Mar Biotechnol (2016) 18:117–123
DOI 10.1007/s10126-015-9676-2

ORIGINAL ARTICLE

Silencing of Gonad-Inhibiting Hormone Transcripts in *Litopenaeus vannamei* Females by use of the RNA Interference Technology

Rubens G. Feijó[1] · André L. Braga[2] · Carlos F. C. Lanes[1] · Márcio A. Figueiredo[1] · Luis A. Romano[2] · Marta C. Klosterhoff[2] · Luis E. M. Nery[1] · Rodrigo Maggioni[3] · Wilson Wasielesky Jr.[2] · Luis F. Marins[1]

Received: 23 April 2015 / Accepted: 6 October 2015 / Published online: 14 November 2015

Abstract The method usually employed to stimulate gonadal maturation and spawning of captive shrimp involves unilateral eyestalk ablation, which results in the removal of the endocrine complex responsible for gonad-inhibiting hormone (GIH) synthesis and release. In the present study, RNAi technology was used to inhibit transcripts of GIH in *Litopenaeus vannamei* females. The effect of gene silencing on gonad development was assessed by analyzing the expression of GIH and vitellogenin, respectively, in the eyestalk and ovaries of *L. vannamei* females, following ablation or injection with dsRNA-GIH, dsRNA-IGSF4D (non-related dsRNA), or saline solution. Histological analyses were performed to determine the stage of gonadal development and to assess the diameter of oocytes throughout the experimental procedure. Only oocytes at pre-vitellogenesis and primary vitellogenesis stages were identified in females injected with dsRNA-GIH, dsRNA-IGSF4D, or saline solution. Oocytes at all developmental stages were observed in eyestalk-ablated females, with predominance of later stages, such as secondary vitellogenesis and mature oocytes. Despite achieving 64, 73, and 71 % knockdown of eyestalk GIH mRNA levels by 15, 30, and 37 days post-injection (dpi), respectively, in dsRNA-GIH-injected females, the expected increase in ovary vitellogenin mRNA expression was only observed on the 37th dpi. This is the first report of the use of RNAi technology to develop an alternative method to eyestalk ablation in captive *L. vannamei* shrimps.

Keywords *Litopenaeus vannamei* · Reproduction · RNAi · GIH · RT-qPCR

✉ Luis F. Marins
dqmluf@furg.br

[1] Molecular Biology Laboratory, Biological Sciences Institute (ICB), Federal University of Rio Grande—FURG, Rio Grande, RS 96203-900, Brazil

[2] Marine Station of Aquaculture, Oceanography Institute, Federal University of Rio Grande, Rio Grande, RS 96 201-900, Brazil

[3] Center of Studies and Diagnosis of Aquatic Organism Diseases (CEDECAM), Marine Sciences Institute, Federal University of Ceara, Fortaleza, CE 60165-081, Brazil

Introduction

During the captive breeding of shrimps, the method usually employed to stimulate gonadal maturation and spawning involves unilateral eyestalk ablation, which results in the removal of the neuroendocrine complex responsible for gonad-inhibiting hormone (GIH) synthesis and release. GIH, also known as vitellogenesis-inhibiting hormone (VIH), is produced and secreted by neuroendocrine cells of the X-organ sinus gland complex (XO-SG), which is present in the eyestalk. This neuropeptide was classified as a member of the crustacean hyperglycemic hormone (CHH) family. Recently, cDNA-encoding GIH was cloned from the eyestalk of *Litopenaeus vannamei* and has been shown to have a nucleotide sequence highly similar (98 %) to GIH from *Penaeus monodon* (Chen et al. 2014).

In addition to reducing the circulating levels of GIH, eyestalk ablation also reduces the effect of other neuropeptides produced in the XO-SG. This procedure leads to a significant hormonal imbalance, which has a considerable impact on the quantity, quality, and survival of the larvae produced (Palacios et al. 1999). Consequently, in order to obtain good quality larvae, the breeding stock is usually discarded every 3 months, which may have a severe impact on the shrimp supply chain

economy. For this reason, in the past couple of decades, research has sought to develop alternative techniques to eyestalk ablation for use during the captive breeding of shrimp. Among these alternative techniques, the most promising are those involving the administration of hormones, neurotransmitters, monoclonal antibodies, optimization of environmental factors (temperature, light exposure, and salinity), and most recently, the use of dsRNA molecules to silence hormonal transcripts by RNA interference (Alfaro and Komen 2004; Balasubramanian 2009; Tinikul et al. 2009; Nagaraju 2010; Treerattrakool et al. 2011).

