



## BACKGROUND OF THE INVENTION

### METHOD OF STIMULATING GROWTH AND RESISTANCE TO DISEASES OF AQUATIC ORGANISMS

5 The invention is related with the chemical synthesis for increase the growth rate in marketable fish and crustacean. The synthesis of peptide analogs of Leu and Met enkephalins was reported and showed that they stimulated growth hormone (GH) release in animals (Bowers C, Momany, G, Reynolds G and A. Hong, 1984, On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the Pituitary to specifically  
10 release growth hormone. Endocrinology . 114: 1537-45).

Studies to investigate structure-activity relationships of GH releasing peptides (GHRPs) continued and led to the identification of GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH<sub>2</sub>), who has been demonstrated to be an extremely potent and safe GH secretagogue (GHS) in animals, including humans, however not previous report has been published using this  
15 compound in aquatic organisms.

We demonstrated as a novel fact, taking into account the lack of evidence crustaceans of the signal cascade to stimulate growth (hypothalamus - pituitary gland - target organ), that the peptide GHRP-6 alone is able to exert a similar biological function in crustaceans than in mammals.

20 Fresh water fish is the principal production in aquaculture, nevertheless in the last decade the increasing of culture of marine algae, mollusks and crustaceans had been significantly. The improving knowledge of the genetic, reproduction, nutrition and the physiology of the organisms to cultivate is the first step to improve the aquaculture production (Gómez-Chiarri M, Smith GJ, de la Fuente J and Powers DA. 1998. Gene  
25 transfer in shellfish and algae. In de la Fuente J and Castro FO, editors. Gene transfer in aquatic organisms. Austin, Texas: RG Landes Company and Germany: Springer Verlag; p.107-125).

The study of the molecular characteristic from the hormone and peptides involved in the growth mechanism in the marketable fish and crustacean species is very important to use  
30 in the improving the aquaculture.

One example to understand that is the use of gonadotropin release hormone (GnRH) and the antagonist of the dopamine receptor used by Silverstein et al., in 1999 (Silverstein JT.,

Bosworth BG. and Wolters WR. 1999. Evaluation of dual injection of LHRHa and the dopamine receptor antagonist pimozide in cage spawning of channel catfish *Ictalurus punctatus*. Journal of the World Aquaculture Society. Vol. 30, No.2, June, 263 – 268) for the regulation and induction of reproduction in catfish (*Ictalurus punctatus*). This species

has high importance in world aquaculture.  
Hashizume et al., reported in 1997 (Hashizume T., Sasaki M., Tauchi S. and Masuda H. 1997. The effect of new growth Hormone-releasing peptide (KP 102) on the release of growth hormone in goats. Animal Science and Technology. Vol. 68, No.3, March, 247-256) the use of a synthetic peptides for the increase of the productivity in animal farms.

They demonstrated the induction of growth hormone in goat by injection with a novel growth hormone release synthetic peptide

Insulin had been administrate to fish orally and demonstrated changes in signal of receptor and hormones involved in carp adaptation to different temperatures (Vera MI., Romero F., Figueroa J., Amthauer R., Leon G., Villanueva. and Krauskopf M. 1993. Oral administration of insulin in winter-acclimatized carp (*Cyprinus carpio*) induces hepatic ultrastructural changes. Comp. Biochem. Physiol. Vol.106A, No.4, 677-682)

In mammals had been tested synthetic variants of the growth hormone release peptide by Patchett et al., in 1995. They probed the peptide MK-0677, designated as potent oral activator of the GH in dogs without effect in the tyrosine and prolactin levels after application (Patchett AA., Nargund RP., Tata JR., Chen MH., Barakat KJ., Johnston DBR., Cheng K., Chan WWS., Butler B., Hickey G., Jacks T., Schleim K., Pong SS., Chaung LYP., Chen HY., Frazier E., Leung KH., Chiu SHL. and Smith RG. 1995. Proc. Natl. Acad. Sci. USA. Vol.92, 7001-7005)

Other application of the growth hormone release hormone had been developed in cattle to increase the milk production, taking into account the stimulation in milk production produced by the increased level of the circulating GH (Soliman EB., Hashizume T., Ohashi S. and Kanematsu S. 1997. Effects of Growth hormone (GH)-releasing hormone and its analogs on GH secretion from cultured adenohypophysial cells in cattle. Domestic animal endocrinology. Vol.14(1), 39-46)

There are not previous reports of the use of GHRP-6 in fish or crustacean. The use of GHRP-6 in fish and crustacean to stimulate growth, improve the larvae quality, and increase the defense against pathogen agents, the dry weight, the protein concentration and the RNA in muscle of fish and crustaceans reported in this document comprise a solution for improve the productivity in the aquatic organisms culture.

