REPORT

Effects of RNA interference on gene functions of aquatic organisms

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ABSTRACT

It was recently discovered that double-stranded RNA (dsRNA) was a potent and specific inhibitor of gene transcription in the Caenorhabditis elegans nematode. Similar results have been reported in *Drosophila melanogaster*, planaria, mice, zebrafish, among other species. The interfering RNA (RNAi) phenomena occurs when a dsRNA is processed by a protein complex, specifically generating small size molecules that anneal to a target sequence of messenger RNA and promote its degradation, while also blocking its expression. The dsRNA-mediated interference is in most cases a post-transcriptional gene silencing mechanism. This paper, demonstrates that gene function can be studied in aquatic organisms by using RNA interference.

Here, we describe the application of this technique in two aquatic organisms:

- Zebrafish (*Danio rerio*), a currently relevant animal model in the field of genetics, developmental biology and biomedicine.

- White shrimp (*Litopenaeus schimitti*), uneconomically relevant shrimp specie from the Atlantic Ocean.

The function of the myostatin growth factor on growth and differentiation of muscular masses and animal growth was studied. Results demonstrated the RNA interference technology as a useful tool to study the biological function of genes involved in zebrafish development. This paper give the first evidence on growth and development of the muscular mass through hyperplasia or hypertrophy of muscle fibers as the main function of myostatin in fish, although its expression is not restricted to the skeletal muscle as is the case in mammals. Furthermore, it was demonstrated that myostatin inhibition promotes a significantly increased muscular mass phenotype. This protein is studied in several laboratories in the world searching for methods or techniques to stably inhibit myostatin. This would be a high impact result for Cuban and wordl aquaculture if it is attained in commercially relevant species.

There were no previous reports in the literature on the functionality of the RNAi technology in crustaceans *in vivo*. Recent studies demonstrated the functionality of gene silencing in cultured shrimp cells, without further demonstrations *in vivo*. Our studies give the first evidence on the usefulness of dsRNA in adult shrimps *in vivo*. Moreover, we isolated, cloned and characterized for the first time the cDNA of the crustacean hyperglycemic hormone (CHH) of the Atlantic Ocean shrimp. Energy regulation is the primary function of this hormone in crustaceans, it is also involved in reproduction, molting, digestion, osmoregulation, and lipid metabolism in different species. The ability of the dsRNA to inhibit the function of the CHH in shrimps was also studied.

*I*ntroduction

The interfering RNA (RNAi) is the double-stranded RNA (dsRNA) that specifically anneals to a target sequence, promoting its degradation and blocking its expression [1]. The dsRNA-mediated RNA interference is a general post-transcriptional gene silencing mechanism (PTGS), preserved throughout evolution. It could be also expressed at transcriptional and translational levels, since the interference could be exerted through processing or the translation of endogenous transcripts; and at the chromatin level, by forming heterochromatin domains in the nucleus, which are critical for genomic organization and homeostasis [2].

This process, the dsRNA inducing sequence-specific gene silencing was formerly discovered in the *Caenorhabditis elegans* nematode [3]. The RNAi has been used for the experimental manipulation of gene expression and to prove the function of certain genes at genomic scale [2].

It has been demonstrated that dsRNA-mediated interference, PTGS and repression mechanisms act generally at the post-transcriptional stage to degrade target mRNA transcripts. Degradation occurs in the nucleus and/or the cytoplasm [4].

The post-transcriptional dsRNA-mediated gene silencing process is a multi-step pathway requiring initiation, facilitated interaction with and degradation of the target mRNA. In some cases, this process could also involve the physical degradation of the activating dsRNA, and the persistence or amplification of gene silencing [2].

