REPORTER GENES FOR TRANSGENIC FISH EXPERIMENTS

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ABSTRACT

The study of regulatory sequences in transient experiments employing reporter genes produce valuable data to design gene constructs for transgenesis. In this report, *Escherichia coli* chloramphenicol acetyl transferase (CAT), hepatitis B surface antigen (HBsAg), *E. coli* ß-galactosidase and the green fluorescent protein (GFP) from the jellyfish *Aqueorea victoria* were assayed as reporter genes for *in vivo* transient expression in tilapia, common carp and zebrafish. In tilapia and common carp, an endogenous ß-galactosidase-like activity was found in embryos, and after hatching, it was localized in the posterior-ventral part of the fry. This ß-galactosidase-like activity could interfere with the use of lacZ as a reporter gene in experiments with tilapia and common carp when the expression is assayed in the early stages of development. In zebrafish, however, lacZ was successfully employed. The HBsAg and CAT genes gave reproducible results in the species tested, thus constituting a choice when a second reporter gene is needed as an internal control. Finally, the GFP provided a simple and powerful mean of monitoring transient gene expression in live zebrafish embryos.

Key words: carp, tilapia, zebrafish, galactosidase, embryo

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RESUMEN

El estudio de secuencias reguladoras empleando genes reporteros en experimentos de expresión transitoria permite diseñar y caracterizar construcciones genéticas para transgénesis. En este reporte, los genes para cloranfenicol acetil transferasa (CAT) y lacZ de *Escherichia coli*, el antígeno de superficie de la hepatitis B (HBsAg) y la proteína fluorescente verde (GFP) del pez *Aequorea victoria* se ensayaron como genes reporteros para ensayos de expresión transitoria en tilapia, carpa común y pez zebra. En embriones de tilapia y carpa común se encontró una actividad tipo galactosidasa ß que se localizó después de la eclosión en la parte postero-ventral de la larva. Esta actividad tipo galactosidasa ß puede interferir con el empleo del gen lacZ como reportero en experimentos con tilapia y carpa común cuando la expresión se ensaye en los estadios tempranos del desarrollo. En pez zebra, no obstante, el gen lacZ se empleó exitosamente. Los genes codificantes para HBsAg y CAT dieron buenos resultados en las especies ensayadas, constituyendo una opción cuando se necesite un segundo gen reportero como control interno. Finalmente, la GFP resultó un medio simple y ventajoso para monitorear la expresión génica transitoria en embriones vivos de pez zebra.

Palabras claves: carpa, tilapia, pez zebra, galactosidasa, embrión

Introduction

Transgenic fish have been generated by DNA microinjection into the germinal disc and, in some cases, the transgene has been expressed and inherited by the progeny of these animals (1-3). However, much remains to be elucidated before gene transfer can be fully manipulated to generate new strains of economically important fish species.

In vivo transient experiments employing reporter genes produce valuable data to design gene constructs for transgenesis (4). The selection of the reporter gene for *in vivo* transient expression experiments requires that the reporter activity be easily detected given good sensitivity and specificity. Furthermore, because in many experiments it is necessary to monitor for two different reporters to employ one as a test signal and the second to normalize for intrinsic variations of the system, several options for reporter genes must be available for fish species suitable for laboratory work.

To select appropriate reporter genes for *in vivo* transient expression experiments in tilapia, common carp and zebrafish fish, species which provide good laboratory models, several genes were assayed.

The *E. coli* lacZ gene is one of the most useful reporter genes because it gives a good sensitivity and quickness. The histochemical staining using X-gal allows the detection of this enzyme in cells and tissues (5). The CAT gene has been widely em-

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ployed as a reporter in *in vivo* and *in vitro* experiments (6, 7). The HBsAg was tested because of the high specificity and sensitivity of the analytical ELISA systems currently used for antigen determination (8) and the GFP for the possibility of employing this system in live fish (9).

In tilapia (*Oreochromis aureus*) and common carp (*Cyprinus carpio* L.), an endogenous ß-galactosidase (ß-gal)-like activity was found in fish fry and embryos. This activity appeared soon after fertilization and continued during the yolk sac resorption. In more advanced developmental stages, the endogenous ß-gal-like activity was confined to the stomach and intestine. This ß-gal-like activity interferes with the use of lacZ as a reporter gene in transgenic fish experiments when the expression is assayed in the early stages of development. However, in zebrafish (*Brachydario rerio*) the lacZ reporter gene was successfully employed. CAT, HBsAg and GFP reporters showed reproducible results in the fish species tested.

