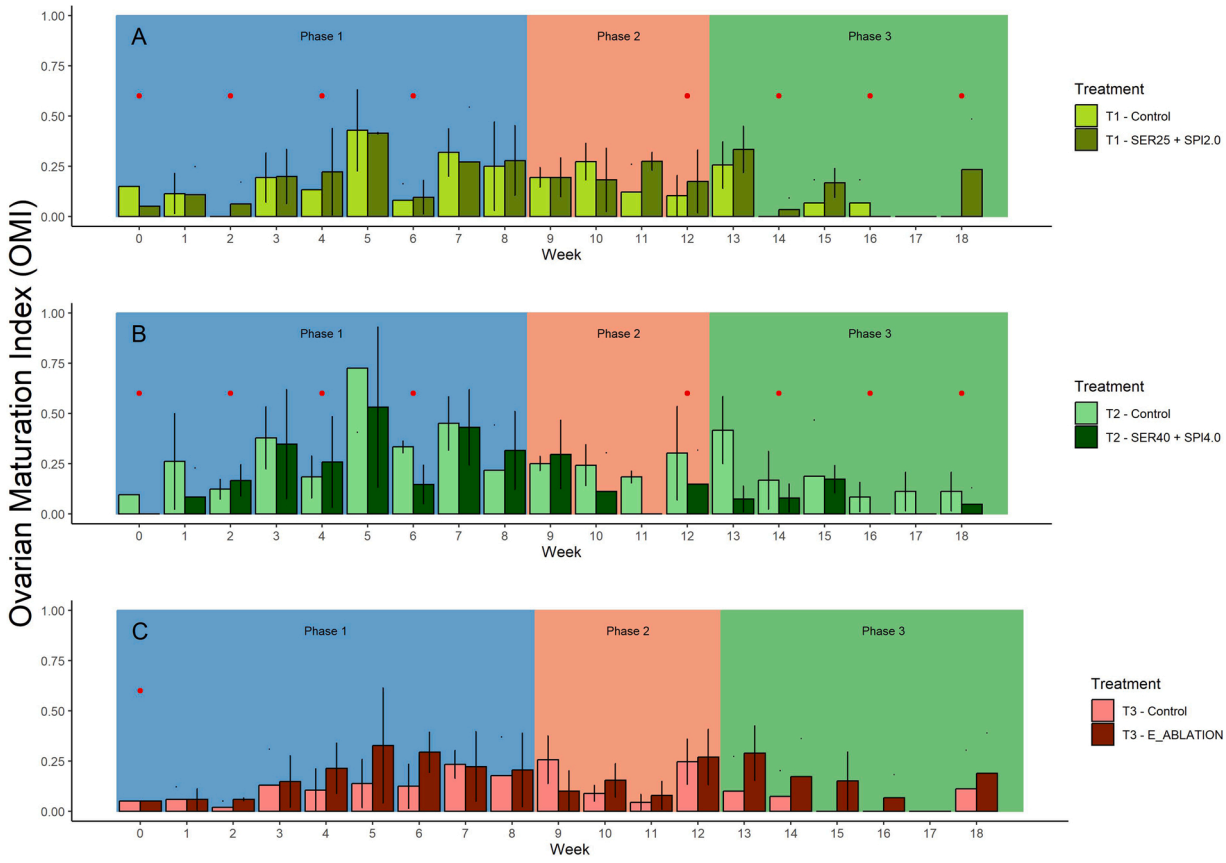

Fig. 3. Average weekly ovarian maturation rate in the three tanks during the 18 experimental weeks. The red dots indicate the application of the treatments (injection and eyestalk ablation for tank 3), and the black lines show the standard deviation.