Gene silencing by RNAi involves the use of long synthetic dsRNA molecules with homology to a specific target gene. When inside the cell, the long dsRNA molecules are processed into smaller RNA fragments (20–28 bp siRNA) by the action of dsRNA-specific RNase III type endonucleases (i.e., DICER enzymes). siRNA can guide the cleavage of complementary RNA due to a multiprotein silencing complex (RNA-induced silencing complex, RISC), leading to the specific degradation of the target mRNA sequence (Meister and Tuschl 2008). Recent studies that have investigated hormonal control in penaeid shrimps have used RNAi in vivo to clarify the role of certain neuropeptides belonging to the CHH family, such as hormones involved in carbohydrate metabolism in *Litopenaeus schmitti* (Lugo et al. 2006), in the molt of *L. vannamei* (Hui et al. 2008), and in *P. monodon* reproduction (Treerattrakool et al. 2008).

The possibility of using RNAi to silence the GIH gene as an alternative method to eyestalk ablation has been recently demonstrated in *P. monodon* (Treerattrakool et al. 2011). Therefore, the present study aimed to investigate the feasibility of using gene-specific dsRNA to stimulate gonadal maturation of female *L. vannamei* through silencing of GIH, which was recently characterized for this species (Chen et al. 2014).

Materials and Methods

Animal Origin and Acclimatization

Post-larval stages of specific pathogen-free (SPF) *L. vannamei* were obtained from Aquatec Ltda (Canguaretama, RN) and reared at the Marine Station of Aquaculture, at the Federal University of Rio Grande (EMA-FURG) for 10 months. Before the experiment, 200 animals were selected taking into account their apparent health condition. Size, lack of melanization in the body, presence of all appendices, and complete filling of the hepatopancreas and digestive tract were the parameters used to evaluate the health condition of the animals. The animals were then separated by gender and placed into two circular maturation tanks (10 m^2 of bottom area, 5000 l of volume), where they were acclimated for 30 days. During the acclimatization, animals were subjected to artificial light conditions (14 h of light and 10 h of dark) and were fed four times per day with *Illex argentinus* squid and a commercial diet (Breed S InveAquaculture, Belgium), which were offered alternately. The seawater was completely renewed every day and maintained at salinity 30, 28 °C, pH 7.2, and 5.5 mg/l DO.

Production of dsRNA Molecules

Fragments from the *L. vannamei* GIH gene (768 bp) (GenBank Accession number: KC962398) and from the gene encoding a *Danio rerio* fish immunoglobulin (IGSF4D, 678 bp) (GenBank Accession number: AL954312), were amplified by RT-PCR using specific primers containing the promoter sequence of the 5′ end of the T7 bacteriophage (Table 1). The IGSF4D amplicons were used to synthesize non-related dsRNA. The identity of the PCR products was confirmed by DNA sequencing before the dsRNA was produced (data not shown).

The PCR products were concentrated by precipitation in absolute ethanol (Merck) (1 V) and sodium acetate 3 M (0.1 V) and were then purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) kit, according to the manufacturer's instructions. The in vitro transcription of the dsRNA molecules was performed with the T7 RiboMAX™ Express Large Scale RNA Production System (Promega) kit with the following incubation steps: 42 °C for 4 h, 70 °C for 10 min, followed by a gradual temperature decrease (0.5 °C every 20 s) until 20 °C. Subsequently, the dsRNA molecules were treated with DNAse I (to remove dsDNA and ssDNA) and RNAse A (for the removal of ssRNA, in the presence of NaCl 0.5 M) and were incubated for 30 min at 37 °C. Proteins, free nucleotides, and residues belonging to degraded nucleic acids that were still present in the transcription reaction were removed using Wizard® SV Minicolumns (Promega) using 4 M guanidine thiocyanate, 0.01 M Tris/HCl (pH 7.5) as the denaturing solution. The presence of both dsRNA-GIH and dsRNA-IGSF4D molecules was quantified using a Qubit™ quantification assay (Invitrogen) fluorometric kit and were then eluted in 150 μl of saline solution (NaCl 330 mM, Tris 10 mM, pH 7.4).