There are preliminary results in the genetic breeding of some shrimp species like *Penaeus japonicus* and *Litopenaeus vannamei* but these are not enough because the lack of knowledge about the genetic and biochemistry of these organisms (Benzie, J.A.H., 1998. Penaeid genetics and biotechnology. *Aquaculture* 164, 23–47 and Fjalestadl, K.T., Carr, W.H., Lotz, J.L., Sweeney, J.N., 1999. Genetic variation and selection response in body weight and diseases resistance in the Pacific White Shrimp *Penaeus* *Oannamei* . *Aquaculture* 173, 10, Abstract only). It is very important to apply the biotechnology, molecular biology, genetic engineering and biochemistry to the culture of shrimp to improve the productivity (Bachere, E., Mialhe, E., Noel, D., Boulo, V., Morvan, A., Rodriguez, J. (1995) Knowledge and research prospects in marine mollusc and crustacean immunology. *Aquaculture*.132, 17-32).

This invention support that the GHRP-6 alone is able to stimulate growth, improve the larvae quality, and increase the defense against pathogen agents, the dry weight, the protein concentration and the RNA in muscle of fish and crustaceans.

#### ESSENCE OF THE INVENTION.

The essence and novelty of this invention support that the peptide GHRP-6 alone is able to stimulate growth, improve the larvae quality, and increase the defense against pathogen agents, the dry weight, the protein concentration and the RNA in muscle of fish and crustaceans. The secretagogue GHRP-6 alone is able to stimulate growth orally, by injection or immersion in fish and crustaceans.

#### Brief description of the figures:

**Figure 1.** Tilapia IGF mRNA levels and serum GH levels in juvenile tilapia injected with the GHRP-6 peptide. Each group intraperitoneally injected was of three animals. Liver and serum sample were taken 15, 30, 60 and 360 minutes post injection. A sample serum was taken from each animal before the treatment. \*Significantly different from the control group ( $p < 0.001$  ANOVA, Duncan test). The standard error bar is showed.

**Figura 2.** Effect on body weight and growth rate of GHRP-6 intraperitoneally injection in juvenile tilapia over a three-week period. \*Significantly different from the control group ( $p < 0.029$ ; Student's t test) Bars indicate standard error. Triangle indicate the mean growth rate.

**Figura 3.** Effect on body weight and growth rate of GHRP-6 non encapsulated orally intubated in juvenile tilapia over a three week period. \*Significantly different from the

control group ( $p = 0.015$ ,  $t$  Test) Bars indicate standard error. Triangle indicate the mean growth rate.

Figure 4. Effect in juvenile tilapia over a three-week period treated orally with GHRP-6 encapsulated on body weight and growth rate. Significantly different from the control group ( $*p = 0.05$ ;  $**p = 0.007$ ,  $t$  Test) Bars indicate standard error. Triangle indicates the mean growth rate.

Figure 5. Growth promoting activity of GHRP-6 in shrimp *Litopenaeus schmitti* treated with GHRP-6 by immersion

A: Increase of weight in milligrams of the three groups treated with the GHRP-6 and the control group. B: Increase of size in millimeters of the three groups treated with the GHRP-6 and the control group. C: Absolute frequency distribution of the gill ramifications of the three groups treated with the GHRP-6 and the control group. D: Absolute frequency distribution of the rostral bone of the three groups treated with the GHRP-6 and the control group. Groups I, II and III were treated with different doses of the peptide. Control: Group treated with BSA. \*\*\*  $p < 0.001$ . Bars indicate standard deviation ( $\pm DS$ ). ANOVA follow by a DUNCAN test were applied for the statically calculation of the difference in weigh and size. Kolmogorov-Smmirnov was applied to rostral bone and gill ramifications.

Figure 6. Dry weigh of the shrimp treated with the GHRP-6 and the control group. \*\*\*  $t$ -test  $p < 0.001$ . Bars indicate standard deviation.