Zebrafish (*Danio rerio*) embryos microinjected with dsRNA specific for *lacZ* and tail-less phenotypes

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genes showed reduced levels of endogenous mRNA, with 20 to 30% of the fish presenting the specific effects of the tail-less phenotype [5]. To determine if the dsRNA could attenuate the expression of endogenous genes [6], Li and coworkers (2000) microinjected zebrafish embryos, at 1-2 cells developmental stages, with dsRNA specific for the Zf-T and Pax 6.1 genes [6]. In this experiment, 80% of the microinjected fish showed specific effects. In this study, no toxicity was reported, suggesting that the RNAi interference is an ideal tool to sequence-specifically inactivate gene expression in zebrafish, in contrast to previous reports on unspecific effects after the injection of dsRNA in this animal model. Due to the scarce information on the function of myostatin in fish, and the subsequent relevance of increasing the muscular mass for aquaculture by myostatin inhibition, we decided to study the effects of myostatin inhibition by RNA interference and its applicability in zebrafish.

In adult shrimps, dsRNA-mediated gene silencing had not been reported before, in spite of being a commercially-relevant species with several laboratories doing research on the molecular mechanisms controlling its growth. Its function would be essential for the understanding of gene regulation in shrimps, in view of its practical application.

Results and discussion

Silencing the myostatin gene by RNAi produces a giant-phenotype zebrafish

Myostatin, also known as Growth and Differentiation Factor 8 (GDF-8), is a member of the type ß transforming growth factor family that functions as a negative regulator of skeletal muscle growth and development in mammals. Abrogating its expression dramatically increases the muscular mass by hyperplasia and/or hypertrophy of muscle fibers [7].

To determine if the Tilapia myostatin gene-specific dsRNA would be able to stimulate growth of the muscular mass in zebrafish, the synthetic dsRNA was injected to zebrafish embryos at the 1-2 cell stage. Two different dosages were employed, one low dosage (5 molecules per embryo) to find the minimal dsRNA dosage that can effect the expression of specific genes, and another higher dosage (5 x 10^6 dsRNA molecules per embryo) similar to those used by other research groups for this purpose. The negative control consisted of embryos from the same spawning for each dosage injected with 1X phosphate-buffered saline (PBS). After two and a half months, the average weight of fish microinjected either with 5 or 5 x 10^6 dsRNA molecules increased nearly 39 and 45%, respectively, compared to their respective control animals. In both cases, a phenotype characterized by a significant increase in muscular mass was obtained (Table 1, and Figure 1 in [8]).

Besides, as shown in figure 2, the dsRNA did not promote unspecific phenotype effects in microinjected fish, which were shown to be healthy.

In mammals, the inhibition of the myostatin gene in different ways produces increased body weight phenotypes due to hyperplasia and or hypertrophy. In fish, specifically in transgenic zebrafish expressing high levels of myostatin pro-peptide, increased the number of skeletal muscle myofibrils; however, there are no significant differences in fiber size [9].

A histological analysis of skeletal muscle was carried out to determine if the increase in the skeletal muscular mass observed in fish microinjected with myostatin-specific dsRNA was due to hyperplasia or hypertrophy of muscular fibers. The number of fibers in a given area was determined by direct count.

Table 1. Comparing the mean body weight of zebrafish microinjected or not, two months and a half after fertilization

	Not microinjected animals		Microinjected animals			
Experiment	No. of fish	Weight (g)[a]	No. of fish	Weight (g)[c]	*P*[d] Valued	Gain in bodyweight
1[a]	145	0.2052 ± 0.0149	130	0.2872 ± 0.0178	< 0.01	39 %
2[b]	55	0.3135 ± 0.0359	55	0.4563 ± 0.0258	< 0.01	45 %

[a] Injection of 5 dsRNA molecules.
[b] Injection of 5 x 10^6 dsRNA molecules.
[c] Weight is presented as the mean ±SD.
P[d] Values correspond to Student's t test.

