Consolidation of Native Species Aquaculture in Southeastern Mexico: Continuation of a Selective Breeding Program for Native Cichlids and Snook Aquaculture

Indigenous Species Development/Experiment/09IND05UA

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INTRODUCTION

One of the most important fisheries resources of Mexico is the snook group. Its economic importance is so big that this group of fish is considered among the most precious organisms for coastal and inland fishermen. This pressure over the resource has generated a high exploitation resulting in capture decreasing, especially for large organisms- mainly composed of adult females. Within most of the countries of the range of the snooks, the issue is the same. As a result, researchers from United States, Central America and Brasil have demonstrated their concern to develop actions that ensure the continuity of the management of this resource (Tucker y Campbell, 1988; Taylor et al., 1998; Álvarez-Lajonchere et al., 2002; Cerqueira y Tsuzuki, 2009). In the US, especially in Florida and Texas, sport fishing constitutes a very important economic industry and there have been isolated efforts for artificially reproducing these specie for restocking (Tucker y Campbell, 1988; McMichael et al., 1989; Taylor et al., 1998; Neidig et al., 1999; Skapura et al., 1999 and Main et al., 2009).

The common snook *Centropomus undecimalis* and the fat snook *Centropomus parallelus* are two fish species distributed from the US to Brasil in the Atlantic Ocean. They are euryhaline and at juvenile stage are found in estuaries (Castro-Aguirre et al., 1999). These fish are difficult to reproduce in captivity (Álvarez-Lajonchere y Hernández-Molejón, 2001) thus sometimes it is necessary to collect them and adapt them to captivity (Álvarez-Lajonchere y Hernández-Molejón, 2001). This adaptation in most of the cases result in holding the fish for long time, feeding them with fish which can result in high costs (Alves et al., 2006), so it is necessary to develop strategies that allow the inclusion of feed based on meals available in the market. Having artificial feed available for snook in captivity will allow us to give all nutrients the fish need at sufficient levels, resulting in an increase in growth rate (Roberts, 2002). In addition, the determination of snook gene expression let the generation of valuable information on initial ontogeny in order to understand the identity of the best feed to establish the snook culture.

The snook *C. poeyi,* on the other hand is one of the species of this genus with fewer studies. However, it is known that is endemic of the Gulf of Mexico from Tampico, Tamaulipas to Centla, Tabasco and have catadromous habits –adults are found in fresh waters, mature organisms in estuarine zones and in low river zones-. While juveniles are located in mixed-haline environments and at the adjacent neritic zone (Chávez, 1963).

The development of aquacultural techniques for native species not only can reduce the pressure over wild populations, but can also provide a reliable supply for the development and implementation of working plans for restocking the overexploited stocks. At the same time, a sustainable fishery of native species could contribute to solve the rural emigration problem providing new and better employment and income opportunities. The present project arises from the necessity of continuing the development of research that let us achieve the correct methodologies in order to obtain snook fry from captive broodstock. The design of right feeding during early development will allow us to obtain fingerling survival for the incorporation of aquacultural activities through strategic evaluation.

METHODS AND MATERIALS

Establishment of Common snook (*C. undecimalis*) and fat snook (*C. parallelus*) broodstock groups from wild and hatchery

Objective 1: To obtain broodstock from wild and hatchery-reared snook juveniles
General Experimental Procedure. Among the proposed studies for the present investigation we proposed the creation of two lines of *C. undecimalis* and *C. parallelus;* one initiated from juveniles captured from the wild at the Laguna de Mecoacan and the Gonzalez River. The other line would be obtained from induced spawnings of broodstock keep in captivity since 2008.

Experiment 1a: Origin of the wild organisms. The organisms were captured at the Laguna de Mecoacán, Paraíso, Tabasco, using a mosquito mesh net of 10 m length and 2 m height. Sampling was conducted close to the edge of the lagoon in places were mangrove roots allow the passage of the net and fishermen, 126 juveniles were captured and transferred to the Marine Aquaculture Station (Universidad Juárez Autónoma de Tabasco) for their adaptation to captivity. These fish mere placed in plastic tanks of 1 m^3 capacity containing filtered marine water and provided with constant aeration. After acclimation, the fish were transferred to the Tropical Aquaculture Laboratory at the Biological Division (UJAT) located in Villahermosa. Fish were acclimated to fresh water in a recirculating system composed of 1,300 L plastic tanks and equipped with sand filter and constant aeration.

The feeding regime was: during the first 14 days, snooks were fed with live cichlids (*Cichlasoma Urophthalmus* and *Oreochromis niloticus*) ad libitum; the day 15 and 16 fish were fasted; day 17 and 18 fish were fed ad libitum with chunks of fresh fish; during days 19 and 20, the fish were fasted and during days 21 and 22, fish were fed with pieces of fresh fish. For days 23 and 24, the fish were fasted again and from day 25 onwards the fish were fed with a semi-humid diet two or three times per day. The diet was designed based on fish meal, grounded fish fillet, shrimp meal, fish oil, soy lecithin, a mix of vitamins and minerals, vitamin C, unflavored gelatin, soy milk and sorghum flour, all these ingredients have been used in similar investigation by other authors (2009; Cerqueira and Tsuzuki, 2009).

Samplings were performed monthly in order to determine growth in weight and length; fish were anesthetized with either Methanesulphonate of tricaine (MS 222) or clove oil.

Experiment 1b: Obtaining and maintaining *C. undecimalis* broodstock. Wild organisms adapted for three years to captivity were used**.** Nine females were selected from this stock with sizes ranging from 60 to 91 cm and a weight between 1.896 and 5.290 kg. Eighteen males with an average length of 83 cm and a weight ranging from 806 and 3,886 g. Organisms were maintained at the Marine Aquaculture Station from UJAT in Jalapita, Centla, Tabasco. The adaptation to captivity was performed in 25 m^3 geomembrane tanks. Fish were fed with wild sardines from the Clupeidae family collected with a seine net along the shore, and Breed-M de INVE® feed.

Experimental design: Due to insufficient number of tanks a randomized block design was used (date of replication) with three treatments (0 µg/fish, 100 y 200 µg/fish de GnRH-a). The experiment was replicated three times.

Spawning induction of *C. undecimalis* through GnRH-a implants. In this experiment the method used was from Álvarez-Lajonchere and Hernández-Molejón, 2001. Geomembrane tanks of 4 m diameter connected to a recirculating system were used. Thanks had an eggs collector adapted; consisting of a cylindrical tank of 100 L containing a 400 µm mesh bag. Seawater was used in all tanks with a daily water exchange of 50%. We measured; dissolved Oxygen, temperature (oxygen meter YSI55®) and salinity (refractometer SR6 Vital Sine Premium®). The average value of temperature, DO and salinity during the spawning trials were; 30.65 ± 0.45 ºC, 4.24 ± 0.53 mg/L and 27.5 ± 4.36 ppm, respectively.

Oocyte samples were taken using female's cannulation with a caliber 5 probe and 90 cm in length. Oocytes were measured with a stereoscope (Zeiss®) in order to determine the right diameter for induction (>300 µm). If females had the right oocyte diameter, a female and two males were placed randomly in each tank. Each fish was measured and weighed (with a conventional ichtiometer and a Torrey® balance of 2 g precision). An implant with 0 (control), 100 or 200 µg/fish of GnRH-a hormone (Argent Labs®) was placed in each female. All males from the treatments with hormone were implanted with 100 µg of GnRH-a/fish expecting maturation with just one dose. Only mature males with fluid sperm were used.

Organisms were anesthetized using Methanesulphonate of tricaine (MS 222) and implants were placed in the intra-peritoneal cavity under the pectoral fin with a sterile implants syringe (AVID®). A gentamicine antibiotic cream was applied to avoid infections (Álvarez-Lajonchere and Hernández-Molejón, 2001). The experimental tanks were checked every four hours during three days in order to observe eggs presence. When spawning occurred, eggs were incubated. Eggs were collected by overflow using the egg collector bags, and then were placed in 40 L-plastic buckets with water of the same salinity, temperature and gentle aeration.

Treatments effectiveness evaluation: Treatment effectiveness was evaluated using the occurrence or not of spawning activity. The quality of the spawning was assessed using the number of eggs obtained and the percentage of fertilization. The number of eggs was estimated by volume, taking three samples of 50 mL in different places of the tank. Multiple countings of each sample were performed using 1 mL sub-samples (Álvarez-Lajonchere and Hernández-Molejón, 2001). The percentage of fertilization was evaluated using presence or absence of embryo development analyzing 100 eggs of each experiment; observation was conduced using a stereoscopic microscope (Álvarez-Lajonchere and Hernández-Molejón, 2001).