Figura 7. Relation between RNA, protein and DNA of the shrimp treated with the GHRP-6 and the control group. The statistical difference show the hypertrophy promoted by peptide in comparison on the control group. \*\*\*  $t$ -test  $p < 0.001$ . Bars indicate standard deviation.

Figure 8. Growth rate of larvae shrimp *Litopenaeus schmitti* in production conditions treated with GHRP-6 by immersion. The control group received BSA. The experiment was follow by six week. \*\*\*  $t$ -test  $p < 0.001$ . Bars indicate standard deviation.

#### DETAILED DESCRIPTION OF THE INVENTION.

Firstly, the biological activity to promote the releasing of GH of the hexapeptide GHRP-6 (Growth hormone release peptide), with the sequence corresponding to: His-D-Trp-Ala-Trp-D-Phe-Lys-NH<sub>2</sub>, was demonstrated like in mammals and birds (Bowers C, Momany,

G, Reynolds G and A. Hong. 1984. On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the Pituitary to specifically release growth hormone. *Endocrinology* . 114: 1537-45).

As the teleost GH levels are very variable along the day serum samples were taken before the treatment and after that per each fish. The result shows there was an increased in serum GH levels in the time coursing experiment at 15 min after the intraperitoneal hexapeptide treatment. In teleost fish, as in other vertebrates, the growth promoting action of GH is mainly mediated by the induction of IGF. The level of hepatic IGF mRNA increased significantly in response to intraperitoneal injection of GHRP-6 at 30 min backing to the normal value during six hours after injection. (Example 1).

It was demonstrated that the peptide GHRP-6 alone exert a similar biological function in fish than in mammals and birds suggesting that this mechanisms is similar.

In vivo, the role of the GHRP-6 in controlling body growth in freshwater juvenile tilapia was assessed. The treatment with GHRP-6 using different ways of administration significantly stimulated growth in comparison with control group as determined by the increase in growth rate after three weeks of treatment by three different ways of administration; a) Intraperitoneal injection, b) Oral and c) Immersion. (Example 2)

In 1g tilapia larvae were demonstrated the effect of the hexapeptide GHRP-6 applied by immersion in the increasing of pathogen resistance and muscle quality. (Example 3). The result showed that the tilapia groups treated with the GHRP-6 increased the weigh and protein concentration and decrease the intensity and extension of pathogen invasion and the water content of the muscle. This results suggest the positive effect of the hexapeptide in the protein synthesis.

GHRP-6 was assayed in shrimp *Litopenaeus Schmitt* demonstrate that alone was able to improve the growth rate in crustaceans by different way of administration: a) Intraperitoneal injection, b) Oral and c) Immersion. (Example 4)

The hexapeptide was assayed in shrimp *Litopenaeus schmitti* demonstrate that alone was able to improve the growth rate by the application of 4-immersion bath in different larvae stage. The quality of the larvae at the final of the larvae culture cycle was improved by the peptide. The larvae showed significantly increase in weigh, size, number of gill ramifications, modification of rostral bone, protein concentration and RNA in the muscle. It was evident an increased metabolic activity.

The improved quality of the larvae was maintain by the adults shrimp yield an increase biomass and more uniformity in the weigh and size of the animals. GHRP-6 was able to promote the growth rate in adult shrimp *Litopenaeus schmitti*, by intramuscle injection. After 15 days of the injection of the GHRP-6 the group of animals treated showed an increase between 100% to 150% respect to a control group. (Example 5)

The secretagogue GHRP-6 administrated orally in the diet or encapsulated in *Artemia salina* improved the growth rate in shrimp *Litopenaeus Schmitt*, between 30% to 40 % respect a control group. (Example 6)

### Examples

#### Example 1. Biological activity demonstration of the GHRP-6 in fish.

Fifteen tilapias with average weight  $71 \pm 28$  g were used for the experiment. The GHRP-6 was injected with hexapeptide at  $0.1 \mu\text{g/gbw}$ . The liver and blood samples were drawn before the treatment and 15, 30, 60 and 360 minutes after the peptide injection (three animals per group). The serum and liver sample were collected and stored at  $-70^{\circ}\text{C}$  until used for total RNA isolation and Northern blot analysis or for measurement of GH level by ELISA. A serum sample was also taken before the treatment in all animals.