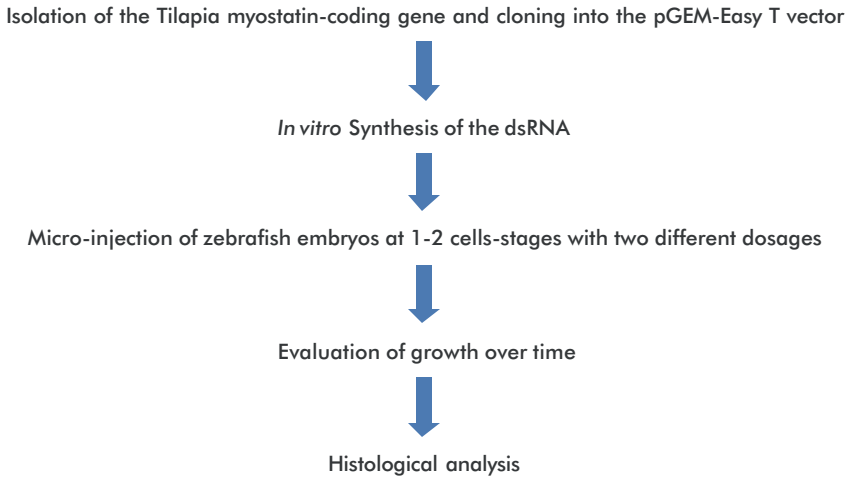

Figure 1. Experimental procedure for obtaining and assaying dsRNA interference against the Tilapia myostatin gene in zebrafish

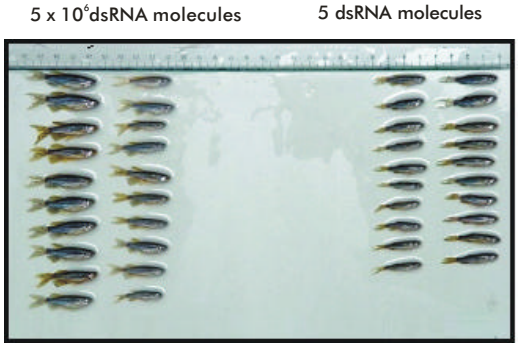

Figure 2. Phenotypic properties of dsRNA-microinjected zebrafish (M), compared to their uninjected controls (C). Fish receiving both dsRNA dosages dsRNA showed normal development.

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6. Li YX, Farrell MJ, Liu R, Mohanty N, Kirby ML. Double-stranded RNA injection produces null phenotypes in zebrafish. Dev Biol (2000); 217:394-405.

The number of fibers in a fixed area was significantly higher in animals microinjected with 5 dsRNA molecules (54.06 ± 9.72; mean ± standard deviation) compared to control animals (44.25 ± 7.27). This increase denotes a hyperplasic growth of muscular fibers derived from dsRNA-specific myostatin silencing (Figure 3).

By analyzing fish microinjected with 5 x 10^6 dsRNA molecules, the mean number of fibers for a fixed area was significantly higher in control animals (40.81 ± 7.33) than in animals injected with the dsRNA (27.43 ± 4.56). The average area of the individual fibers increased in 48.7% in microinjected fish over the negative controls. The significantly lower number of muscular fibers in a given area for dsRNA microinjected zebrafish, compared to their controls, indicates an increased number of fibers, denoting hypertrophy. It is relevant to notice that the number of fibers in negative control animals for both dosages of dsRNA showed no statistically significant differences in the number of muscular fibers (Figure 4).

Finally, to demonstrate that the resulting phenotype was caused by myostatin silencing at a molecular level, a semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was carried out by using the Max gene to control PCR efficiency. The highest dsRNA dosage was used. The RT-PCR showed a drastic reduction in myostatin mRNA in zebrafish, 24 hours after fertilization (hpf), compared to the controls (Figure 2).

The RNA interference technology employed to silence the myostatin gene in zebrafish induced a drastic increase in the muscular mass due to hyperplasia or hypertrophy of muscle fibers. RNA interference technology is a supplementary method to study gene function in fish. These results suggest that molecules blocking the activity of myostatin could be very useful for increasing the muscular mass in relevant aquaculture species.