The present study took into account some parameters described by Álvarez-Lajonchere and Hernández-Molejón (2001) and Hernández-Vidal (2002), in order to determine egg quality considering the following: egg diameter, egg number, fertilization rate, percentage of hatching, and

larvae length. The egg diameter was determined using a sample of 100 eggs from each spawning. They were measured with a ocular micrometer calibrated in a stereoscopic microscope (Zeiss®; Hernández-Vidal, 2002). Pelagic eggs (considered viable) were incubated from each spawning (Álvarez-Lajonchere and Hernández-Molejón, 2001). After this, hatching percentage was evaluated following the same method for determination of fertilization rate. The number of larvae was obtained through direct counting (Hernández-Vidal, 2002). The hatched larvae were maintained in clean containers with the same conditions (Hernández-Vidal, 2002). Percentage of survival was also determined. Larvae length was registered at the point where yolk sac was absorbed.

Statistic analysis: the presence or absence of spawning was recorded and egg diameter analyzed using a covariance analysis, blocking the possible effect of the pseudo-replication (replication date). Hormone concentration was used as the factor of interest. Female weight and oocyte initial diameter were used in the analysis as covariates. The variables fertilization rate and percentage of hatching were analyzed using a Chi-square. There was no data analysis for larvae number and size due to there was just data for one replicate of each treatment. All tests were performed using the software STATGRAPHICS™ V 5.1. The statistic differences were considered using a confidence level of $p< 0.05$.

Experiment 1c: For the spawning induction with *C. parallelus* the same method was used as described for experiment 1 using wild and captivity organisms in 2 m diameter tanks.

RESULTS

Objective 1a: Obtaining and maintaining of a C. undecimalis broodstock from the wild.
According with observations, snooks can be adapted easily to captivity conditions; these organisms did not showed problems ingesting live preys, hold or pieces of fresh feed. With the semi-humid feed the adaptation was effective after fasting days.

The statistics analysis indicates the existence of statistics differences (KW; $p <0.01$) among the different months of sampling for weight and for length. The initial average weight was 26.52 ± 24.59g, while final weight was 137.83 ± 79.40 g (Fig. 1), having a gain of 111.31 g in twelve months of sampling (9.2 g per month). For length the initial average was 14.60 cm and the final average 27.04 cm (Fig. 2). The gain of 12.44 cm (1.03 cm per month)

Experiment 1b: Obtaining and maintaining of a *C. undecimalis* broodstock.
After implantation, *C. undecimalis* broodstock behavior was monitored for two days, observing that spawned fish made a courtship swimming in circles going up to the surface forming a spiral. All fish went back to the bottom and restarted this behavior. Was determined that fish spawned 27 hours after implantation. Spawning was obtained from 2 females of each hormonal treatment tested. Given that only the females treated with hormone spawned (4 of 6), in this analysis only those cases were included. There were no statistical differences (ANOVA; $p = > 0.05$) for the number of eggs produced by female among the treatments 100 and 200 µg/fish (1.98 ± 1.27 and 2.46 ± 0.93 million of eggs, respectively).

Results for the diameter of the fertilized eggs indicated that there is no effect of the factors or from the covariables used in this study (ANCOVA; $p > 0.05$). However, the highest average was obtained with the dose of 200 µg/fish with 746.50 ± 2.12 µm and the lowest average for dose of 100 µg/fish with 681.50 ± 23.50 µm. Te results of the statistics analysis for fertilization indicated that there are statistic differences among treatments (X2; $p > 0.001$), the implants with better results were the 200 µg/fish with 76.84%. By other hand the fish induced with 100 µg/fish had a 60.47% fertilization. Was observed that hatching occurred four hours post fertilization. For this variable, the statistics

analysis indicated high statistics differences (X2; p > 0.001), having a percentage of 100 ± 0.00% hatching for 200 µg/fish treatment and 50 ± 60.10% for the treatment of 100 µg/fish.

From all spawned females, only the larvae from one female from each hormonal treatment survived to the first feeding. 540,000 larvae were obtained for the treatment of 100 µg/fish and 3,119,999 larvae for the dose of 200 µg/fish. The larvae from the high dose measured in average 1.56 ± 0.09 mm and the larvae from the low dose 1.98 ± 0.05 mm.

Experiment 1c: In the case of the stock that would be obtained from captivity broodstock with *C. parallelus* wild and captivity females were induced to spawn. The obtained eggs were not viable.

METHODS AND MATERIALS

Mexican snook (*Centropomus poeyi*) reproduction through GnRH-a implants

Objective 2: to obtain spawning from Mexican snook in captivity

General Experimental Procedure. 14 wild organisms (*C. poeyi)* were collected during an annual reproduction season (June - September). Initially were maintained at the Marine Aquaculture Station in quarantine tanks of 25 m^3. Spermatic activation was carried out under microscopic examination in order to confirm that spawning occurs in marine waters using salinities of 0.15 and 35 ppm. Also intraovarian cannulation were performed to obtain oocytes, its diameter was taken before transported to experimentation tanks. Spawning was induced using GnRH-a implants (Argent Lab). Females were implanted with vehicle (pellet without GnRH-a) 100 µg/fish, ó 200 µg/fish. All males were implanted with 100µg/fish. The floating eggs were collected and transported to a density of 100 eggs/L. The 99.5% of the obtained larvae was released in the spawning sites. Marine water was used (31 ppm; salinity found in the sea at the experiment moment) in all tanks; water exchange was done at 80% daily. The DO, salinity and temperature were 5.3 mg/L, 31 ppm and 27 ºC, respectively. After implantation, spawning tanks were monitored every 2 hours in order to observe eggs/embryo presence.

Statistics analysis: The diameter and number of eggs were analyzed through ANOVA. Eggs quality was evaluated through fertilization and hatching rate; these results will be compared among treatments using a Chi square test with contingency tables.

RESULTS

Objective 2: Obtain Mexican snook spawning in captivity. Cannulation of mature females and males were performed during June and July. However, sexual products were not obtained which was used as an indicator to wait for the next month (August), in which no oocytes were obtained in some cases. However, implants were placed to three females with dose of 100 y 200 µg/female. In the cases were oocytes were obtained these had an average measure of 332.5 µm. Spawning occurred around 29 hours post implantation in three of the implanted females, 4 million eggs were obtained with the females with the high dose. For the low dose the number of eggs was around 2 million eggs, however, these eggs were not viable. The average eggs diameter was 541.29 µm for the high dose and 620.31 µm for the low dose. The fertilization percentage was among 18 and 100% and hatching rate among 29 and 68%. Were obtained between 1 and 3 million of viable larvae. For larvae feeding was used the rotifer *Brachionus plicatilis* and microalgae *Nannochloropsis oculata*. Larvae measured an average of 1.5 mm length.

No statistical analysis was performed due to insufficient fish for the application of a experimental design.

METHODS AND MATERIALS

Identification of native plankton for snook feeding

Objective 3: Identification of native plankton used as feed during snook early development
General Experimental Procedure. Phytoplankton and zooplankton was collected from the common snook spawning zones close to the Gonzalez River. The snook spawning sites were marked and three sampling sites were selected: the first site on the fishermen capture area, the second between the capture sites and the coastal line and the third in front of the coastal line. Samplings were performed for 10 minutes with plankton nets of 20, 64 and 120 µm with a boat of 25 ft length at low speed. The samples were fixed with formaline at 4% and were analyzed under stereoscopic microscope.

RESULTS

To date we have made five monthly samples, finding a great variety of organisms: copepods, cladocerans, chaetognaths, brachyuran, clams, polychaetes, appendicularians, nematodes, luciferase, among others. However, the most abundant was registered for copepods, follow by cladocerans, meanwhile the lowest abundance was registered for caridean, nauplii and eggs (Fig. 3-7).

METHODS AND MATERIALS

Objective 4: Determination of the digestive enzyme gene expression during different life stages of the common snook (*Centropomus undecimalis)*.

General Experimental Procedure. Larvae and juveniles of *C. undecimalis* and *C. parallelus* were collected in quantities that allow obtaining a minimum of 150 mg of stomach, intestine and pancreatic tissue. The sampling was carried out after 12 hours of fasting for larvae and 48 hours for the juveniles and adults. Organisms were euthanized through a cold-water shock. Tissue was collected and washes with distilled water, later were submerged in an Rnasa inhibitor (RNA, ambion, AM7020, Austin, TX, USA) and were storage in liquid nitrogen until processed.