Experimental Design

After acclimatization, 80 female and 80 male shrimp (♀=45–55 g; ♂=35–45 g), in the intermolting stage, were selected from the acclimatized population. Females were weighed and labeled with individual numbers through elastomers subcutaneously implanted in the last abdominal segment. After the labeling process, the females were evenly distributed, along with the males, into two circular maturation tanks at a density of eight shrimp/m^2 (male to female ratio=1:1). Four groups of 20 females were treated according to the following protocol: group I control group, intramuscular injection of 150 μl saline

Table 1 Primers used to amplify fragments for in vitro synthesis of dsRNA and for gene expression analysis in *Litopenaeus vannamei*

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon (bp)	GenBank (accession no.)
GIH-T7	[a]***TAATACGACTCACTATA***GT TCCTGATGTGCCTCCACT	***TAATACGACTCACTATA***CCAAT CAGTTCCCCTTGAAA	768	KC962398
IGSF4D-T7	***TAATACGACTCACTATA***GTTT GTCTGTCCCTCGTGGT	***TAATACGACTCACTATA***AGG GTGCAGGTTTTCCTTTT	678	AL954312
GIH (real time)	TGATGCTTCGACGCCTTGGCT	CCTTGAACATAATAGGACG CCCTTCCC	74	KC962398
Vitellogenin (real time)	AATACAAGAACGTGAG GGATAGGAA	AGGCAATCACACTTGT ATATTTGTATTTC	72	AY321153
Ef1α (real time)	CCACCCTGGCCAGATTCA	GCGAACTTGCAGGCAATGT	75	DQ858921
β-actin (real time)	GACCGAGGCTCCCCTTCAA	TCTCGAACATGATCT GTGTCAT	114	AF300705

[a] T7 promoter sequence *(bold and italic)*

solution, group II intramuscular injection of 2.8 μg/g of dsRNA-GIH, group III intramuscular injection of 2.8 μg/g of dsRNA-IGSF4D, group IV unilateral eyestalk-ablation. All dsRNA was diluted in 150 μl of saline solution. All rearing conditions used during the acclimatization period were maintained throughout the 38 days of experimental testing.

Gonadal development was assessed daily in females from all groups through visual examination with the aid of a flashlight. Macroscopically, the stages of gonadal development were classified according to the following criteria: *S0*, undeveloped ovaries (immature), *S1*, early development of the ovaries in the cephalothorax region (early maturation), *S2*, presence of a yellowish ovarian line along the abdominal back (intermediate maturation), and *S3–4*, enlarged cephalothorax and the ovaries start to become orange-colored, appearing as a double thick line along the abdominal back (mature) (adapted from Tan-Fermin and Pudadera 1989). Females identified as S3–4 were separated, and the spawning was duly registered.

At the 15th, 30th, and 37th days post-injection (dpi), five females in the intermolting stage were randomly collected from each group, dissected, and the optical ganglions and ovaries were harvested for subsequent molecular analysis. Posterior ovarian fragments were also collected at the 15th, 30th, and 37th dpi for quantitative histological analysis.

Gene Expression Analysis

The extraction of total RNA from the optical ganglion and ovaries was performed using TRIzol (Invitrogen) reagent, according to the manufacturer's instructions. The extracted RNA was treated with DNAse I Amplification Grade (Life Technologies) and incubated for 30 min at 37 °C. The quality of the extracted RNA was confirmed by electrophoresis in a 1 % agarose gel, prepared in 60 ml of TAE (1×) (0.04 M Tris-Base, 0.04 M acetate, and 0.001 M EDTA) containing 0.1 μg/ml of ethidium bromide. The RNA was then quantified by fluorometry using a Quant-iT RNA Assay Kit (Life Technologies) and a Qubit™ fluorometer (Life Technologies). cDNA synthesis was performed with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) in a final reaction volume of 20 μl, containing 2 μl of RT Buffer (10×), 0.8 μl of dNTPs mix (25×), 2 μl of RT random primers (10×), 1 μl of Multi Scribe™ Reverse Transcriptase (50 U), and 1 μg of total RNA diluted into ultra-pure water (Invitrogen). The samples were incubated in a thermocycler at 25 °C/10 min, followed by 37 °C/120 min and 85 °C/5 min. The cDNA was then stored at −20 °C until it was used in the gene expression analysis.

Analysis of GIH-mRNA expression was performed to assess the effect of RNAi silencing in the optical ganglion samples, while the analysis of vitellogenin-mRNA expression was performed in the harvested ovarian samples. After validation with Data Assist v3.0 (Applied Biosystems) software, the means between the expression of the *β-actin* and *Ef1α* genes were used as a reference to normalize the expression of the *GIH* and *vitellogenin* genes. Expression of *β-actin* and *Ef1α* have been recently suggested as the most stable controls for reproduction experiments on *P. monodon* (Leelatanawit et al. 2012). For the expression analysis, we used the ABI 7500 Real-Time Systems (Applied Biosystems) platform and the SYBR® Green qPCR SuperMix-*UDG* (Invitrogen) detection system. The primers used for amplification of the analyzed genes are listed in Table 1.