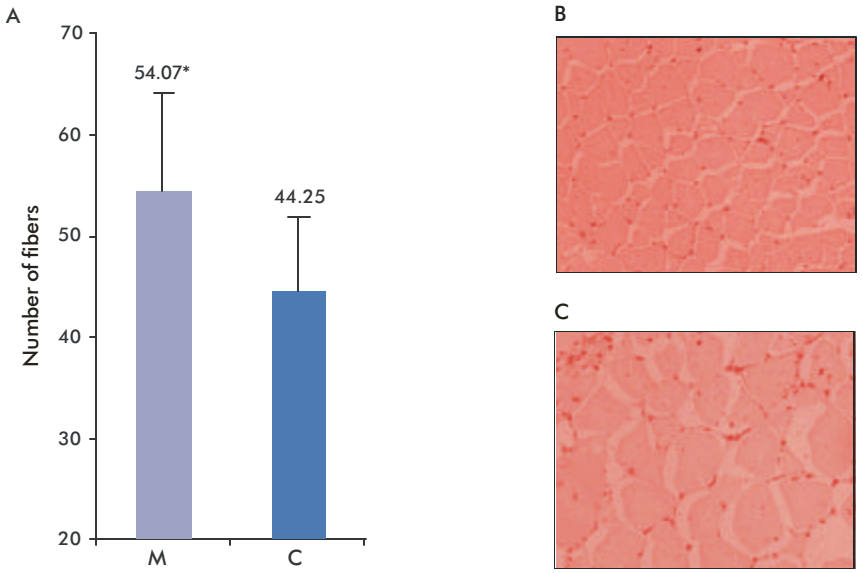

Figure 3. Effect of microinjecting dsRNA on the number of muscular fibers.
(A) Average number of fibers in a given area, in animals microinjected (M) with 5 dsRNA molecules compared to their controls (C).
Hematoxilin/eosin staining of transversal sections in microinjected animals and their controls (B) and (C) respectively.
* Indicates statistically significant differences..

Cloning and characterization of the hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique

The crustacean hyperglycemic hormone (CHH) is the first hormone of the sinus gland of the Atlantic Ocean shrimp *L. schmitti* characterized [10]. Its amino acid sequence was obtained by Edman automatic degradation, enzyme digestion and mass spectrometry [10]. The CHH is mainly synthesized in the X-organ-sinus gland complex in the eyestalk and it shows different expression levels during gonad development [10]. The main CHH activity is to elevate glucose concentration in the hemolymph by a process of glycogen degradation in the hepatopancreas [11]. Besides its primary role in energetic regulation, CHH has been demonstrated to be pleiotropic. It also participates in reproduction, molting, digestion, osmoregulation and lipid metabolism in different species [12]. The CHH family is found exclusively in arthropods. Sequence analysis comparison shows a high percent of identity among CHHs in penaeid shrimps. This property has led to design degenerate primers from conserved regions of these hormones in order to isolate CHH genes family. In the shrimps farming, the developed techniques to stimulate shrimps reproduction are mainly focused in the CHH genes family. However, the *L. schmitti* CHH family has been little characterized. In this study, we have applied the molecular biology techniques to this important species of industrial exploitation. We have isolated, cloned and characterized the first CHH cDNA from an Atlantic Ocean shrimp, *L. schmitti*. This work

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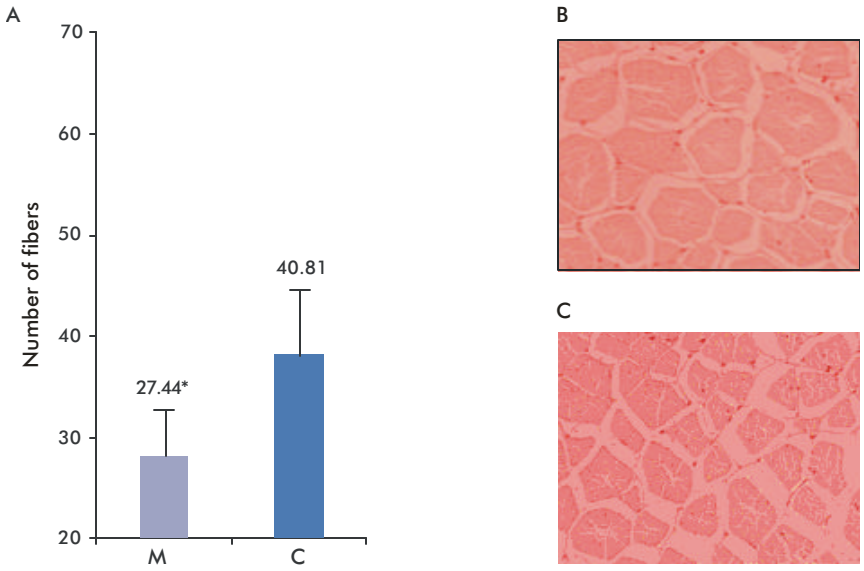