Sampling was performed before first feeding by triplicate from the fertilization (400 embryo, 0 DAH) and from 1, 3, 5 and 12 DAH (400 organisms per day). The embryo an larvae samples were rinsed with distilled water and transferred to Eppendorf tubes containing 1.0-1.5 mL of RNALater and storage at -80 ºC for later process.

RNA extraction. The total RNA from each of the samples from embryo, larvae and the portions of intestine, stomach and pancreas which were storage in RNALater at -80 °C, was isolated according to the method of Valenzuela et al. (2005). The concentration of RNA was estimated using the equation of the Lambert-Beer law after reading in the spectrophotomer (Jenway, Genova Ltd, Felsted, United Kingdom) the absorbance (230 nm) of each of the samples. For knowing the integrity of the nucleic acids an electrophoresis was performed in an agarose gel at 1% using an aliquot of 1 µl of mix sample with 1 µl of running buffer of each of the samples at 100 volts in a electrophoresis chamber Mini Sub Cell GT (Bio-Rad, Hercules, CA, USA) for 30 minutes, the resulting bands were visualized in a documentation system of Molecular Image gel Gel Doc XR+ Imaging System (Bio-Rad, Hercules, CA, USA). Once we evaluated the integrity of the genetic

material of each of the samples the aliquots were made from each of the dissected organs according with the obtained concentration, resulting in a final concentration of 0.5– 1µg/µl of RNA.

Reverse transcription of the Polymerase Chain Reaction (RT-PCR). One microgram of the total RNA of each of the samples of intestine, stomach and pancreas was reversibly transcribed according to the instructions from the kit SuperScript III One-Step RT-PCR System con Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) using the specific primers for the ubiquitin (UB), pancreatic lipase (LIP) y trypsin (TRY) genes in a total volume of 25µl in a real time thermocycler iCycler IQ5 Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The nucleotide sequence and the alignment temperature for each enzyme used in this study are detailed in Table 1.

The PCR conditions for the reverse transcription in one step of the enzymes were: 1 cycle of 30 minutes at 55 °C; 1 cycle of 94°C; 30 cycles of 1 minute at 94°C, one minute to the specific temperature for each enzyme, two minutes at 72°C; ten minutes at 72°C. The amplify products were visualized in an agarose gel at 2%, using a stair of 100 pb with marker (Promega, Madison, WI, USA). The observed bands were cropped from the gel and purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) kit. The purified bands were send to the Unidad de Síntesis y Secuenciación from the Instituto de Biotecnología de la UNAM for its sequenced.

The different obtained sequences of digestive enzymes were analyzed using BLAST (www.ncbi.nlm.nih.gov/BLAST) for identification according with reported in the database.

Real Time Polymerase Chain Reaction (qRT-PCR). The total RNA from the common snook larvae from days 0, 1, 3, 5 and 12 after fertilization, which was stored in RNALater, was isolated as mentioned above. The reverse transcription was performed according with the kit Improm-II Reverse Transcriptase (Promega). The experiments of qRT-PCR were carried out in order to observe the RNAm expression during the digestive system development. The specific primers for the ubiquitin (UB), pancreatic lipase (LIP) and trypsin (TRI) (trypsinogen) were designed based on the obtained sequence in this study (Table 2).

β-actin amplification was carried out using specific primers choose from the comparison of different ARNm and represented a very conserved region of nucleotide in order to confirm the expression level of a domestic gen and provide a intern control (Table 2). For this, aliquots of 20µl were taken from the reaction mix, which was prepared with iQ SYBR Green Super Mix (Bio-Rad) and the specific primers (UB5' and UB3' of ubiquitin, LIP5' and LIP3' of pancreatic lipase, TRY5' and TRY3' of trypsin, ACT5' and ACT3' of β-actin for qRT-PCR) for a final concentration of 1µM. The qRT-PCR protocol consisted in one cycle at 95°C for 15 minute follow by 35 cycles of 1 minute at 94°C, one minute at the specific temperature of each gen and 3 minutes at 72°C. The reactions were performed by duplicate and each level of expression was calculated according to the 2- ΔΔCt (Livak y Thomas, 2001) method. The mixture of transcribed genes was made in a real time thermocycler StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

RNA extraction: the total extraction of RNA was performed according with TRIZOL protocol. Later the RNA will be treated with Dnasa I for eliminate the genomic DNA. The reverse transcription will be performed using the commercial kit from Improm II.

RESULTS

PCR amplifications. The amplification by PCR using the primers of Table 1 had as result products of 700, 350 and 200 pb for RTAMI (amylase) in intestine, which were named RTAMI1, RTAMI2 Y RTAMI3 correspondingly, 1200 pb for TLPCA (lipase) in pancreas and 800 pb for TRINA (trypsine) in the intestine (Fig. 8). In the case of TAPSA (aminopeptidase) and PEPNOT (pepsinogen) nothing was observed in the agarose gel.

Sequence analysis. From the five PCR products with their primers send to sequence only three of them were possible of obtain sequence products (Table 3). When doing the sequence analysis on BLAST, was observed that in the case of the sequencing products of RTAMI3, the sequence had a similitude of 90% with the partial pancreatic lipase of the Japanese snapper (*Pagrus major*). In the alienation of the TRINAR sequencing a maximum identity (95%) was found with the trypsinogen of the Blowfish (*Takifugu rubripes*). In table 4 we can observed with more detail the similitude found for RTAMI3, TLPCA y TRINAR en el BLAST.

Genes relative quantification. For the determination of the expression levels of ARNm in the different days of collection of *C. undecimalis,* a relative quantification was performed of PCR-TR (Table 5). For the ubiquitin was observed the gen expression from day 0 with respect to the intestine, expressing 0.32 copies of RNAm, this is that the expression was less than in the intestine. The day 5 (0.76 copies RNAm) was expressed a little more than double than in day 0 although, in less quantity than in the intestine. For days 1, 3 and 12 was not possible to calculate the expression level due to values for Ct en el PCR-TR were not obtained. For trypsinogen was observed the gen expression from day 3 (0.33 copies RNAm) with respect to the intestine and increased for day 5 to 0.47 copies RNAm. No values of Ct from days 0, 1 and 12 in trypsinogen gen were obtained. In the case of the pancreatic lipase we observed that in day 0 there were 8.05 copies greater than in the pancreas.

METHODS AND MATERIALS

Objective 4: Determination of the digestive enzyme gene expression during different life stages of the fat snook (*Centropomus parallelus*).

Five adult fish (150 g mean weight) were captured in the coastal shore of Jalapita, Paraiso, Tabasco, Mexico. Fish were transported to the Laboratorio de Acuicultura Tropical de la DACBIOL-UJAT and maintained in one 2000-L circular plastic tank for 48 h without feeding. To obtained samples of intestine, fish were sacrificed after anesthetized with MS-222, and individual dissection of anterior intestine was obtained. The sampling of the fish was realized to the 7:30 A.M. The intestines were rinsed with distilled water, placed in vials and freeze introducing them immediately in liquid nitrogen. The samples collected, were dissected in cold on a Petri dish sterilized and placed on a cold plate. The fresh, individual and total weight was registered, and they were finally submerged into inhibiting buffer of RNAses (RNA Later, Ambion, AM7020, Austin, TX, USA) for they transport and processing in Biological Research center of the Northwest in La Paz, B.C.S.

Extraction of RNA

The total extraction of RNA took place from the homogenized of the intestines (125 mg of wet tissue), according to the protocol of the reagent of TRIZOL Invitrogen, (Life Technologies SKU# 15596-018, California, USA). Later, the RNA was dealt with DNAse I (Deoxyribonuclease I, Amplification Clay, Invitrogen Cat. No. 18068-015 the USA, Tree-lined avenue, CA, USA) to eliminate the genomic DNA. For the retro-transcription use kit commercial Improm II (Promega, A3800, Wisconsin, USA).

Design of primers
To performed the PCR, the primers used for this study were design for the European sea Dicentrachus labrax (Peres et al. 1998), registered in www.ncbi.nhl.nih.gov (Table 6). In addition electrophoresis was realized in agarose gels (1%) to determine the best temperature for amplification, and bands were stained with ethidium bromide. These bands were recovered using the protocol of kit GENE CLEAN SPIN KIT (BIO 101 INC., Californian, USA) and sent to Macrogen Inc. Korea for their sequencing.

Amplification of cDNA
The amplification of cDNA was realized in a thermocycler Icycler (BIO-RAD ICYCLER, California, USA) with system of temperature gradient, optimizing itself in terms of temperature of alignment and concentration of Mg^{2+}.