Northern blot analysis was used to measure the relative IGF mRNA levels in tilapia injected with the peptide. Total RNA was purified from liver samples as described by Chomczynski and Sacchi (Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-59). Twenty  $\mu\text{g}$  of RNA were fractionated on 1% formaldehyde agarose gels and transferred to nylon membranes (Hybond N, Amersham UK) and hybridized with a tilapia IGF-I cDNA probe and subsequently rehybridized with a human glyceraldehyde 3 phosphate dehydrogenase cDNA (GAPDH, kindly given by Dr. Bryan Williams, Cleveland Foundation, OH, USA) to normalize the signals. Hybridization signals were quantified by digital image processing of scanned gels with a Hewlett Packard Scanjet Plus scanner. Images were processed with the Bandleader computer program. Results were expressed in RNA arbitrary units.

Serum GH levels were determined by ELISA using two monoclonal antibodies against tilapia GH (Muñoz et al, in preparation).

As the teleost GH levels are very variable along the day serum samples were taken before the treatment and after that per each fish. Figure 1 shows there was an increased in serum

GH levels in the time coursing experiment at 15 min after the intraperitoneal hexapeptide treatment. In teleost fish, as in other vertebrates, the growth promoting action of GH is mainly mediated by the induction of IGF. The level of hepatic IGF mRNA increased significantly in response to intraperitoneal injection of GHRP-6 at 30 min backing to the normal value during six hours after injection. (Figure 1)

## Example 2. Growth promoting activity of GHRP-6 in juvenile (*Oreochromis sp*) tilapia

### 2.1 Intraperitoneal injection

GHRP-6 (BACHEM, Switzerland) was diluted in PBS and injected twice a week during three weeks at 0.1  $\mu$ g per gram of fish body weight (gbw). The peptide was administered to a group of eight male tilapia with average body weight of  $61.41 \pm 14.36$  g and seven male tilapia with average body weight of  $61.58 \pm 29.67$  g were injected with PBS as a control group.

Body weight in grams was measured weekly. In all the experiments the animals were labeled with microchips (Stoelting Co. Wood Dale, U.S.A.). (Figure 2)

### 2.2 Oral administration

Other ways of peptide releasing were evaluated orally intubated peptide (Figure 3) and encapsulated intubated peptide (Figure 4), the growth rate was also statistically significantly in both treatments to compare with the respective controls groups, and in the later case there were statistically differences in the body weight to the end of the treatment. A comparison between both orally intubated treatment showed a higher body weight gain with the encapsulated peptide treatment one.

#### 2.2.1. Liquid peptide solution

GHRP-6 peptide was diluted in phosphate buffered saline (PBS) and administered through a plastic tube to the pharyngeal cavity to a group of seven male tilapia with average body weight  $84.66 \pm 12.2$  g. PBS was delivered to the control group (n=7, all male tilapia) with average weight  $86.38 \pm 6.26$  g. The treatment was done twice a week

during three weeks at 0.1  $\mu\text{g}$  / gbw. Body weight in grams was measured weekly. (Figure 3)

### 2.2.2. Encapsulated peptide

For the encapsulation of peptide the capsules were obtained as previously reported by Knorr et al., 1988; using chitosan and alginic acid encapsulated peptide. The hexapeptide GHPR-6 was administered through a plastic tube to the pharyngeal cavity to seven male tilapia with average body weight  $89.09 \pm 8.38$  g. Polymer beads without peptide were administered to seven male tilapia with average weight  $89.86 \pm 13.54$  g as a control group. The treatment was performed twice a week at 0.1  $\mu\text{g}$  / gbw. (Figure 4)

### Example 3. Growth promoting activity of GHRP-6 in juvenile (*Oreochromis sp*) tilapia by immersion

Tilapias (*Oreochromis aureus*) of 1.5 g of mean weigh were treated by immersion with two different doses of GHRP-6 (10  $\mu\text{g}$  /100ml and 100 $\mu\text{g}$  /100ml). A similar control group was treated with physiological solution.

In the experiment was measured the weigh (Table 1), biochemical blood parameters (Table 2), number of parasite in gill (*Trichodinicos* (Table 3) y *Helmintos mongeneos* (Table 4)) and humidity and protein concentration in muscle

Fifteen animals per group (3x) with mean weigh of 1g were selected for the assay. In 9 tank of 40 liters were developed the experiment. One a week application was done for 45 days.