The amplification efficiency of each primer was determined according to the method described by Pfaffl et al. (2002), and the specificity of the amplification was confirmed by melting curve analysis. The RT-qPCR reactions were performed in a final volume of 10 μl, containing 5 μl of Platinum SYBR Green® qPCR SuperMix-UDG (Invitrogen), 0.3 μl (10 μM) of each primer, 0.2 μl (50×) of Rox reference dye, 1 μl (10 ng) of cDNA, and 3.2 μl of ultra-pure water. The thermocycling conditions used were 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. RT-qPCR reactions were performed as technical duplicates and were only considered valid when both amplicons from each

replicate could be detected. The mean Ct values obtained were then normalized and converted into a linear form by the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001).

Quantitative Histology

Each posterior ovarian fragment was fixed by immersion in Davidson's AFA solution (11.5 % acetic acid, 22 % formalin, and 33 % ethanol) for 24 h. The samples were processed in automatic equipment (LUPE PT05) and were embedded with paraplast. Transverse and longitudinal histological sections of 4-μm thickness were obtained with the aid of a microtome (LUPE MRP03) and were subsequently stained with hematoxylin-eosin. These sections were examined under a light microscope (Olympus BX 45) coupled to a digital camera (Olympus DP 72) for image acquisition. The parameters considered for the quantitative histological analysis were gonadal development stage and mean diameter of the oocytes from each female. The oocytes were classified according to Arcos et al. (2011), into nine types: (I) chromatin nucleolus oocyte (cn), (II) early perinucleolus oocytes (epn), (III) late perinuclear oocytes (lpn), (IV) oil globule oocyte 1 (og1), (V) oil globule oocyte 2 (og2), (VI) yolkless oocyte (yl), (VII) yolk granule oocyte (yg), (VIII) prematuration oocyte (pm), and (IX) maturation oocyte (m). Depending on the oocyte type, the stages of gonadal development were defined as pre-vitellogenesis (oocytes type I, II, and III), primary vitellogenesis (oocytes type IV, V, and VI), secondary vitellogenesis (oocytes type VII and VIII), and mature (oocytes type IX). The diameter of the oocyte was measured through the LAS EZ (Leica) software in 15 microscope fields (20×) for each examined female and were analyzed in accordance with the methodology described by Arcos et al. (2011).

Statistical Analysis

In order to analyze the differences in oocyte diameter and gene expression, one-way analysis of variance (ANOVA) was employed with paired comparisons to the experimental group containing females injected with saline solution (control group). The statistical tests were performed using GraphPad Prism 3.0 software for Windows (GraphPad Software, USA).

Results

Gonadal Development and Spawning

Among the female groups examined in this study, spawning occurred on the 2nd, 3rd, 4th, and 5th weeks, in 25, 15, 15, and 5 % of eyestalk-ablated females, respectively. However, only one spawning event (5 %) was observed for each group containing females injected with dsRNA-IGSF4D or saline solution during the 2nd experimental week. Macroscopically, 10 % of individuals in the groups injected with dsRNA-IGSF4D or saline solution were identified in developmental stages S3–4 on the 2nd experimental week. Only early and intermediate gonadal developmental stages (S1 or S2) were observed in females injected with dsRNA-GIH (Fig. 1).

Gene Silencing and Expression Analysis

When compared to females injected with saline solution, levels of the GIH transcript in the eyestalks of females injected with dsRNA-GIH were reduced by 64, 73, and 71 % at the 15th, 30th, and 37th dpi, respectively ($p<0,0001$). Throughout the experiment, no statistically significant difference was detected between the level of GIH transcript present in the eyestalks of females that were ablated, injected with dsRNA-