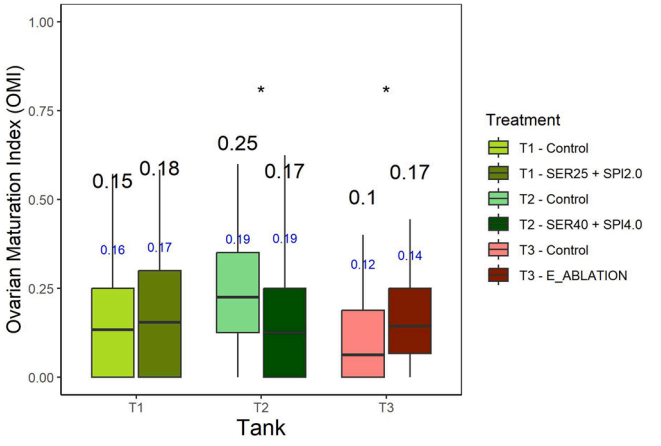

Fig. 4. Comparison of the average ovarian maturation index according to the tank and the treatment applied. The limits of each box indicate the upper and lower quartiles, horizontal bar is the median. Values in black indicate average values and values in blue show standard deviation. The asterisks indicate significant differences between the two treatments of each tank according to the Kruskal-Wallis test.

in a dose dependent manner. Oogenesis was activated in our experimental females, leading to the development of stages III-IV of maturation at a higher rate for Ser40+Spi4.0 and its control group (Tank 2); statistically superior for the control ($p \leq 0.05$). The weekly evaluation of OMI (Fig. 3) demonstrates that combined administration of Ser+Spi directly activates an endocrine cascade in females, leading to immediate oogenesis and fully reaching the mature stage IV. In *M. rosenbergii*,

Table 1
Wilcoxon post-hoc peer testing for the six experimental treatments distributed in three experimental tanks (T).

	T1		T2		T3
	Control	Treatment	Control	Treatment	Control
T1 - SER25 + ESP2.0	1.000	-	-	-	-
T2 - Control	0.024**	0.625	-	-	-
T2 - SER40 + ESP4.0	1.000	1.000	0.100	-	-
T3 - Control	1.000	0.143	5.8e-05**	0.794	-
T3 - E_ABLATION	1.000	1.000	0.288	1.000	0.089

**: significant differences ($p < 0.05$).
Self-comparisons are excluded.

Tinikul et al. (2009), using treatments based on a similar number of replicates (n =28) and comparing against three control groups, reported that Ser+Spi shortened the ovarian maturation period (~24 days) and embryonic development period (~16 days) even more than in Ser-treated, Spi-treated, and control groups.

The maturing females induced by Ser+Spi exerted a remarkable impact on non-treated females, as control individuals exhibited similar or even superior maturation rates compared to treated females. On the contrary, control non-treated females co-cultured with unilateral ESA females displayed a notably low rate of ovarian maturation. The spontaneous maturation of *L. vannamei* occurs at low rates, depending on culture conditions and genetics. Previous research demonstrates that unilateral ESA induction leads to higher spawning frequency compared to unablated pond-grown *L. stylirostris* and *L. vannamei* maintained in the same culture system (Chamberlain and Lawrence, 1981a,b). Moreover,