The groups and doses were distributed as followed:

Group I 10  $\mu\text{g}$ /100ml (Treatment 1)

Group II 100  $\mu\text{g}$  /100ml (Treatment 2)

Group III Control (Physiological solution)

Table 1. Mean weigh measured in the different groups of tilapia treated with GHRP-6 by immersion

| Treatment                       | n  | Mean weigh<br>(g) $\pm$ DS | Comparison<br>between groups | Statistically<br>difference |
|---------------------------------|----|----------------------------|------------------------------|-----------------------------|
| Grupo I<br>10 $\mu$ g /100 mL   | 25 | 4.56 $\pm$ 1.07            | I-II                         | 0.01454                     |
| Grupo II<br>100 $\mu$ g /100 mL | 25 | 4.54 $\pm$ 1.38            | II-III                       | 1.07177*                    |
| Grupo III<br>Saline solution    | 25 | 3.47 $\pm$ 1.52            | III-I                        | 1.08632*                    |

\*Significantly difference. Multiple rank tests

5

Table 2. Hematocrite values in the different groups of tilapia treated with GHRP-6 by immersion

| Treatment                       | n  | Mean weigh<br>(g) $\pm$ SD | Comparison<br>between groups | Statistically<br>difference |
|---------------------------------|----|----------------------------|------------------------------|-----------------------------|
| Grupo I<br>10 $\mu$ g /100 mL   | 15 | 27.46 $\pm$ 4.53           | I-II                         | (2.4)*                      |
| Grupo II<br>100 $\mu$ g /100 mL | 15 | 25.06 $\pm$ 5.25           | II-III                       | (1.0)*                      |
| Grupo III<br>Saline solution    | 15 | 26.46 $\pm$ 4.08           | III-I                        | (1.4)*                      |

10

\* There is not significantly difference. Multiple rank tests

Table 3. Invasion intensity (I) and extension (E) in the tilapia treated with GHRP-6 by immersion for the protozoo *Trichodina* sp.

| Treatment                    | n  | I <sup>a</sup> | E (%) <sup>b</sup> | Comparison between groups | Statistically difference |
|------------------------------|----|----------------|--------------------|---------------------------|--------------------------|
| Grupo I<br>10 µg /100 mL     | 25 | 7.73           | 100                | I-II                      | (4.42)                   |
| Grupo II<br>100µg /100 mL    | 25 | 2.80           | 84.6               | II-III                    | (5.84)*                  |
| Grupo III<br>Saline solution | 25 | 8.76           | 92.30              | III-I                     | (1.42)                   |

<sup>a</sup>I: Invasion intensity

<sup>b</sup>E: Invasion extension

\*Significantly difference. Multiple rank tests

Tabla 4. Invasion intensity (I) and extension (E) in the tilapia treated with GHRP-6 by immersion for *Helminos monogeneos* in the gill per fish.

| Treatment                    | n  | I <sup>a</sup> | E (%) <sup>b</sup> | Comparison between groups | Statistically difference |
|------------------------------|----|----------------|--------------------|---------------------------|--------------------------|
| Grupo I.<br>10 µg /100 mL    | 25 | 0.39           | 34.7               | I-II                      | (0.304)                  |
| Grupo II<br>100µg /100 mL    | 25 | 0.66           | 50                 | II-III                    | (0.521)                  |
| Grupo III<br>Saline solution | 25 | 1.07           | 46                 | III-I                     | (0.826)*                 |

<sup>a</sup>I: Invasion intensity

<sup>b</sup>E: Invasion extension

\*Significantly difference. Multiple rank tests

Table 5. Humidity mean values in muscle of the tilapia treated with GHRP-6 by immersion.

| Treatment                    | n  | Mean humidity<br>± SD (%) | Comparison<br>between groups | Statistically<br>difference |
|------------------------------|----|---------------------------|------------------------------|-----------------------------|
| Grupo I<br>10 µg /100 mL     | 24 | 82.96±3.63                | I-II                         | (0.791)                     |
| Grupo II<br>100µg /100 mL    | 24 | 83.5±3.31                 | II-III                       | (2.666)*                    |
| Grupo III<br>Saline solution | 24 | 86.42±3.23                | III-I                        | (3.458)                     |

\*Significantly difference. Multiple rank tests

5

Table 6. Protein concentration mean values in the tilapia treated with GHRP-6 by immersion.

| Treatment                    | n  | Mean humidity<br>± SD (%) | Comparison<br>between groups | Statistically<br>difference |
|------------------------------|----|---------------------------|------------------------------|-----------------------------|
| Grupo I<br>10 µg /100 mL     | 23 | 6.10                      | I-II                         | (1.160)*                    |
| Grupo II<br>100µg /100 mL    | 23 | 4.94                      | II-III                       | (1.38)*                     |
| Grupo III<br>Saline solution | 23 | 3.55                      | III-I                        | (2.64)*                     |

\*Significantly difference. Multiple rank tests

10

**Example 4. Growth promoting activity of GHRP-6 in shrimp *Litopenaeus schmitti* treated with GHRP-6 by immersion.**

Three different doses of GHRP-6 were applied by immersion to groups or shrimp larvae.