Materials and Methods

Fish embryos and fry

Adult tilapia were obtained from the Empresa Nacional de Acuicultura (Cotorro, La Habana) and placed, separately according to sex, in 1 L tanks at 18-20 °C. Tilapia were fed *ad libitum*. Mature tilapia were selected by visual examination and placed in a 500 L glass aquarium with a biological filter under controlled temperature and photoperiod (28-30 °C and 14 h light x 10 h dark). After spawning and fertilization, the female was carefully removed from the aquarium and the eggs were placed in a glass cylinder (Ø 20 cm) with a 500 mL incubation solution (0.1 % NaCl, 0.004 % $CaCl_2$ $2H_2O$, 0.003 % KCl, 0.016 % $MgSO_4$ $7H_2O$, 0.0001 % methylene blue). Embryos were incubated with gentle shaking (60 rpm) and the solution was changed every 8 h until hatching when shaking was stopped for further incubation. For common carp, embryos and fry were obtained as described by Hernández, *et al.* (10). Microinjection of tilapia and carp embryos was conducted as described by de la Fuente, *et al.* (11) and Hernández, *et al.* (10), respectively. Zebrafish embryos were obtained and microinjected as reported by Stuart, *et al.* (12).

Detection of ß-galactosidase

Tilapia and common carp fry and embryos were fixed for 1 h in 0.1 M $NaHPO_4$ containing 4 % paraformaldehyde and 0.2 % glutaraldehyde at 0 °C. Then, the samples were washed three times at room temperature with PBS, and incubated for 1 h in PBS containing 0.2 % BSA (fraction V, Sigma) and 0.1 % Triton X-100. Then, they were incubated for 14-16 h at 37 °C in a solution of 1 mg/mL X-gal in PBS containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$ and 1.3 mM $MgCl_2$. Before staining, adult organs were washed with sterile water for ß-galactosidase activity. Then, they were processed as described for fry and embryos but including 0.02 % NP 40 in the fixing solution and incubating with X-gal for 18 h at 30 °C. In zebrafish embryos, ß-galactosidase activity was detected as described by García del Barco, *et al.* (4).

Detection of CAT and HBsAg

CAT activity was detected in zebrafish embryos as described by García del Barco, *et al.* (4). For tilapia and carp embryos, the procedure is emphasized in Lleonart, *et al.* (7). Briefly, two plasmids were used in the experiments: pE300, which has the CAT gene under the control of the human cytomegalovirus (CMV) enhancer-promoter region and 3' polyadenylation sequences from the SV40 genome (11), and pUCHBV (13), which contains the entire hepatitis B virus (HBV) genome except for the core sequence inserted in pUC19. Plasmids were diluted to 50 µg/mL in injection buffer (10 mM TrisHCl pH 7.4, 0.25 mM EDTA, 0.1 % Phenol Red) and about 1 nL was injected into one cell embryos 30 to 60 min after fertilization. The injected embryos were incubated for two to four days and, before hatching, they were transferred to eppendorf tubes, resuspended in 100 µL of 250 mM TrisHCl pH 8, and about 50 µL of glass beads (0.5-1 mm) were added. Tubes were vortexed for 1 min, freeze-thawed three times (-70 °C, +37 °C) remaining 5 min at each temperature, and after the addition of 1 µL of 0.5 M EDTA, the tubes were heated 10 min at 60 °C. CAT assay was done by standard procedures and the HBsAg was determined by ELISA in embryo homogenates.

Detection of GFP

One cell zebrafish embryos were microinjected with the plasmid pRSGFP-C1, which contains the jellyfish GFP gene under the control of CMV promoter and SV40 polyadenylation signal (14). Thirty hours after the injection, embryos were placed under UV light in an Olympus IMT2 microscope (Japan) and the GFP visualized as described by Kevin, *et al.* (15).

Results and Discussion

The use of reporter genes to study regulatory sequences *in vivo*, allows the design of appropriate gene constructs for gene transfer experiments in fish (4). The possibility of employing the *E. coli* lacZ and CAT genes, the HBsAg and the jellyfish GFP as reporters in zebrafish, tilapia and common carp, was studied.

With the procedure described in this paper, tilapia embryos were reproducibly obtained with a high rate of normal development (Figure 1).