Figure 4. Effect of microinjecting dsRNA on the number of muscular fibers.
(A) Average number of fibers in a given area, in animals microinjected (M) with 5 x 10^6 dsRNA molecules compared to their controls (C).
Hematoxilin/eosin staining of transversal sections in microinjected animals and their controls, B) and C), respectively.
* Indicates statistically significant differences.

also constitutes the first evidence that the dsRNA technique is functional in adult shrimps *in vivo*.

To isolate by RT-PCR the gene coding for the CHH mature peptide from *L. schmitti*, two degenerate primers, corresponding to the N-terminal and C-terminal regions of the CHH [10]. A complementary DNA (cDNA) fragment of 216 bp (primers included) was properly amplified using these primers (Figure 6).

The nucleotide sequence obtained for the CHH cDNA from *L. schmitti* was compared to other CHH nucleotide sequences reported in penaeid shrimps by ClustalW analysis (http://www.ebi.ac.uk/Tools/clustalw). The highest nucleotide identity (89%) was with *Marsupenaeus japonicus* CHH (Pej-SGP-II). It possesses more than a 70% identity with other eyestalk CHHs of penaeid shrimps such as *Penaeus monodon* (80%), *Metapenaeus ensis* (77%) and *Litopenaeus vannamei* (73%) (Figure 1 in [13]). The deduced aminoacid sequence of the obtained cDNA [10]. This is 72 amino acid residues long and possesses six conserved cysteine residues at the same position as that of other CHHs of penaeid shrimps (Figure 1 in [13]).

The size and expression of the mRNA of CHH in the different tissues were determined by Northern blot analysis of the total RNA isolated from the ocular peduncle, muscle and stomach of *L. schmitti*. A fixed amount of each sample RNA (10 µg) was transferred to a nitrocellulose membrane. The cDNA of the mature CHH peptide from organ X in the ocular peduncle of *L. schmitti*. The quality of RNA samples was corroborated by hybridizing the same nitrocellulose membrane with the beta actin gene cDNA, its transcript being observed as a defined band in all the RNA samples analyzed. The expression of the CHH mRNA was observed in organ X of the ocular peduncle, but not in the muscle or the stomach. The estimated size of the CHH transcript was of 1 kb (Figure 2 [13, 14]). These results agree with to those reported in the literature, describing the complex ocular peduncle-organ X-sinus gland as the main source for the synthesis of the CHH family of peptides [15]. These results agree with reports showing the ocular peduncle as the only structure producing the translated form of organ X CHH, except the pericardium organ that produces alternative variants of the translated CHH.

Moreover, the CHH expression was determined in tissues by RT-PCR, by using CHH specific primers. A DNA fragment of the expected size was isolated from the ocular peduncle and the stomach, and another less intense fragment was isolated from the muscle (Figure 2 in [13]). These results suggest a lower-differential expression of the CHH in stomach tissues, conditioned by the molting stage. The weak band observed in the muscle suggests a similar expression pattern in that tissue.

To investigate the ability of the dsRNA to interfere in the CHH gene function in adult shrimps, the dsRNA was synthesized *in vitro* from the mature CHH peptide cDNA as the template, by a procedure similar to that previously described (Figure 7). The group receiving 20 µg of dsRNA in the abdominal cavity showed a significant decrease ($p < 0.05$) in the hemolymph glucose concentration (43% less) 24 hrs after the injection (Figure 3 in [13]).