15 The application was made each three days. Four applications were made for each group,

including one control group receiving bovine serum albumin (BSA). The treatment was made per 1 hour to a concentration of GHRP-6 per liter of seawater as follow:

Group 1: 0.001 mg/ L

Group 2: 0.01 mg/ L

5 Group 3: 0.1 mg/L

Group 4: 1 mg of BSA / L

The group treated with 0.1 mg / L of GHRP-6 improve the quality of the shrimp larvae showing an increase mean weigh in 125 - 153%, mean size in 15 - 26%, number of gill ramifications and the number of bone rostral. In all cases the difference was statistical significantly. (Figure 5)

The entire treated animal showed lower water content in the muscle and increased the value in the relation between RNA/DNA, Protein/DNA suggesting the activation of the metabolism in the muscle of the larvae by the GHRP-6. (Figure 6 and 7)

15 These results are very important taking into account the relevancy of the quality of the larvae for the culture of crustaceans.

In production condition the survival rate of the larvae was increased in 20% in the group treated with the GHRP-6 in comparison with the non-treated group. In the same assay the weigh was increase in 115% and the size in 37%. On the other hand the uniformity of the animals was higher in the group treated with the hexapeptide and the variation coefficient was lower in weigh and size (Figure 8). The variation coefficient for the treated group was of 24% and 9% of the weight and size respectively, in comparison to 77% and 30% in the control group.

**Example 5. Growth promoting activity of GHRP-6 in adult shrimp *Litopenaeus schmitti* treated with GHRP-6 by intramuscle injection**

25 Injection of 50 µl of the GHRP-6 was made between the second and third abdominal segment in adult shrimp. One injection each three days was made with the doses of 1µg of GHRP-6 per grams of weigh of the shrimp. One control group was injected with BSA at the same concentration. Fifteen animals per group were used. The variable measured were weigh and size. Significantly difference were observed in the groups of animals  
30 treated with the GHRP-6 ( $p < 0.001$ ) with an increase between 100% - 150%.

The animals were maintained in a net (2 x 2 x 1 meters) into a land pond. The pond water temperature was 25°C and the photoperiod natural. The salinity was 30g/L.

**Example 6. Growth promoting activity of GHRP-6 in shrimp *Litopenaeus schmitti* treated with GHRP-6 included in the diet**

5 The GHRP-6 was included at 1% in the diet of post larvae shrimp *Litopenaeus schmitti*. The peptide was encapsulate by a method reported by Knorr (Knorr D. and M.Daly. (1988). Mechanics and diffusional changes observed in multi-layer chitosan/alginate coacervate capsules. Process Biochemistry; 48-50). One control group was feed with the diet with 1% of BSA. The mean weigh and size were measured at the beginning and at  
10 the end of the experiment. The duration of the experiment was 30 days.

The GHRP-6 included in the diet of the post larvae of shrimp improves the growth rate in 30% - 40% respect to a control group. Significantly difference. ( $p < 0.001$ ).

**6.1. *Artemia salina* encapsulation**

15 The GHRP-6 was encapsulated in *Artemia* to feed the post larvae of shrimp *Litopenaeus schmitti* and *Litopenaeus vanamei*. For the encapsulation the peptide was added in *Artemia salina* in a concentration of 10 mg/L for an hour and the artemia was harvested and washed in saline solution. Four time a days were feed the post larvae of shrimp during a month. The control group received BSA encapsulated in artemia at the same concentration.

20 The GHRP-6 encapsulated in *artemia salina* improve the weigh and size of the shrimp larvae in 30%-40%. Respect to the control group. Significantly difference. ( $p < 0.001$ ).