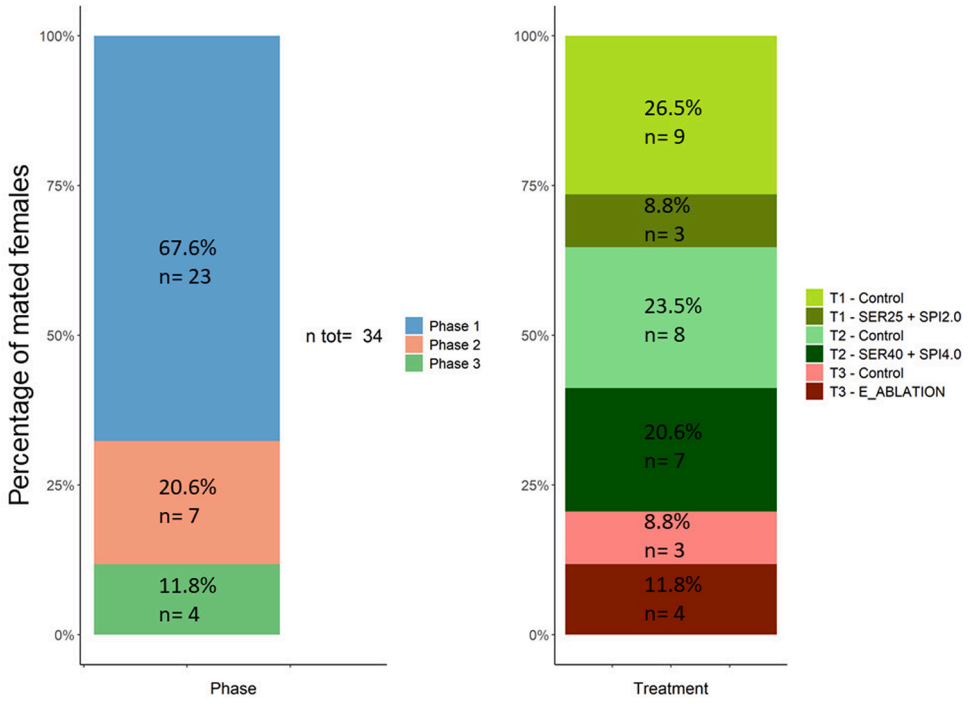

Fig. 5. Percentage and number of mated females according to the phase and treatment applied.

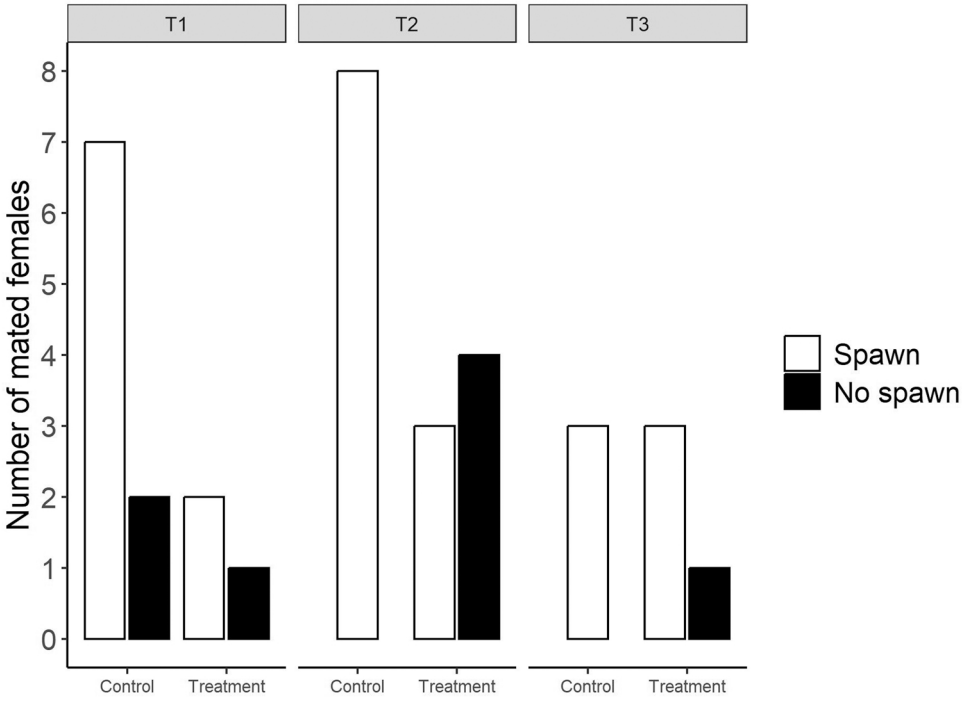

Fig. 6. Number of mated females that spawned and did not spawn based on tank (T) and treatment.

Vaca and Alfaro (2000) used wild *L. vannamei* in a commercial hatchery and employed a statistical block design with Ser injection, ESA, and control in the same tank, reporting only 2 spawns out of 36 females for the control group. However, recent studies have demonstrated that commercial hatcheries may rely on non-ablated lines of *L. vannamei,* yielding higher fecundity parameters than ablated females (Zacarias et al., 2019).

Alfaro et al. (2004) initially observed the positive effect of Ser+Spi injected females over non-treated females, proposing the hypothesis that metabolites released into the water act as ovarian-maturation inducers in neighboring females. This maturation inducing pattern was observed in two penaeid species, *L. vannamei* and *L. stylirostris.* In wild *L. stylirostris* (b.w. = 55 g), Alfaro et al. (2004) collected more spawns from vehicle injected control females than ESA and Ser+Spi females; hatching rates were similar among treatments (50–60 %). However, in young and pond-grown *L. vannamei* (b.w. = 37 g), maturation was induced but mating and spawning activity as well as hatching rates were low in treated and non-treated females. The present study supports this

Table 2
Spawning quality based on average number of eggs, nauplii and hatching percentage according to each treatment.