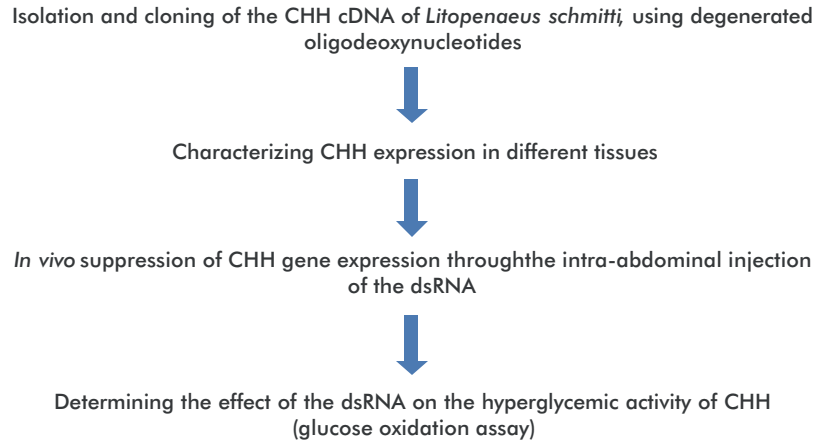

Figure 5. Experimental procedure to obtain and test the interfering dsRNA against the gene of the Atlantic Ocean shrimp *Litopenaeus schmitti* CHH

To demonstrate the specificity of the dsRNA gene silencing mechanism, a group of shrimps was injected with 20 µg of a dsRNA unrelated to the CHH. The unrelated dsRNA was synthesized from a transcript isolated from the stomach of a *L. schmitti* shrimp, coding for a protein homologous to chitinase. As expected, this experimental group did not show any significant decrease of glucose concentration in the hemolymph ($p > 0.05$) (Figure 3 in [13]), the CHH activity being unaffected. Additionally, the silencing of the unrelated gene was corroborated 24 hrs after treatment by Northern blot. This demonstrated the appropriate functioning of the silencing mechanism. In the mixture of total RNA from shrimps of the control group, receiving the same volume of saline, a defined signal at the expected size was observed (500 bp). No signals were observed in the total RNA samples from stomach tissues in shrimps treated with the CHH-unrelated

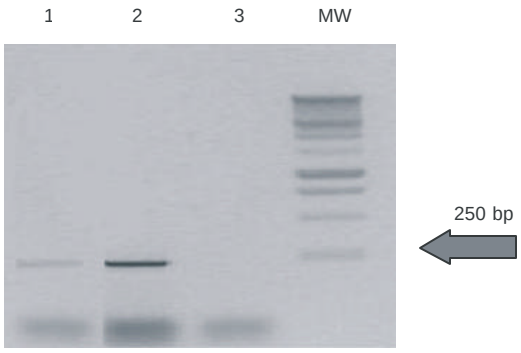

Figure 6. Electrophoresis in agarose gel (1.5% w/v), that shows the results of the RT-PCR prepared to amplify the CHH cDNA from *L. schimitti*.
Lane 1. RT-PCR using as template 5 µg of eyestalk total RNA from *L. schmitti*.
Lane 2. RT-PCR using as template 10 µg of eyestalk total RNA from *L. schmitti*.
Lane 3. Negative control (RT-PCR without template).
Lane MW. Molecular weight marker 1 kb DNA ladder (Promega, USA).

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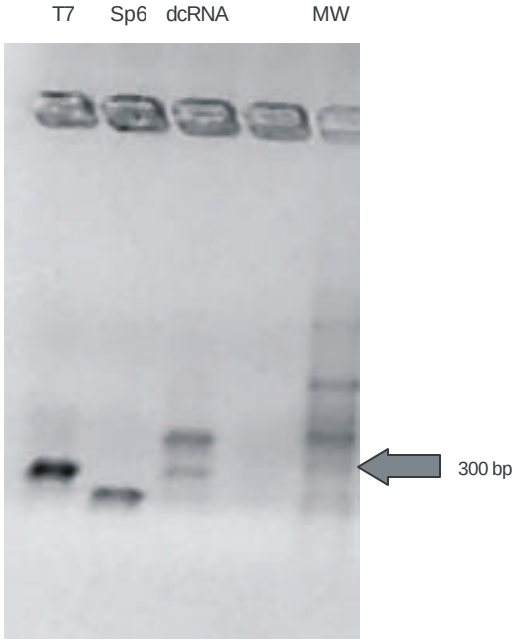

Figure 7. *In vitro* synthesis of the dsRNA. T7- synthesis of the first RNA strand with the T7 oligonucleotide. Sp6- synthesis of the second RNA strand with the Sp6 oligonucleotide. dsRNA- formation of the double stranded hybrid. MW-RNA molecular weight marker.

dsRNA. This evidenced the satisfactory gene silencing at the transcriptional level (Figure 4 in [13]).