The reactions for a PCR were carried out in 50 µl, containing 50 pmol of forward and reverse primers of the trypsin gene, 1 µl of cDNA, 0,5 Or of Taq polymerase (Invitrogen), 100µM of dNTP and 1X of buffer solution (Invitrogen), under the following conditions: 30s to 95°C; 35 cycles of 1 min. to 95°C, 1:30 s to 60°C, 2 min to 72°C; 7 min to 72°C; 4°C ∞.

The purity of cDNA was quantified by means of biophotometer (Eppendorf, 22331), being the standard of reading of double chain (dsDNA) for cDNA higher to 1,6 and lower to 2.1 µg ml^{-1}. The amplification products were separated by electrophoresis in agarose to the 1,5% (A5054 Sigma, Missouri, USA) and later analyzed in a photodocumentator (transiluminator Gel-Pro, **C-62**, CA, USA). The recovery of the DNA was obtained cutting to the bands of interest with a sterile bistoury in the transiluminator and for the purification use the protocol of kit GENE CLEAN SPIN KIT (BIO-RAD 101, CA, USA).

Finally, the amplified segments by PCR were cloned according to the protocol of kit TOPO TA CLONING (Invitrogen n° K4550-40, California, USA) using competent cells of *E. coli* Top 10F' and the plasmid pCR 2,1 like a vector. Once cloned, plasmids of the cells of *E. coli* were obtained using the procedure of kit RPM (BIO 101, California, USA) to be sequence and obtain their homologies with the banks of sequences.

Polymerase chain reaction (PCR)
The PCR of trypsin gene cDNA, was realized according to the protocol suggested by Applied Biosystems user's guide using specific Taqman® probes for the genes and the method of standard curve with plasmids. This was carried out in a thermocycler (Applied Biosystem, Abi Prism® 7000 Sequence Detection System, 4330087, California, USA). The PCR reactions were carried out in plates of 96 wells under the following conditions: 1 cycle to 50°C for 2 min; 1 cycle to 95°C for 10 min; 40 cycles to 95°C for 15 sec and 60°C for 1 min.

A set of sequential dilutions (0, 10, 100, 1000, 10 000, 100 000 and 1 000 000) of the plasmid containing inserted of the trypsin gen at 50 µg ml^{-1} and the 18S rRNA gene (Applied Biosystems), which was used like endogenous control.

Simultaneously, amplification of the trypsin gene of each individual intestine samples were realized (M1D0-M16D30), which were prepared using cDNA, corresponding to every day of the sample, with an approximated concentration of 1000 µg ml^{-1}, TaqMan® Universal PCR Masters Mix (4331348 CA, USA) and a specific Taqman probes (4331348, Custom Taqman (R) Gene Assay Service TRYTG2007-TTD, CA, USA) for trypsin gene for *C. parallelus.* The corresponding calculations were realized on the basis of the protocol *User Bulletin #2 of ABI PRISM 7700 Sequence Detection System.*

Analysis of similarity
Combined to the study of the amplification of the trypsin, an analysis of similarity was realized comparing our sequence with other sequences of fish species. The sequences were obtained from the GenBank/EMBL/DDBJ using Educative Surroundings of "Biology Workbench" on line to perform the blast. The species selected for this analysis were: *Pleurogrammus azonus* (AB441709), *Tribolodon hakonensis* (AB445492), *Salvelinus leucomaenis* (AB447372), *Sparus aurata* (AF316852) *Danio rerio* (AJ297822), *Dicentrarchus labrax* (AJ006882), *Paralabrax maculatofasciatus* (AJ344566), *Oreochromis niloticus* (AY510093 and AY737394), *Oreochromis aureus* (AY510094), *Chelon labrosus* (AY628239), *Symphysodon aequifasciatus* (AY690664), *Cebidichthys violaceus* (AY973822), *Anoplarchus purpurescens* (AY986477), *Xiphister mucosus* (AY986478), *Xiphister atropurpureus* (AY986479), *Spinibarbus sinensis* (DQ839550), *Myxocyprinus asiaticus* (EF493027), *Menidia estor* (FJ859998), *Salmo salar* (X70071, X70073, X70074 and X70075) and *Paranotothenia magellanica* (X82223). The alignment of these sequences allowed calculates the percentage of similarity and the dendrogram.

RESULTS

Amplification of cDNA
The amplification of cDNA from *C. parallelus* trypsin gen gave us a product of 525 pair of bases pair (pb) (Fig. 10). Additionally, in figure 11 we showed the gene sequence and the amino acid sequence (75 amino acids) for the partial trypsin gene of this species.

Analysis of similarity
An alignment of the nucleotides sequences of the trypsin gene in relation to a great variety of fish species was conducted. This analysis revealed that the trypsin gene enough is low conserved with species such as *Sparus aurata* (12.04%), *Chelon labrosus* and *Myxocyprinus asiaticus* (12.10% and 20.08% respectively); on the other hand, the highest similarity percentage was determined with *Myxocyprinus asiaticus* (39.86%) and *Dicentrarchus labrax* (39.40%) (Table 7).
On the basis of the pre-alignment of the sequences, a dendrogram was created, which demonstrates the close relation of the trypsin genes in several fish species, since it can be observed that they come from a common ancestor. With respect to the *C. parallelus* it can be observed that one is in the same group that *S. aurata* (Fig. 12).

DISCUSSION

The study of obtaining a broodstock of common snook suggests that the growth of juveniles from the coast of Tabasco, have a similar growth in captivity similar to reports from Sánchez-Zamora *et al*., (2003) they suggested in their study that this specie have a growth of 0.8 g in a year and our study indicates a growth of 0.7 g per month. For the case of growth in length studies like Aliume (2000) mention that common snook could have an increment of 0.04 cm. Our study suggest a gain in length pretty similar, with data of 1.03 cm/day.

With respect to the reproduction induction of *C. undecimalis* and *C. poeyi* through implants, was possible to obtain spawns and larvae using GnRH-a, with 100 µg/fish with oocytes diameter of 333.00 ± 54.08µm and salinities from 21 ppt in *C. undecimalis.* The spawning obtained in our study match the reproductive season reported for *C. undecimalis* by Taylor et al. (1998) in the coast of Florida and by Perera *et al*. (2008) in the coast of Tabasco. Was also possible to identify that in *C. poeyi* the spawning season is similar for *C. undecimalis* in captivity conditions. This indicate that common snook and Mexican snook populations maintained in captivity completed the oogenesis through a hormonal stimulus. Our results match with other studies of common snook, where they have obtained spawn with viable larvae in fish keep in captivity using implants. However, the

effective dose (50 µg/kg de GnRH-a) varies in the method to apply, which is according to fish weight (Soligo, 2007). In this study, the percentage of fertilization was 6% and survival 7.5%. Skapura et al. (1999) evaluated implants in *C. undecimalis* with dose of 10 µg/kg/day during five days, using a control group and a placebo group. Results indicated that the used of the mGnRH, sGnRH and cGnRH-II implant allow the success in ovulation and Neidig *et al* (1999) determined that the used of Human Chorionic Gonadotropin (GCH) in *C. undecimalis* with dose of 500 IU/kg of weight produced ovulation, good quality eggs and larvae survival. Our results are also similar to the obtained with fat snook (*C. parallelus*) using the same dose (100 y 200 µg/fish) of GnRH-a with the implants technique (Contreras-García, 2011). The percentage of fertilization with 100% in some experimental units and hatching of 10% in some cases, were also similar, in addition successful spawning occurred in *C. poeyi* with results of 100% fertilization.

Cerqueira (2009) with fat snook used LHRHa implants and obtained four consecutive spawning with the same female. That study allowed the observation that the larvae survival until juvenile stage was only of 1%, increasing in later inductions to 30%.

The above suggest that the use of GnRH-a gives a balance stimulus and probably a better integration in the reproductive event with other physiologic functions, affecting direct or indirectly hormone release necessary for the success in final oocytes maturation, the spermiation and the spawning (Zohar y Mylonas, 2001).

In other fish of the Centropomidae family such as *Lates calcarifer* (barramundi), Almendras et al. (1988) obtained multiples spawning using implants of GnRH-a. A female spawned around 7 million eggs with a percentage of fertilization and hatching of 90% in both cases. The larvae survival in this study was 60.8%. Also was observed that only one male was able of fertilize the eggs of a four consecutive spawning from one female. In our study, the percentage of fertilization is also similar to the barramundi data, only for the number of larvae was different being our less. Also, Ibarra-Castro et al. (2009) obtained larvae in barramundi, from lower dose than our dose (50 µg/kg per female and 25 µg/kg per male) with fertilization percentage (90%) also similar to our study.