Tank	Treatment	No. mated females	Eggs/ spawn	Nauplii/ spawn	Hatching rate (%)
T1	SER25+SPI2.0	3	22055 ±25186	14666 ±25403	66.50±0.86
	Control	9	24370 ± 24917	14148 ± 20074	58.05 ±19.44
T2	SER40+SPI4.0	7	13333 ±29517	7238 ±16399	54.29 ±44.44
	Control	8	21500 ±19595	9250 ±10980	43.02 ±43.96
T3	E_ABLATION	4	21125 ±23889	9750 ±19168	46.15 ±19.76
	Control	3	5222 ±4141	333±577	6.38±86.06

Values are expressed as mean ± standard deviation. No statistically significant differences were observed ($p > 0.05$).

hypothesis with statistical validation based on a high number of replicates per treatment (n = 20 females), three control groups, and long-term study (18 weeks). In our experimental findings, a high rate of maturation was induced in non-treated females co-cultured in the same environment.

The observed reproduction phenomenon is a novel finding in penaeid shrimp. As previously mentioned, ESA has no discernible effect on non-ablated females, implying the activation of a different endocrine mechanism for the release of maturation inducing pheromone(s). Tinikul et al. (2009) did not observe this pheromone effect on control non-treated *M. rosenbergii* females, likely due culturing of control groups separately. The discovery and molecular characterization of a potential sex pheromone serving as chemical signals triggering ovarian maturation requires novel model species for bioassays, such as *L. vannamei* and *Marsupenaeus japonicus*. These species are suitable due to their commercially cultured status and the availability of their complete genome sequences (Kamio et al., 2022). Antennal glands from maturing and immature females treated with Ser+Spi were collected for a comparative transcriptomic and biochemical study, that is being executed and will be submitted separately.

Gutiérrez-Vera et al. (2024) demonstrated that female contact pheromones modulate courtship behavior of male *L. vannamei*. Significant correlations were observed between male courtship behavior and exposure to four different cuticular extracts from maturing and immature females. The behavior observed in males against artificial females provides evidence of the existence of sex pheromones located primarily in the ventral portion of the abdomen exoskeleton of shrimp females.

Our study displays elevated mating and spawning in tanks treated with serotonin plus spiperone when compared to ESA, as indicated in Figs. 5 and 6. Courtship behavior and spermatophore transfer to mature females, resulting in the spawning of fertilized eggs, was evident. It appears that the active reproductive status of females stimulated the courtship behavior in males. This complementary phenomenon was also observed by Alfaro et al. (2004) in wild *L. stylirostris*, but not in pond-grown *L. vannamei*. Therefore, it is suggested that Ser+Spi administered to females also enhances the reproductive capabilities of males, including courtship behavior and improved spermatophores quality. This new hypothesis is supported by the spawning activity registered in this contribution and preliminary data showing that 30.7 ± 4.2 g males cultured under our maturation protocol had spermatozoa counting of 26 375 000 ± 2 792 288 and abnormalities of 30 ± 5 % compared to wild 36 g male *L. vannamei* (spermatozoa counting = 12 million, abnormality = 38 %; Rendón et al., 2007). However, this hypothesis warrants further statistical validation.

For this study, pond-grown shrimp were obtained from a local commercial farm, cultured to young brooder size (females: 40 g and males: 34 g) to fulfill our research goals. However, it is worth noting that the size and quality of our brooders were suboptimal compared to those used in commercial facilities. This is evident from the low yields in eggs and nauplii, as well as reduced reproduction activity in phase 3 of the experiment. This may be attributed to the factors such as exhaustion and mortality, as documented in previous research (Palacios et al., 1999; 2000). The mortality rate in our experiment ranged between 40 % and 50 % across all treatments after 12 weeks of culture.