Silencing of the CHH gene was also checked by Northern blot analysis and semi-quantitative RT-PCR. All shrimps were sacrificed 24 hrs after treatment and their ocular peduncle removed for total RNA extraction. Twenty micrograms of each total RNA sample were transferred to the nitrocellulose membrane.

The Northern blot analyses were carried out using the CHH cDNA amplified by PCR as the radioactive probe, showing a defined signal of the expected 1 kb size from the ocular peduncle total RNA of shrimps treated with saline. In contrast, no signal was evidenced in RNA samples from shrimps treated with the CHH-specific dsRNA (Figure 5 in [13]), demonstrating that the dsRNA was able to completely silence the CHH gene. The levels of CHH transcripts were compared to those of the β-actin control gene in the same nitrocellulose membrane. The band corresponding to the β-actin control gene was observed as defined in all the ocular peduncle total RNA samples analyzed. This result evidenced once more that gene silencing is a target-specific mechanism (Figure 5 in [13]).

Similar results were obtained in the semi-quantitative RT-PCR assays. A defined fragment of DNA of 216 bp in size was observed, corresponding to the CHH cDNA, only in those total RNA samples from shrimps treated with saline (Figure 6 [13]). As expected, a DNA fragment corresponding to the β-actin control gene was also amplified from all the ocular peduncle RNA samples analyzed (Figure 6 in [13]).

All the above-mentioned results corroborated the specific suppression of the CHH gene expression by injecting a dsRNA specific for the CHH, with the injection of an unrelated dsRNA not diminishing glucose levels in the hemolymph and the normal expression of the β-actin control gene after the injection of the dsRNA molecules.

Our results show for the first time that the intra-abdominal injection of dsRNA could be used to interfere in RNA expression and cause gene silencing *in vivo* in adult shrimps.

Scientific relevance

These are pioneering studies in our country, and are of great significance worldwide. They demonstrate the potential of the RNAi mechanism, and its ability to demonstrate gene function and concepts, to generate new biotechnological products applied in different fields of research, or to generate information to design new drugs.

The RNA interference technology used for silencing the myostatin expression in zebrafish, promoted a dramatic increase of the muscular mass due to hyperplasia or hypertrophy in muscle fibers. We demonstrated the functionality of the RNA silencing technique as being complementary to the myriad of pre-existing methods to study gene function in fish. Additionally, our results suggest that the molecules blocking myostatin activity could be very useful agents for increasing the muscular mass in relevant aquaculture species .

The CHH gene of the Atlantic Ocean shrimp*Litopeneaus schmitti* was cloned and sequenced for the first time, also demonstrating that the intra-abdominal injection of dsRNA molecules can be used for RNA interference and gene silencing *in vivo* in adult shrimps. These findings could evolve as a powerful tool for studying gene function in crustaceans.

This is the first report in Cuba on the use of RNA interference to study gene function in aquatic organisms.

These results have a great impact in Aquatic Biotechnology, characterizing the function of genes relevant for scientific and productive purposes.

Conclusions

1. The RNA interference mechanism is useful to study gene function in fish and crustaceans

2. The RNAi-mediated gene silencing of myostatin in zebrafish drastically increases the muscular mass due to hypertrophy of muscle fibers, demonstrating the myostatin function in zebrafish as negative regulator of muscle growth.

3. The RNAi-mediated silencing of the CHH gene in shrimps significantly decreases the glucose levels in the hemolymph, demonstrating the function of the CHH in the energy metabolism in *L. schmitii*.

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