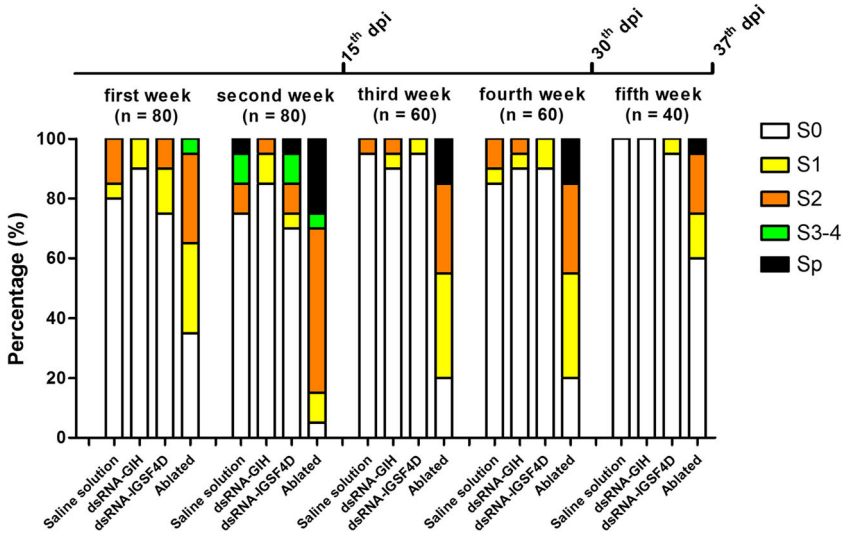

Fig. 1 Proportion of *Litopenaeus vannamei* females at each gonadal development stage as revealed through macroscopic observation of shrimp ovaries, over 5 weeks of experimental testing. *S0* immature, *S1* early maturation, *S2* intermediate maturation, *S3–4* mature, *Sp* spawning, dpi, days post-injection

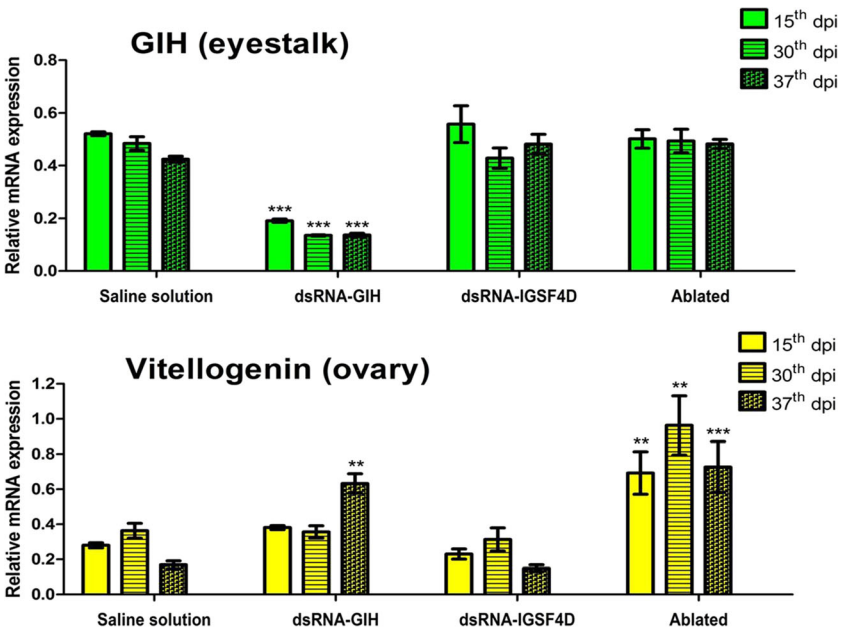

Fig. 2 Levels of GIH and vitellogenin transcripts detected in eyestalk and ovaries, respectively, in *Litopenaeus vannamei* females that were ablated, injected with dsRNA-GIH, dsRNA-IGSF4D, or saline solution. The *vertical bars* represent the mean normalized expression (± SEM) (n=5). Statistically significant differences to the control group (saline solution) are represented by ***(p<0.0001) and **(p<0.001)

IGSF4D, or injected with saline solution. Expression of vitellogenin mRNA increased by 2.5-, 2.7-, and 4.4-fold in the ovaries of ablated females collected on the 15th, 30th, and 37th dpi (p<0.0001), respectively. On the 37th dpi, a 3.8-fold increase in the level of vitellogenin transcript was detected in the ovaries of females injected with dsRNA-GIH when compared with females injected with saline solution (p<0.001). No statistically significant differences in the levels of vitellogenin transcript were observed in the ovaries of dsRNA-IGSF4D (non-related dsRNA) injected females when compared to the group of females injected with saline solution (Fig. 2).

Histology

Only oocytes belonging to the pre-vitellogenesis (oocytes type I, II, and III) and primary vitellogenesis (oocytes type IV, V, and VI) stages were identified in females injected with dsRNA-GIH, dsRNA-IGSF4D, or saline solution. On the other hand, oocytes from all gonadal developmental stages were observed in eyestalk-ablated females, with a predominance of later stages such as secondary vitellogenesis (oocytes type VII) and mature oocytes (oocytes type IX) (Fig. 3).