Currently, there are commercial hatchery operations utilizing genetically selected families of *L. vannamei* that exhibit spontaneous maturation and spawning (Corral-Rosales et al., 2018; Zacarias et al., 2019). However, despite these advancements, ESA continues to be widely applied worldwide (Ren et al., 2020). The reproduction of both non-genetically and genetically superior *L. vannamei* brooders holds potential for improved maturation and spawning responses than those observed in the present study. The transfer of this therapy to the shrimp industry, injecting a fraction of the female population, and evaluating the environmental possible adverse effects is a step that must be implemented soon. Furthermore, the identification of maturation-inducing pheromones remains a subject of significant basic and applied importance for the shrimp industry, requiring future research efforts.

5. Conclusion

Oogenesis was activated in experimental females generating stages III-IV of maturation at a higher rate for SER40+SPI4.0 and its control group (Tank 2); being statistically superior for the control ($p \leq 0.05$). The ovarian maturation index (OMI) and mating peaked during phase 1 of the experiment. Notably, among the control groups, tank 2 exhibited a statistically higher OMI, suggesting that Ser+Spi at high dose also induced maturation in control females. Tanks 1 and 2 displayed a higher number of mated females and spawning events among the control groups. These findings support the potential of Ser+Spi as inducer of ovarian maturation and spawning for commercial usage. Overall, the results support the hypothesis that Ser+Spi injected females likely attributes to the release of a metabolite into the water, acting as an ovarian-maturation inducer in neighboring females.

CRediT authorship contribution statement

Jorge Alfaro-Montoya: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Conceptualization. **Isabel Quesada-Ávila:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Tomer Ventura:** Writing – review & editing, Methodology, Conceptualization. **Marvin Ramírez-Alvarado:** Methodology, Investigation. **André Braga:** Writing – review & editing, Methodology, Conceptualization. **Rodolfo Umaña-Castro:** Writing – review & editing, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

This research is part of the post-graduate studies of Isabel Quesada-Ávila. We thank the Ministry of Science, Innovation, Technology and Telecommunications (MICITT, project FI 057B 19) from the government of Costa Rica and the Research Vice-presidency of UNA (project SIA

0413-20), for the financial support for this research. To CONAGEBIO (MINAE) and SINAC for given us the permission for executing this research under the legal frame of Costa Rica, based on resolution CPI-SINAC-PNI-ACT-004-2021. To unknown referees for their valuable comments and to Katherine R. Weber from University of Florida for English editing.

References

Alfaro, J., Zúñiga, G., Komen, J., 2004. Induction of ovarian maturation and spawning by combined treatment of serotonin and a dopamine antagonist, spiperone in *Litopenaeus stylirostris* and *Litopenaeus vannamei*. Aquaculture 236 (1–4), 511–522.

Bell, T.A., Lightner, D.V., 1988. A Handbook Of Normal Penaeid Shrimp Histology, 114. The World Aquaculture Society, Baton Rouge.

Benzie, J.A., 1998. Penaeid genetics and biotechnology. Aquaculture 164 (1-4), 23–47.

Bray, W.A., Lawrence, A.L., Lester, L.J., 1990. Reproduction of eyestalk-ablated *Penaeus stylirostris* fed various levels of total dietary lipid. J. World Aquac. Soc. 21 (1), 41–52.

Cahansky, A.V., Medesani, D.A., Chaulet, A., Rodríguez, E.M., 2011. *In vitro* effects of both dopaminergic and enkephalinergic antagonists on the ovarian growth of *Cherax quadricarinatus* (Decapoda, Parastacidae), at different periods of the reproductive cycle. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 158 (1), 126–131.

Chamberlain, G.W., Lawrence, A.L., 1981a. Effect of light intensity and male and female eyestalk ablation on reproduction of *Penaeus stylirostris* and *P. vannamei*. J. World Maric. Soc. 12 (2), 357–372.

Chamberlain, G.W., Lawrence, A.L., 1981b. Maturation, reproduction, and growth of *Penaeus vannamei* and *P. stylirostris* fed natural diets. J. World Maric. Soc. 12 (1), 209–224.