In other fish, Basaran et al. (2009) increased the success in the reproduction of the drum fish through injections and implants obtaining spawning 70 to 72 hours post injection and 89 to 90 hours post implant. The oocytes diameter obtained with the first technique was 767.5±1.0 µm and 753.5±1.3 µm under the second technique. For our study, the oocytes diameter was similar to the reported for this fish through implants.

Mylonas et al. (2004) used GnRH-a through implants in *Seriola dumerili* (greater amberjack). In this study, spawnings were obtained around 36 hours post implant and 15 days later. The fertilization was around 100%. In the common snook the used of GnRH-a also is effective through implants for the success in reproduction obtaining spawning in similar times to the reported for greater amberjack. Marino et al. (2003) also used implants of D-Ala6, Pro9, NEt]-GnRH (GnRH-a) with dose of 30.5 to 68.3 µg/kg in *Epinephelus marginatus* (dusky grouper). The 85% percentage of the organisms responded to the induction and ovulated between 60 to 238 hours post implant (eggs were obtained with abdominal pressure), while the control group did not mature. The percentage of fertilization was between 48.2% and 52.2%.

Results showed that GnRH-a through implants is an effective method for produce eggs of good quality in greater amberjack in captivity. In the present study, spawned the 66.7% of the females being the spawning times less than the reported for greater amberjack. As well as with the greater amberjack, we could not obtain spawning with the control group therefore the used of GnRH-a is ideal for obtain viable larvae of these fish.

In *C. undecimalis* and *C. poeyi* we obtained similar average in larvae size to those obtained by Peters et al. (1998) they identified that larvae hatched in Florida measured 1.4 to 1.5 mm. The obtained larvae only survived to the fourth day after the absorption of the yolk sac. To this respect, Witenrich et al. (2009) mention that snook larvae mortality can be explain to the lack of adequate prey in the early life stages. In these stages larvae have rudimentary skeletal elements and at the first feeding the larvae have a digestive system little developed, which can restrict its ability to consume preys.

Witenrich et al. (2009) mentions that the mortality of snook larvae is attributed a la carencia de una presa adecuada en las primeras etapas de vida. En estas etapas las larvas presentan elementos esqueléticos rudimentarios y a la primera alimentación las larvas presentan un aparato digestivo poco desarrollado que puede restringir su capacidad para consumir las presas.

Finally, we can mention that the success in the present study is due to mainly to the maintenance of the broodstock for several years under captivity with perfect feeding and handling strategies therefore the used of GnRH-a implants was efficient to allow the completion of the final oocytes maturation with a slowly release of the hormone, even when the oocytes diameter before the treatment application was less to the ideal diameter reported for snooks.

By other hand, in the identification of the native plankton, we found that in the different sampling zones the most abundant organisms were copepods. This will be a probably indicator that snook larvae consume copepods in their first life stages. This abundance increases importantly for the month of May which match with the natural reproduction season of common snook, reported for several authors.

This suggest that even when copepods have sizes from 0.5 to 3 mm snook larvae could be consuming any of the life stages of this crustacean, such as nauplii stages with sizes of 50 µm. Authors like Streble y Krauter (1987), consider these microcrustaceans together with rotifers the most important feed for many organisms for their early life stages. The follow most abundant class in the spawning sites was cladocerans in this study, which indicate that could be the second feed used by larvae in later life stages. Other authors such as Stottrup y Norsker (1997) indicate that the inclusion of copepods in the marine fish larvae culture sustain a normal development and a good larval growth.

Payne y Rippingale (2000) mention that copepods constitute the natural feed of the marine species larvae and represent a high content of the essential fatty acids and antioxidants. All of the above reinforce the probability of snook larvae are feeding of these organisms either in their nauplius or copepodite stages.

As the study of the gene expression, we could identify three of them which act during the initial ontogeny of the common snook; ubiquitin, trypsinogen and pancreatic lipase. This study give new information, due to this is the first work where digestive enzymes sequencing and PCR-TR for the specie *C. undecimalis.*
Given that there is no knowledge of the common snook genome the β-actin was selected as the normalize gene for the experiments with PCR-TR considering the reported for other species (Darias et al., 2006; Murray et al., 2004; Oku et al., 2006). However, according with the observations in our study (Fig. 9) the β-actin was not constant thus exist changes in the expression in all the sampled days, suggesting that it is necessary to explore new possibilities of reference genes for future studies with *C. undecimalis.*

According with other studies (Murray et al., 2004) the trypsinogen is detected since day 5. The levels of ARNm not only are influenced by the regulation of the gene expression, there are more

conditions such as: nutrition, size differences and the tissue components which could influence the ARNm levels from the gene working on (Hibbeler et al., 2008) thus it is important to keep investigating the effects of feeding or the age in the gene expression in this specie. The activity of the pancreatic lipase is not only regulated at the transcriptional level but also at the posttranscriptional level (Birk and Brannon, 2004).

CONCLUSIONS

The adaptations to captivity conditions in *C. undecimalis* is factible, using as feed semihumid balance diets

The use of GnRH-a through implants in common snook *C. undecimalis* and Mexican snook *C. poeyi* allow the oocytes maturation induction and generate spawning in captivity.

Besides, viable larvae were obtained from dose of 100 µg per female independently of its weight. The spawning could be obtained in salinities of 21 ppt for the common snook; a value under the reported in the literature for this specie and from 30 ppt for the Mexican snook.

The use of life feeds such as copepods could be viable for the survival of the snook larvae.

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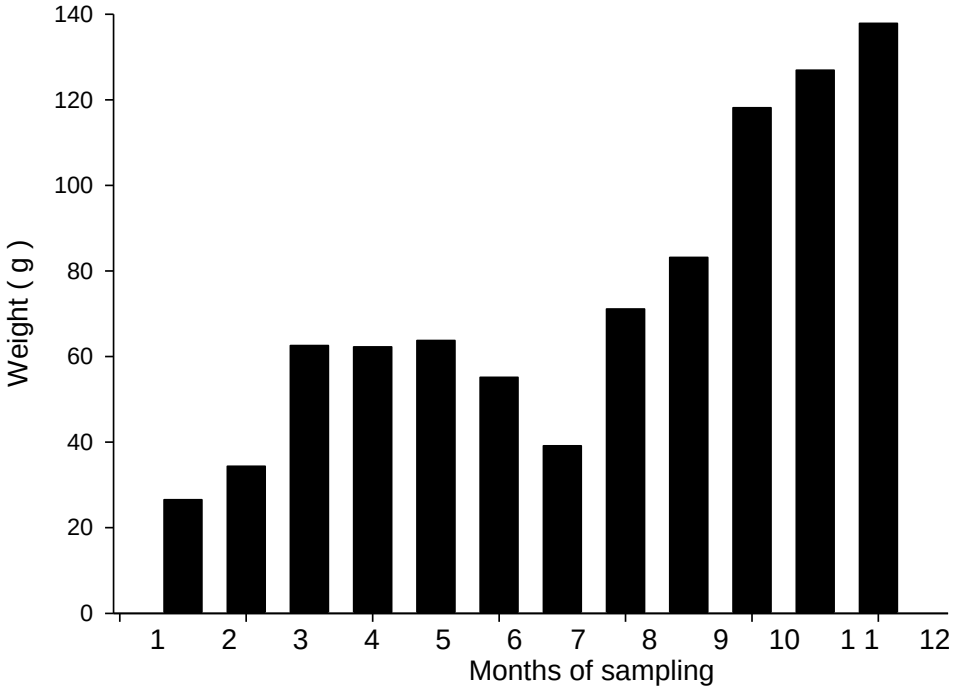

Figure 1. Average weight of common snook juveniles in captivity during a year.