The diameters of oocytes observed in the ovaries of eyestalk-ablated females were significantly different to those

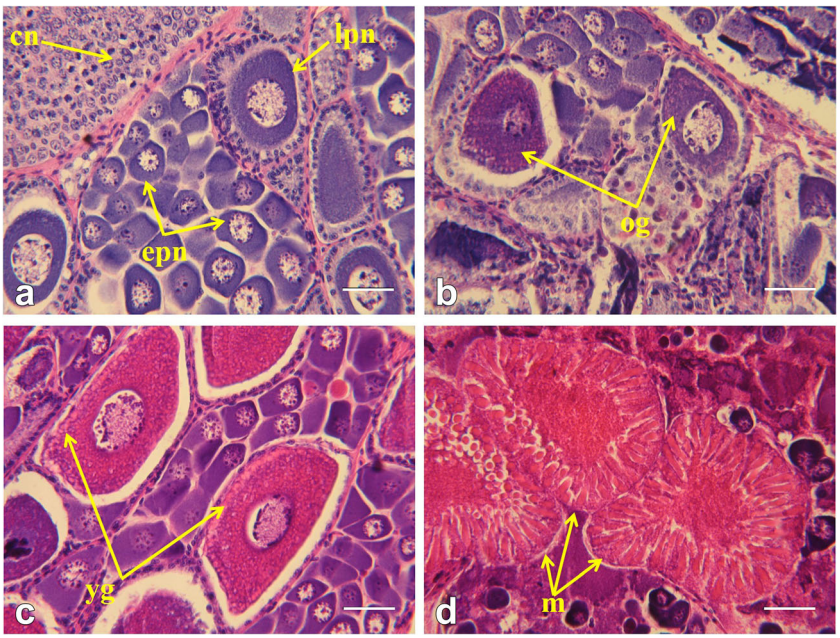

Fig. 3 Microscopic images of histological sections of ovary (stained with hematoxylin-eosin) from *Litopenaeus vannamei* females. *Arrows* indicate the oocyte types characteristic to the pre-vitellogenesis stage (**a**), primary vitellogenesis stage (**b**), secondary vitellogenesis stage (**c**), and mature stage (**d**) observed during the 38 days of experimental testing. *Oocyte types*: (cn) chromatin nucleolus oocyte, (epn) early perinucleolus oocyte, (lpn) late perinucleolus oocyte, (og) oil globule oocyte, (yg) yolk granule oocyte, and (m) maturation oocyte. Scale bar: 25 μm

observed in the ovaries of females injected with saline solution on the 15th, 30th, and 37th dpi ($p<0.001$). There was also a significant ($p<0.05$) increase in the diameter of oocytes from females injected with dsRNA-GIH on the 37th dpi. No statistically significant differences were observed in the diameter of oocytes derived from females injected with dsRNA-IGSF4D throughout the experimental period (Fig. 4).

Discussion

The most common method used to stimulate gonadal maturation and spawning during captive breeding of *L. vannamei* shrimps is unilateral eyestalk ablation, which in addition to causing severe injury to the animal, is also responsible for a significant hormonal imbalance that has a considerable impact on the quantity, quality, and survival of the larvae produced, especially during the first post-ablation spawning events (Palacios et al. 1999). Recently, Chen et al. (2014) cloned a hormone with similar characteristics to GIH from the *L. vannamei* eyestalk. A recombinant protein from the cloned neuropeptide has shown inhibitory effect on the expression of vitellogenin mRNA in the hepatopancreas (Chen et al. 2014). According to Raviv et al. (2006), vitellogenin transcripts can be detected in both the hepatopancreas and ovaries of *L. vannamei* shrimps. However, the levels of expression are higher in the ovaries throughout the molting cycle when compared to those detected in the hepatopancreas. In this study, we performed gene expression analysis and quantitative histology to assess gonadal development in *L. vannamei* shrimps in captivity following inhibition of the GIH transcript by RNAi.

The results of the gene silencing study showed that expression of GIH was reduced 64, 73, and 71 % in the eyestalks of females injected with dsRNA-GIH on the 15th, 30th, and 37th dpi, respectively. However, ovarian vitellogenin mRNA expression was observed only on the 37th dpi, when the transcript levels were overexpressed 3.8-fold when compared to that detected in females injected with saline solution (Fig. 2). This result is consistent with the increase in oocyte diameter observed on the 37th dpi in females injected with dsRNA-GIH (Fig. 4). However, only the primary vitellogenesis stage of gonadal development was observed in the ovaries of females injected with dsRNA-GIH, dsRNA-IGSF4D, or saline solution throughout the experimental procedure. On the other hand, oocytes characteristic of secondary vitellogenesis and mature stages were predominantly observed in the ovaries of ablated females. According to Palacios and Racotta (2003), measuring the diameter of oocytes by histological analysis in *L. vannamei* breeding stocks is the best method to evaluate the maturation stage of the female in breeding systems. The late response of RNAi GIH silencing on the expression of vitellogenin mRNA might be due to residual concentrations of GIH in the sinus gland and circulating in the hemolymph, since mRNA silencing by dsRNA only inhibits the synthesis of new hormone molecules.