Chandrakant-Sitaram, P., 2001. Role of putative male pheromones in the ovarian maturation of the fresh water prawn *Macrobrachium idella* (Hilgendorf). [Master's Thesis, Department of Fishery Biology, College of Fisheries, Kerala Agricultural University, Panangad, India].

Chen, T., Zhang, L.P., Wong, N.K., Zhong, M., Ren, C.H., Hu, C.Q., 2014. Pacific white shrimp (*Litopenaeus vannamei*) vitellogenesis-inhibiting hormone (VIH) is predominantly expressed in the brain and negatively regulates hepatopancreatic vitellogenin (VTG) gene expression. Biol. Reprod. 90 (3), 47, 1-10.

Corral-Rosales, D.C., Palacios, E., Ricque-Marie, D., Cruz-Suárez, L.E., 2018. Enhancement of reproductive performance in shrimp *Litopenaeus vannamei* (Boone, 1931) by supplementation of *Ulva clathrata* meal in maturation diet in two commercial hatcheries. Aquac. Res 49, 1053–1059. https://doi.org/10.1111/are.13554.

Diggles, B.K., 2019. Review of some scientific issues related to crustacean welfare. ICES J. Mar. Sci. 76 (1), 66–81.

Dineshan, S., Sudha Devi, A.R., 2022. Effects of serotonin and dopamine on ovarian development in the freshwater crab *Travancoriana schirnerae* (Brachyura: Gecarcinucidae). Acta Zool. 103 (2), 167–181.

Fingerman, M., Nagabhushanam, R., Sarojini, R., Reddy, P.S., 1994. Biogenic amines in crustaceans: identification, localization, and roles. J. Crustace Biol. 14 (3), 413–437.

Gutiérrez-Vera, J.A., Ponce-Rivas, E., Braga, A., Paniagua-Chávez, C.G., Alfaro-Montoya, J., Rosales-Leija, M., 2024. Evidence of the existence of site-specific female contact pheromones involved in the sexual interaction behavior of the pacific whiteleg shrimp *Penaeus vannamei*. Animals 14, 1523. https://doi.org/10.3390/ani14111523.

Harlıoğlu, M.M., Farhadi, A., Harlıoğlu, A.G., 2020. Roles of Neurotransmitters in Decapod Reproduction. Thalass.: Int. J. Mar. Sci. 36 (2), 633–639.

Kamio, M., Yambe, H., Fusetani, N., 2022. Chemical cues for intraspecific chemical communication and interspecific interactions in aquatic environments: applications for fisheries and aquaculture. Fish. Sci. 88, 203–239.

Kannan, D., Thirunavukkarasu, P., Jagadeesan, K., Shettu, N., Kumar, A., 2015. Procedure for maturation and spawning of imported shrimp *Litopenaeus vannamei* in commercial hatchery, South East coast of India. Fish. Aquac. J. 6 (4), 1–5.

Okumura, T., 2007. Effects of bilateral and unilateral eyestalk ablation on vitellogenin synthesis in immature female kuruma prawns, *Marsupenaeus japonicus*. Zool. Sci. 24 (3), 233–240.

Palacios, E., Ibarra, A.M., Racotta, I.S., 2000. Tissue biochemical composition in relation to multiple spawning in wild and pond-reared *Penaeus vannamei* broodstock. Aquaculture 185, 353–371. https://doi.org/10.1016/S0044-8486(99)00362-2.

Palacios, E., Pérez-Rostro, C.I., Ramírez, J.L., Ibarra, A.M., Racotta, I.S., 1999. Reproductive exhaustion in shrimp (*Penaeus vannamei*) reflected in larval biochemical composition, survival and growth. Aquaculture 171 (3-4), 309–321.

Prasad, G.L.V., Naik, B.R., Ko, J.E., Nagaraju, G.P., 2014. Effects of naloxone, serotonin, and dopamine on reproduction of the freshwater crab *Barytelphusa guerini*. J. Exp. Zool. Part A: Ecol. Genet. Physiol. 321 (3), 173–182.