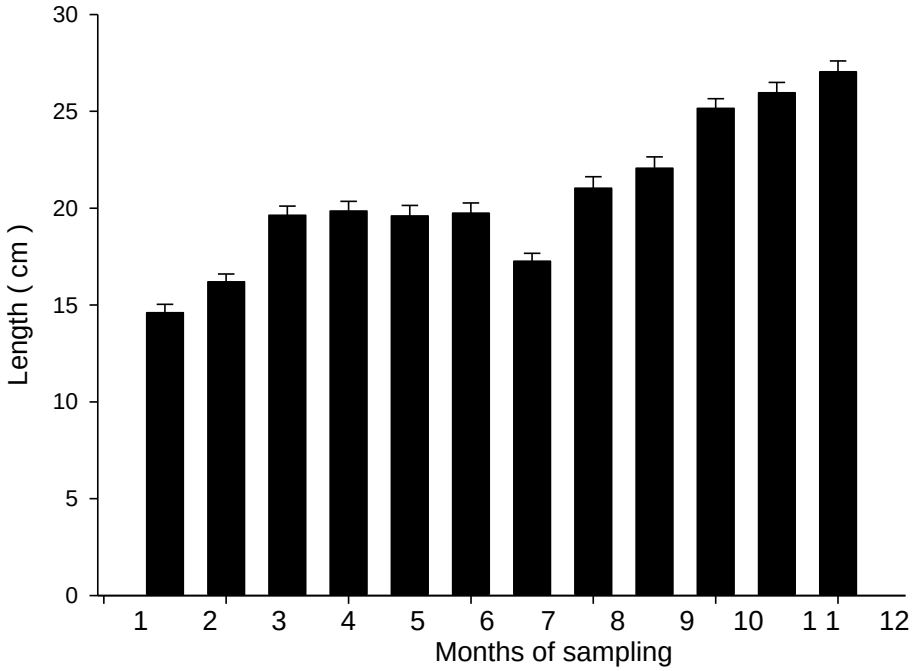

Figure 2. Average total length of common snook juveniles in captivity during a year.

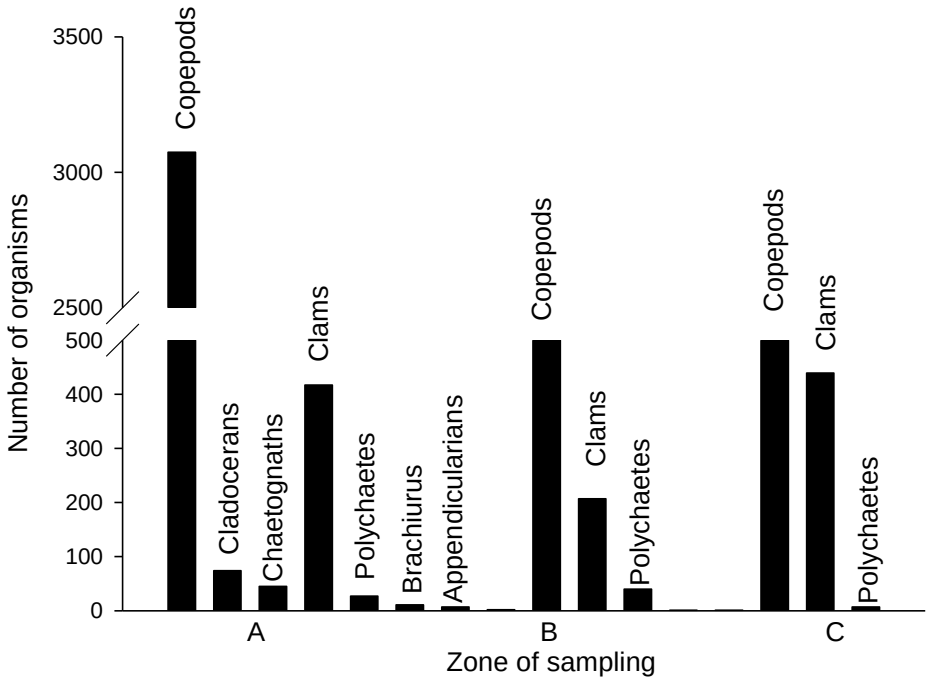

Figure 3. Abundance of groups of zooplankton in April in the coast of Centla, Tabasco.

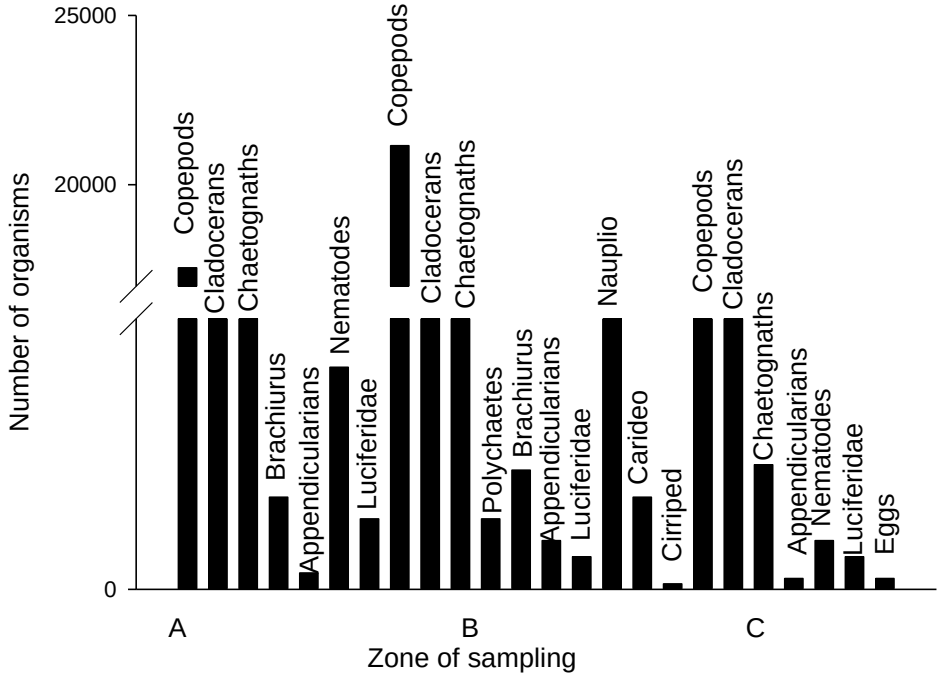

Figure 4. Abundance of groups of zooplankton in May in the coast of Centla,Tabasco.

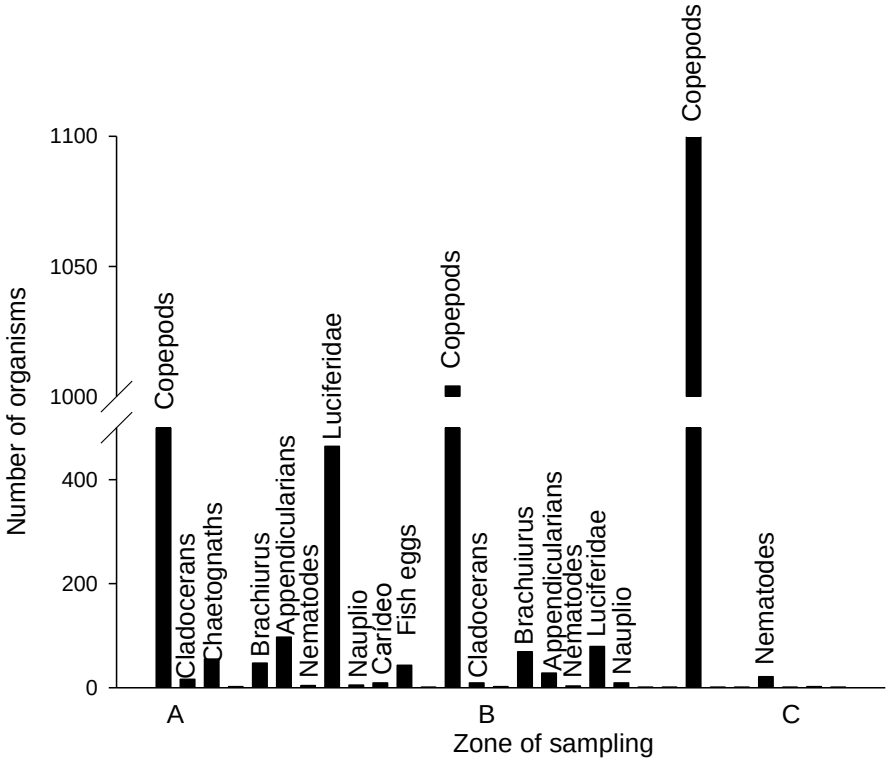

Figure 5. Abundance of groups of zooplankton in June in the coast of Centla, Tabasco.