In domesticated *P. monodon*, Treerattrakool et al. (2011) demonstrated that inhibition of GIH transcripts in the eyestalk following RNAi persisted for 30 days. Despite being able to observe spawning events for 14 % of the dsRNA GIH-injected females, the authors found no significant gonadal development in more than 50 % of them. However, the authors showed that RNAi could stimulate gonadal development and spawning in wild *P. monodon*. The difference in RNAi efficiency between domesticated and wild females was attributed to factors such as size, age, water quality (salinity and temperature), nutrition, and rearing density used in the experimental procedures (Treerattrakool et al. 2011). A similar set of factors may have affected RNAi knockdown efficiency in the *L. vannamei* from the dsRNA-GIH group in the present study.

Interestingly, in this study, no change in GIH expression was detected in eyestalks (15th, 30th, and 37th dpi) from unilaterally ablated *L. vannamei* females. According to Chen et al. (2014), the eyestalk of *L. vannamei* is not the only structure able to synthesize GIH, which can also be synthesized by the brain. However, the underlying mechanisms of GIH synthesis involved in breeding control in *L. vannamei* females are not yet fully understood.

In this study, the low breeding efficiency observed in female shrimp that were treated with dsRNA-GIH molecules might have been caused by the combined action of several forms of GIH. For example, some neuropeptides isolated from the sinus glands of *L. vannamei* were also identified as possible GIH candidates, and were named Liv-SGP-A, -B, -C, -E, -F, and –G. Among the isolated hormones, Liv-SGP-G shared 100 %

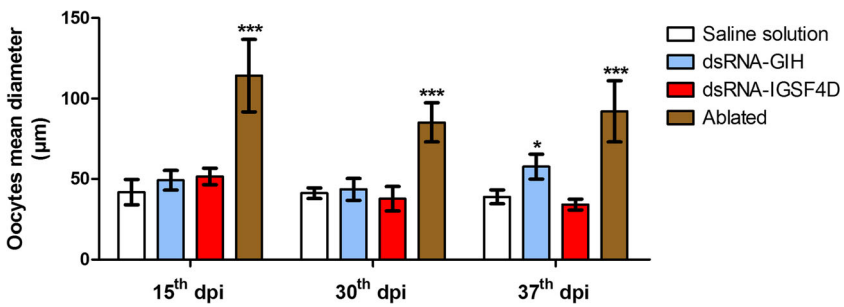

Fig. 4 Mean oocyte diameters (± SD) observed in *L. vannamei* females following ablation or injection with dsRNA-GIH, dsRNA-IGSF4D, or saline solution at the 15th, 30th, and 37th dpi. Statistically significant differences are represented by *($p<0.05$) and ***($p<0.001$)

nucleotide homology with *L. vannamei* CHH and also exhibited the highest inhibitory effect on the expression of vitellogenin mRNA when subjected to in vivo incubation tests with *Marsupenaeus japonicus* ovary fragments (Tsutsui et al. 2007). Similarly, the differential action of the GIH candidates over *Jasus lalandii* lobster vitellogenesis has also been described through in vivo heterologous assays using *P. semisulcatus* ovaries (Marco et al. 2002). These authors also demonstrated that some of these neuropeptides, in addition to their effect on breeding, also have a hyperglycemic effect. In fact, previous studies have suggested that all members of the CHH family are derived from a common ancestor and subsequently diverged through successive mutation and gene duplication events throughout the evolutionary process, which might have contributed to the multiple biological functions that are widely reported for neuropeptides from this family (Wilcockson et al. 2002; Chan et al. 2003; Webster et al. 2012).

The results presented in this study clearly demonstrate the potential of RNAi technology for silencing hormonal transcripts in domesticated *L. vannamei* in order to achieve gonadal development. However, further studies on GIH forms and physiological mechanisms are necessary in order to achieve an efficient RNAi-based technique to stimulate gonadal development and spawning in *L. vannamei*, alternative to the traditional method of eyestalk ablation.