Ren, S., Mather, P.B., Tang, B., Hurwood, D.A., 2020. Comparison of reproductive performance of domesticated *Litopenaeus vannamei* females reared in recirculating tanks and earthen ponds: an evaluation of reproductive quality of spawns in relation to female body size and spawning order. Front. Mar. Sci. 7, 560. https://doi.org/10.3389/fmars.2020.00560.

Rendón, S.R., Macías, E.R., Calderón, S.P., Núñez, A.P., Solís, R.I., 2007. Comparison of some reproductive characteristics of farmed and wild white shrimp males *Litopenaeus vannamei* (Decapoda: Penaeidae). Rev. Biol. Trop. 55 (1), 199–206.

Rotllant, G., Nguyen, T.V., Aizen, J., Suwansa-ard, S., Ventura, T., 2018. Toward the identification of female gonad-stimulating factors in crustaceans. Hydrobiologia 825, 91–119. https://doi.org/10.1007/s10750-017-3497-4.

Ryan, L.W., 2018. An investigation of sexual reproduction and development in the giant tiger prawn, *Penaeus monodon*. [Ph.D. Thesis, University of the Sunshine Coast, Australia].

Santhoshi, S., Sugumar, V., Munuswamy, N., 2009. Serotonergic stimulation of ovarian maturation and hemolymph vitellogenin in the Indian white shrimp, *Fenneropenaeus indicus*. Aquaculture 291 (3-4), 192–199.

Sarojini, R., Nagabhushanam, R., Fingerman, M., 1995. Mode of action of the neurotransmitter 5-hydroxytryptamine in stimulating ovarian maturation in the red swamp crayfish, *Procambarus clarkii*: An *in vivo* and *in vitro* study. J. Exp. Zool. 271 (5), 395–400.

Swetha, C.H., Sainath, S.B., Reddy, P.R., Reddy, P.S., 2011. Reproductive endocrinology of female crustaceans: perspective and prospective. J. Mar. Sci. Res. Dev. S 3, 1–13.

Tinikul, Y., Soonthornsumrith, B., Phoungpetchara, I., Meeratana, P., Poljaroen, J., Duangsuwan, P., Soonklang, N., Mercier, A.J., Sobhon, P., 2009. Effects of serotonin, dopamine, octopamine, and spiperone on ovarian maturation and 56 embryonic development in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879). Crustaceana 1007–1022.

Tropea, C., Lavarías, S.M.L., López-Greco, L.S., 2018. Getting ready for mating: The importance of male touching as an accelerator of ovarian growth in a caridean shrimp. Zoology 130, 57–6.

Uawisetwathana, U., Leelatanawit, R., Klanchui, A., Prommoon, J., Klinbunga, S., Karoonuthaisiri, N., 2011. Insights into eyestalk ablation mechanism to induce ovarian maturation in the black tiger shrimp. PloS One 6 (9), e24427.

Uengwetwanit, T., Ponza, P., Sangsrakru, D., Wichadakul, D., Ingsriswang, S., Leelatanawit, R., Klinbunga, S., Tangphatsornruang, S., Karoonuthaisiri, N., 2018. Transcriptome-based discovery of pathways and genes related to reproduction of the black tiger shrimp (*Penaeus monodon*). Mar. Genom. 37, 69–73.

Vaca, A.A., Alfaro, J., 2000. Ovarian maturation and spawning in the white shrimp, *Penaeus vannamei*, by serotonin injection. Aquaculture 182 (3-4), 373–385.

Wongprasert, K., Asuvapongpatana, S., Poltana, P., Tiensuwan, M., Withyachumnarnkul, B., 2006. Serotonin stimulates ovarian maturation and spawning in the black tiger shrimp *Penaeus monodon*. Aquaculture 261 (4), 1447–1454.

Zapata, V., López-Greco, L.S., Medesani, D., Rodríguez, E.M., 2003. Ovarian growth in the crab *Chasmagnathus granulata* induced by hormones and neuroregulators throughout the year. *In vivo* and *in vitro* studies. Aquaculture 224 (1-4), 339–352.

Zacarias, S., Carboni, S., Davie, A., Little, D.C., 2019. Reproductive performance and offspring quality of non-ablated Pacific white shrimp (*Litopenaeus vannamei*) under intensive commercial scale conditions. Aquaculture 503, 460–466.