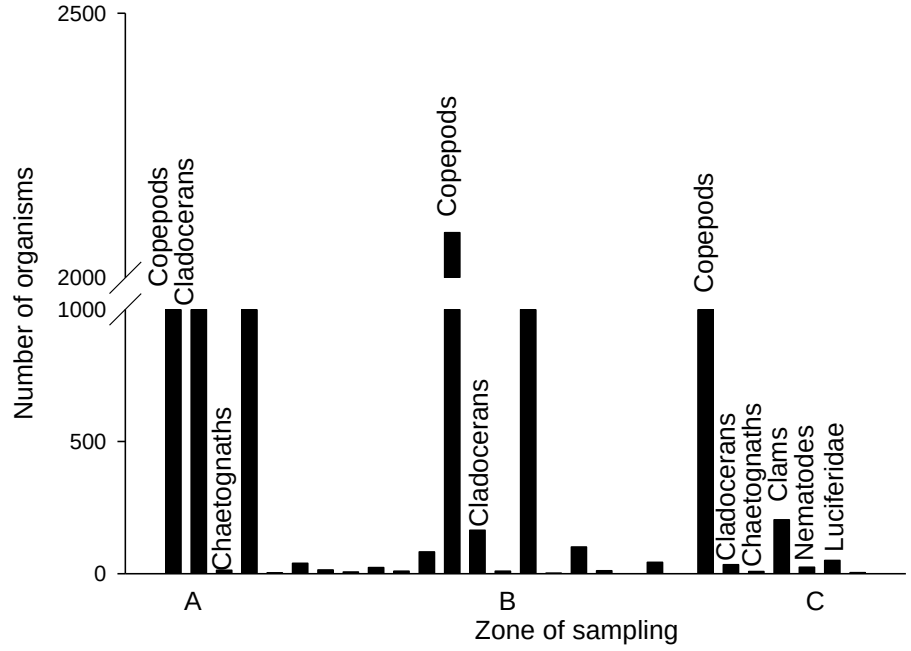

Figure 6. Abundance of groups of zooplankton in July in the coast of Centla, Tabasco.

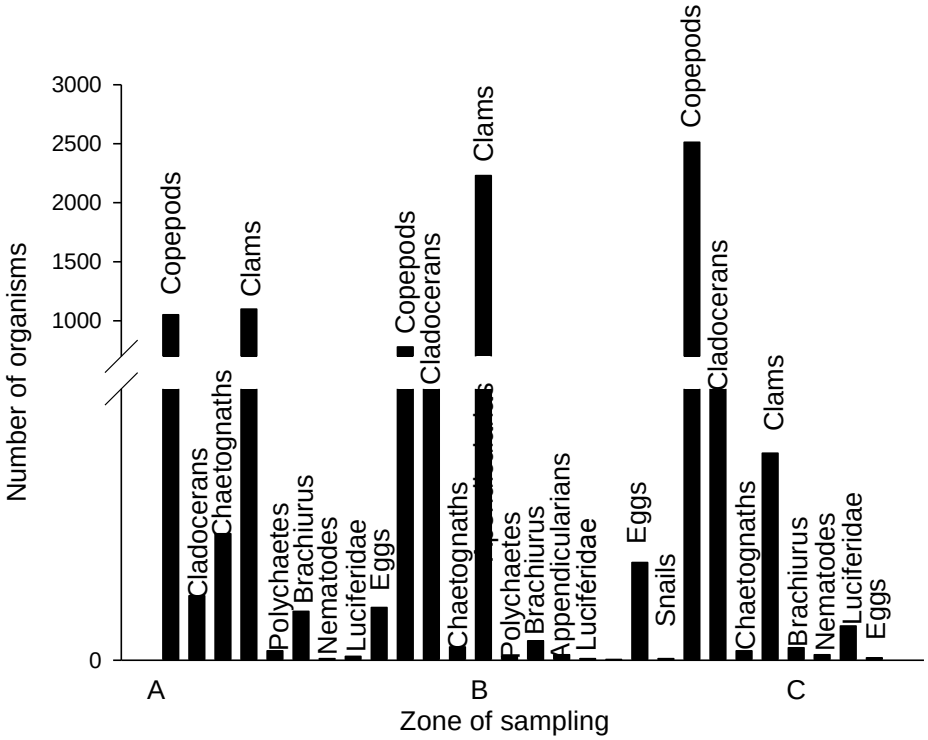

Figure 7. Abundance of groups of zooplankton in August in the coast of Centla, Tabasco.

Table 1. Sequence of primers and alignment temperature of RTAMI (amylase), TAPSA (aminopeptidase), TLPCA (lipase), PEPNOT (pepsynogen) and TRINA (trypsine) used in the reactions of Reverse Transcription-PCR

Primer	Nucleotides sequence (5'→3')	Alignment Temperature (°C)
RT-PCR RTAMI		
RTAMI-F	TTCATATTGGCGTTAGTCCT	59.2
RTAMI-R	TTACAATTTGGAGTCCAAGAC	
RT-PCR TAPSA		
TAPSA-F	TACATCAGTAAAGCTGTCGG	60.7
TAPSA-R	AAGGCTCTCATGACCAAGAC	
RT-PCR TLPCA		
TLPCA-F	AGAAGAACCGCTACTACCAG	61
TLPCA-R	GATCTCATTCTCCTCCACCT	
RT-PCR PEPNOT		
PEPNOT-F	GAGTGCCTCCATAAGATTCC	59.3
PEPNOT-R	ACCAATGTACTTAGACTGGG	
RT-PCR TRINA		
TRINA-F	**TCTCTTGTATTCGTTCTGCT**	**60**
TRINA-R	**AGAGGATGATGGAAGAAAGG**	

Table 2. Primers sequence and alignment temperature of RTUbi (Ubiquitine), RTLip (lipase) and RTTri (tripsynogen) which were used in the PCR-TR reactions of snook larvae

Primer	Nucleotides sequence (5'→3')	Alignment Temperature (°C)
PCR-TRUbi		
TRUbi5'	GCAACACACCTGACCTGAGG	55
TRUbi3'	CGTCCTGCTGATTGTATCCC	
PCR-TRLip		
TRLip5'	TGATGCCTGCAATCACGTCC	59
TRLip3'	TTAGCCACTGGGCCATCAGG	
PCR-TRTri		
TRTri5'	GCTCCACTGCTGACAGGAAC	55
TRTri3'	CAGAGTCACCCTGGCAAGAG	
PCR-TRAct		
TRAct5'	**CGCGACCTCACAGACTACCT**	**59/55[1]**
TRAct3'	**GATTCCGCAGGACTCCATAC**	

[1] Alignment Temperature in the PCR-RT for the actine changed according with the studied gen

Table 3. Results from the sequence of the send enzymes to the Instituto de Biotecnología de la UNAM

Enzyme	# de bp	
	Forward	Reverse
RTAMI1	---	---
RTAMI2	---	---
RTAMI3	186	192
TLPCA	870	870
TRINA	---	519

Table 4. Results from the sequence alignment of RTAMI3, TLPCA and TRINA-R in the BLAST

Organism	e Value	Matching percentage
RTAMI3		
Salmo salar ARNm for the clon ssal-rgf-520-156 of the hydrolase ubiquitin carboxiterminal 2, cds pseudogene	5e-27	85
PREDICTO: *Danio rerio* ARNm similar for specific ubiquitin of peptidasa 2	1e-16	79
Oryzias melastigma ARNm for ubiquitin specific of peptidase 2, cds partial	6e-13	88
TLPCA		
Pagrus major ARNm for pancreatic lipase, cds partial	0.0	86
Epinephelus coioides ARNm of colipase-dependt of pancreatic lipase, cds complete	0.0	85
TSA: Hippoglossus hippoglossus all_halibut.1371.C1 mRNA sequence	3e-158	88
TRINA-R		
Takifugu rubripes ARNm for trypsinogen, cds partial	0.0	95
Siniperca chuatsi ARNm for trypsinogen 1, cds complete	0.0	94
Solea senegalensis ARNm for trypsinogen 1b, cds complete	0.0	94
Pleurogrammus azonus ARNm for trypsine, cds complete	0.0	94
Solea senegalensis ARNm for trypsinogen 1c, cds complete	0.0	94
Sparus aurata ARNm for trypsinogen, cds complete	0.0	94
Paralichthys olivaceus ARNm for trypsinogen 2, cds partial	0.0	94
Sparus aurata ARNm precursor for trypsinogen II, cds complete	0.0	94
Dissostichus mawsoni ARNm of the clon 25 for the trypsogen precursor, cds complete	0.0	93
Solea senegalensis ARNm for the trypsinogen 1a, cds complete	0.0	93
Tautogolabrus adspersus ARNm for retripsynogen, cds complete	0.0	93
P.magellanica ARNm for trypsine	0.0	93
Paralichthys olivaceus ARNm for trypsinogen 1, cds complete	0.0	92
Epinephelus coioides ARNm for trypsinogen 1a, cds partial	0.0	91