Acknowledgments This work was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico—Proc. No. 405519/2012–5). Rubens Galdino Feijó is supported by a scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Carlos F. C. Lanes is a holder of a scholarship from Science Without Borders Program (CNPq). Luis Fernando Marins, Luis Alberto Romano, and Wilson Wasielesky Jr. are research fellows from CNPq (Proc. No. 304675/2011–3, 301002/2012–6, and 310993/2013–0, respectively).

References

Alfaro JGZ, 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 236:511–522

Arcos FG, Ibarra AM, Racotta LS (2011) Vitellogenin in hemolymph predicts gonad maturity in adult female *Litopenaeus* (*Penaeus*) *vannamei* shrimp. Aquaculture 316:93–98

Balasubramanian CP (2009) Physiology in aquaculture with special reference to penaeid shrimp aquaculture. Winter School on Recent Advances in Breeding and Larviculture of Marine Finfish and Shellfish 29:161–169

Chan SM, Gu PL, Chu KH, Tobe SS (2003) Crustacean neuropeptide genes of the CHH/MIH/GIH family: implications from molecular studies. Gen Comp Endocrinol 134:214–219

Chen T, Zhang L, Wong N, Zhong M, Ren C, Hu C (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):1–10

Hui JH, Tobe SS, Chan SM (2008) Characterization of the putative farnesoic acid O-methyltransferase (LvFAMeT) cDNA from white shrimp, *Litopenaeus vannamei*: evidence for its role in molting. Peptides 29:252–260

Leelatanawit R, Klanchui A, Uawisetwathana U, Karoonuthaisiri N (2012) Validation of reference genes for real-time PCR of reproductive system in the black tiger shrimp. Plos One 7(12):e52677

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25:402–408

Lugo JM, Morera Y, Rodriguez T, Huberman A, Ramos L, Estrada MP (2006) Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique. FEBS J 273:5669–5677

Marco HG, Avarre JC, Lubzens E, Gäde G (2002) In search of a vitellogenesis-inhibiting hormone from the eyestalks of the South African spiny lobster, *Jasus lalandii*. Invertebr Reprod Dev 41:143–150

Meister G, Tuschl T (2008) Mechanisms of gene silencing by double-stranded RNA. Nature 431:343–349

Nagaraju GPC (2010) Reproductive regulators in decapod crustaceans: an overview. J Exp Biol 214:3–16

Palacios E, Racotta IS (2003) Effect of number of spawns on the resulting spawn quality of 1-year old pond reared *Penaeus vannamei* (Boone) broodstock. Aquac Res 34:427–435

Palacios E, Perez-Rostro CI, Ramirez JL, Ibarra AM, Racotta IS (1999) Reproductive exhaustion in shrimp *Penaeus vannamei* reflected in larval biochemical composition, survival and growth. Aquaculture 171:309–321

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:1–10

Raviv S, Parnes S, Segall C, Davis C, Sagi A (2006) Complete sequence of *Litopenaeus vannamei* (Crustacea: Decapoda) vitellogenin cDNA and its expression in endocrinologically induced sub-adult females. Gen Comp Endocrinol 145:39–50

Tan-Fermin JD, Pudadera RA (1989) Ovarian maturation stages of the wild giant tiger prawn, *Penaeus monodon* Fabricous. Aquaculture 77:229–242

Tinikul Y, Soonthornsumrith B, Phoungpetchara I, Meeratana P, Poljaroen J, Duangsuwan P, Soonklang N, Mercier AJ, Sobhon P (2009) Effects of serotonin, dopamine, octopamine, and spiperone on ovarian maturation and embryonic development in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879). Crustaceana 82(8):1007–1022

Treerattrakool S, Panyim S, Chan SM, Withyachumnarnkul B, Udomkit A (2008) Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. FEBS J 275:970–980

Treerattrakool S, Panyim S, Udomkit A (2011) Induction of ovarian maturation and spawning in *Penaeus monodon* broodstock by double-stranded RNA. Mar Biotechnol 13:163–169

Tsutsui N, Ohira T, Kawazoe I, Takahashi A, Wilder MN (2007) Purification of sinus gland peptides having vitellogenesis-inhibiting activity from the whiteleg shrimp *Litopenaeus vannamei*. Mar Biotechnol 9:360–369

Webster SG, Keller R, Dircksen H (2012) The CHH-superfamily of multifunctional peptide hormone controlling crustacean metabolism, osmoregulation, moulting, and reproduction. Gen Comp Endocrinol 175:217–233

Wilcockson DC, Chung SJ, Webster SG (2002) Is crustacean hyperglycaemic hormone precursor-related peptide a circulating neurohormone in crabs? Cell Tissue Res 307:129–138