**Example 7. Growth promoting activity of GHRP-6 in larvae of salmon (*Salmon salar*) treated with GHRP-6 by immersion**

25 There took three groups of larvae of salmon (*Salmon salar*) and six immersion baths were applied to them, one every three days of an hour of duration with different concentrations of the peptide GHRP-6 to two of them and a group was taken as a control. The used concentrations of the peptide were: 0.01 and 0.1 mg/L, in the groups I and II respectively, to the control group it was given the same frequency of immersion baths with BSA to 1  
30 mg/L.

As result observed, that in the group treated with the major concentration of peptide 0.1

mg/L, was increased in weight of 120-145 %, taking 100 % of the group as a reference control and observed an increase of 15-26 % in the size with regard to the group control. On the other hand it was found that the animals treated with GHRP-6 had minor contained of water in the muscle, increase in the values of the relationships RNA/DNA, protein/DNA, giving an idea of the activation of the metabolism in the muscle of these larvae.

These results perform great importance since on having accelerated the growth of the larvae of salmon in this stage; a higher survival is obtained in this phase of culturing what is vital to increase the productivity of this species so important in the trade of fish in the world.

**Example 8. Effectiveness of the peptide GHRP-6 over the ectoparasite "sea lice" *Caligus sp.* infection in trout (*Oncorhynchus mykiss*).**

Trout treated in their stage of larvae by means of given immersion baths every three days, in a whole of 6 baths, with the peptide GHRP-6 to concentrations of 0.01 mg/L, 0.1 mg/L and a group control, were put in cages of go on in a region affected by the ectoparasite *Caligus sp.* Of every cage there were tested five times 20 fishes to intervals of one week. Both groups treated in their larvae stages with the peptide GHRP-6 reduced significantly ( $p < 0.05$ ) the infestation of *Chalimus* and *Caligus* (two important types of "sea lice") adults in relation with the group control, not existing difference between the treated groups.

## METHOD OF STIMULATING GROWTH AND RESISTANCE TO DISEASES OF AQUATIC ORGANISMS.

### Claims:

5

10

15

20

25

30

1. A Method for the growth stimulation and resistance to diseases of fish and crustaceans characterized by the use of a peptide GHRP-6 with the sequence: His-D-Trp-Ala-Trp-D-Phe-Lys-NH<sub>2</sub>.
2. A method as claim 1, characterized by the use of the GHRP-6 in formulated feed for the growth stimulation and resistance to diseases of fish and crustaceans in concentration of 0.01 – 50 µg of peptide per gram of wet weight of the animal.
3. A method as claim 1, characterized by the use of the GHRP-6 in periodic injections for the growth stimulation and resistance to diseases of fish and crustaceans in concentration of 0.01 – 50 µg of peptide per gram of wet weight of the animal.
4. A method as claim 1, characterized by the use of the GHRP-6 by immersion for the growth stimulation and resistance to diseases of fish and crustaceans in fresh water and seawater at concentration between 10 – 500 µg of peptide per liter in interval between 1 to 7 days.
5. A method as claim 1, characterized by the use the GHRP-6 in feed encapsulated for the growth stimulation and resistance to diseases of fish and crustaceans at concentration between 0.05 – 10 µg of peptide per gram of wet weight of animal in intervals between 1 to 7 days
6. A method as claim 1 to 5 characterized by their application in tilapia *Oreochromis sp.*
7. A method as claim 1 to 5 characterized by their application in *Salmon sp.*
8. A method as claim 1 to 5 characterize by their application in *Litopenaeus schmitti*, *Litopenaeus vanamei*.
9. A method as claim 8 characterize by their use in shrimp *Penaeus sp.*

10. A method as claim 1 to 9 characterize by the use for the preventive and therapeutic treatment in parasites infection and other diseases.
11. A method as claim 10 characterized by the use for the preventive and therapeutic treatment in parasites infection produced by *Trichodina sp.* y Helmintos monogeneos.
12. A method as claim 10 characterized by the use for the preventive and therapeutic treatment in parasites infection produced by sea lice.
13. A method as claim 10 characterized by the use for the preventive and therapeutic treatment in parasites infection produced by ectoparasites.
14. A veterinary formulation for the growth stimulation and resistance to diseases of fish and crustaceans characterized by the use of peptide GHRP-6 with the sequence: His-D-Trp-Ala-Trp-D-Phe-Lys -NH<sub>2</sub>
15. Usage as in claim 14, characterize by its use as injection, oral or immersion administration.