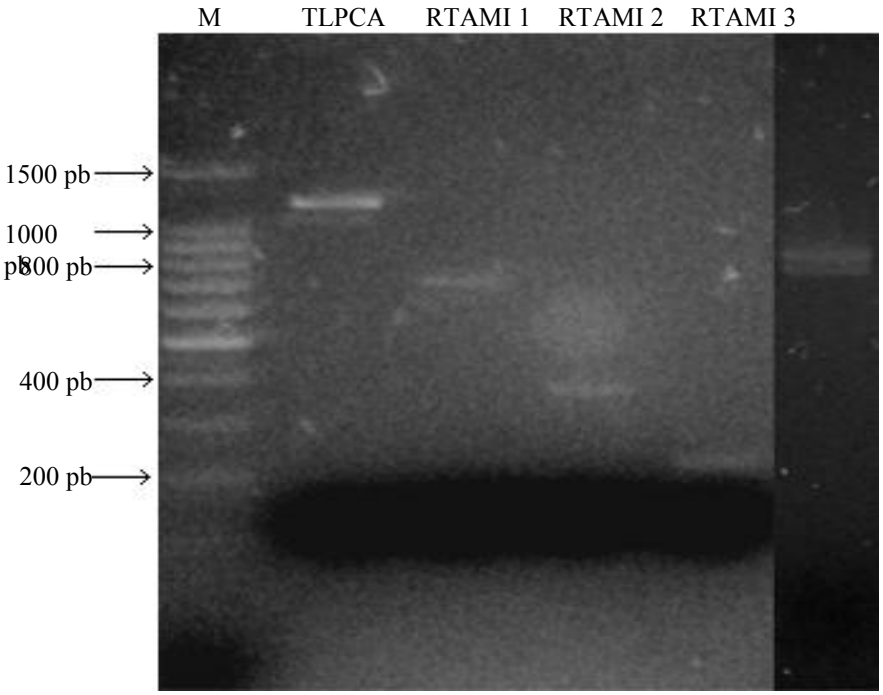

Figure 8. Amplified products for the Lipase (TLPCA), Amilase 1 (RTAMI1), Amilase 2 (RTAMI2), Amilase 3 (RTAMI3) and Trypsine (TRINA) of *C. undecimalis*. Marker (M).

Table 5. Relative expressions of the ubiquitine, trypsinogen and pancreatic lipase in the days of results of Ct for common snook.

Gene	$2^{-\Delta\Delta Ct}$	Control
Ubiquitin day 0	0.32	Intestine
Ubiquitin day 5	0.76	Intestine
Trypsinogen day 3	0.33	Intestine
Trypsinogen day 5	0.47	Intestine
Pancreatic lipase day 0	8.05	Pancreas

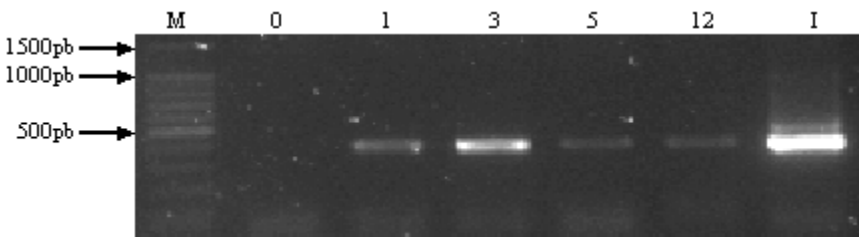

Figure 9. Agarose gel electrophoresis at 2% of the PCR-TR products of the actine gen of common snook (*C. undecimalis*). Larvae age (post fertilization) is indicated above each well. Intestine (I) and Marker (M).

Table 6. Sequence of degenerate primers of trypsin of *Dicentrarchus labrax* (Péres et al. 1998) and temperature of amplification.

Primers	Forward	Reverse	Temperature of amplification ºC
Trypsin	CAggTgTTCTgAAC	CCC (Ag) gACACAACACCT	60

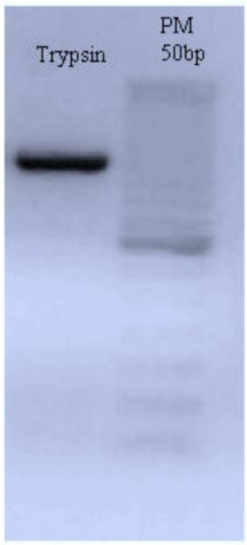

Figure 10. Gel of Agarose in which is the band of trypsin with 525 bp of the intestine of *C. parallelus* adults. PM: Marker of molecular weight of 50pb.

Figure 11. Partial sequence of *C. parallelus* trypsin gene.

Table 7. Percentage of similarity in comparison with *C. parallelus* trypsin gene.

Species	Percentage of similarity
Myxocyprinus asiaticus	39,86
Dicentrarchus labrax	39,40
Cebidichthys violaceus	39,21
Paralabrax maculofasciatus	38,34
Xiphister mucosus	35,85
Anoplarchus purpurencens	35,59
Menidia estor	34,68
Symphysodon aequifasciatus	34,00
Oreochromis niloticus	26,18
Danio rerio	26,04
Paranotothenia magellanica	24,67
Salmo salar	24,62
Salmo salar	24,17
Salmo salar	23,52
Tribolodon hakonensis	23,50
Salvelinus leucomaenis	23,38
Salmo salar	23,36
Oreochromis aureus	23,31
Oreochromis niloticus	23,18
Pleurogrammus azonus	23,08
Spinibarbus sinensis	21,81
Myxocyprinus asiaticus	20,08
Chelon labrosus	14,10
Sparus aurata	12,04

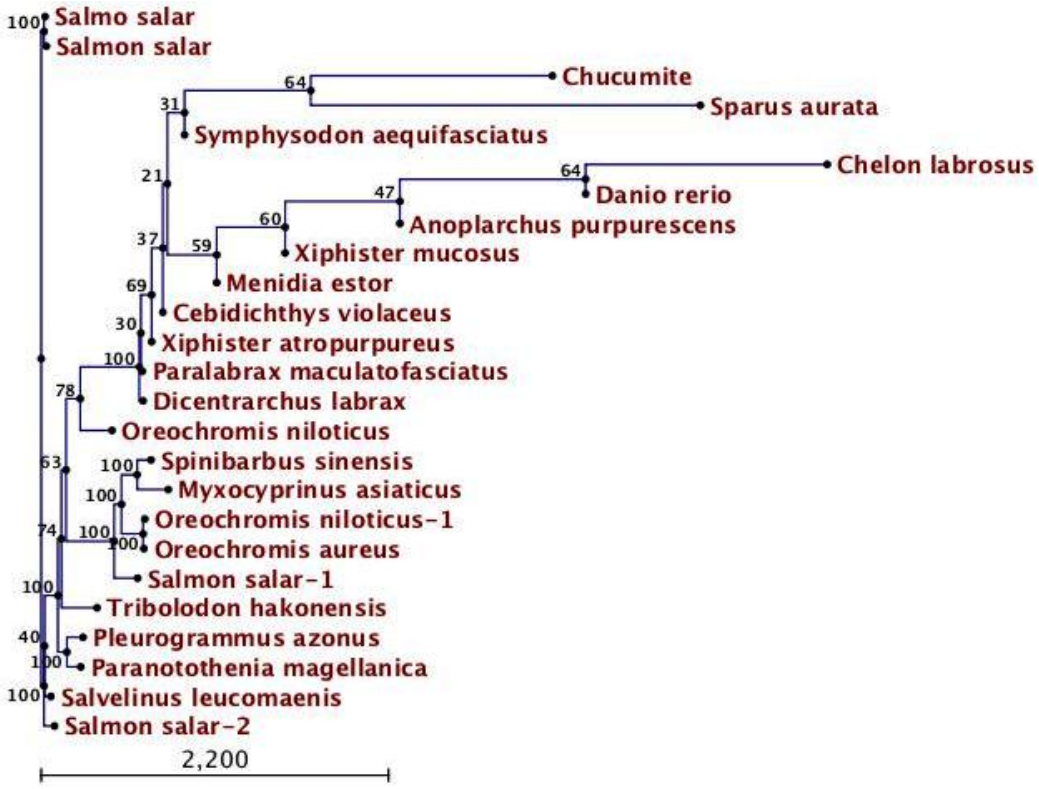

Figure 12. Dendrogram of the alignment of the similarity analysis, where is the relation of the trypsin gene in different fish species.