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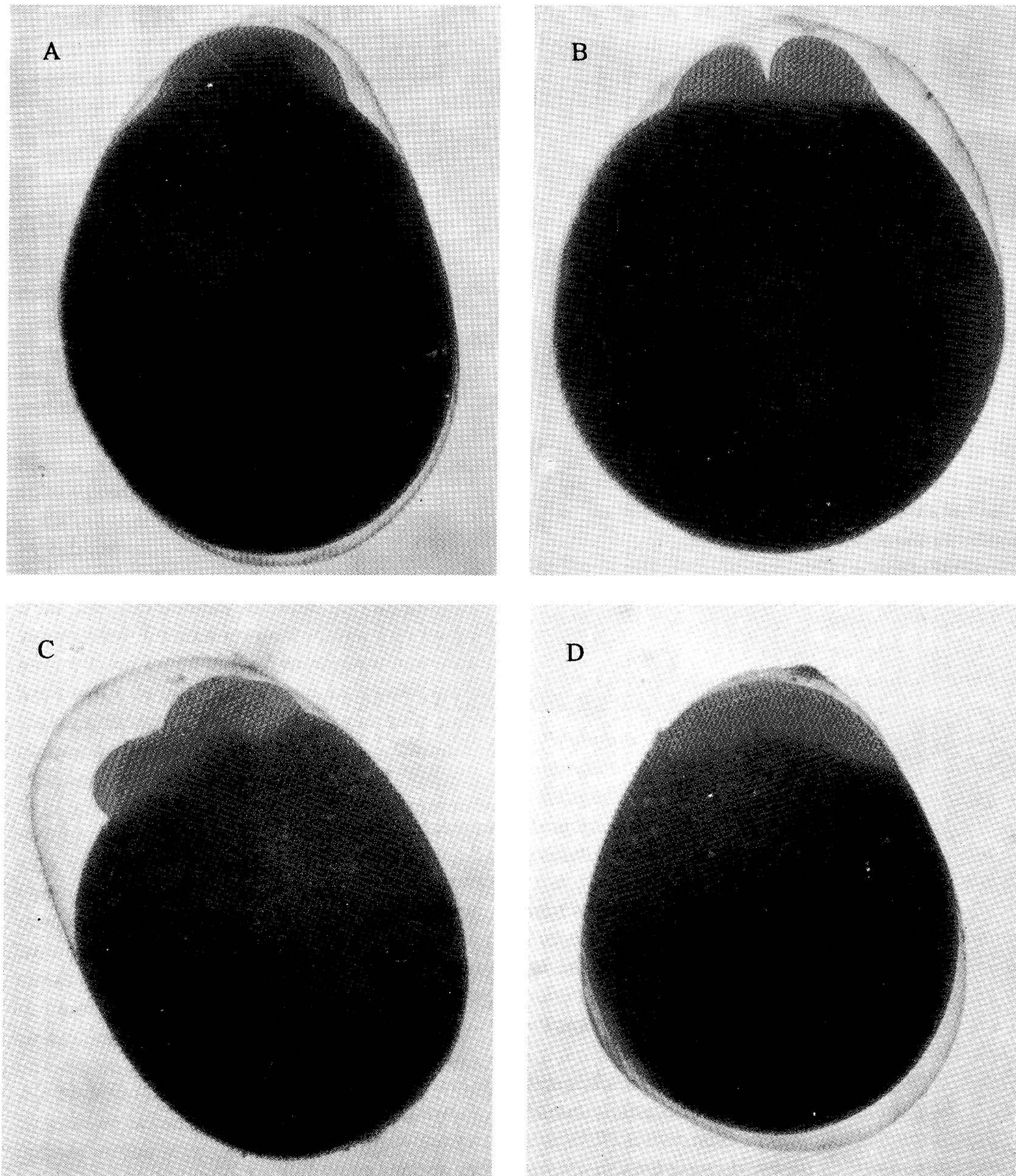

Figure 1. *Microphotograph of tilapia embryos obtained with the procedure described in Materials and Methods. A) 30 min after fertilization (one cell), B) 1.5 h after fertilization (two cells), C) 2 h after fertilization (four cells) and D) 12 h after fertilization (gastrula).*

In tilapia, an endogenous ß-gal-like activity was found in the fertilized egg associated with the embryos (Figure 2 A). Soon after hatching, this activity was localized in the posterior-ventral part, in the region between the stomach and the yolk sac (Figure 2 B). In the adult animal, the activity was present only in the stomach and intestine (Figure 3).

In carp, the endogenous ß-gal activity was found in fertilized eggs. This activity continued later in development and was found in four days old fry showing different expression patterns (Figure 4). This variegated gene expression may represent a form of gene regulation (16), and could be related to the age of the cell, its health or its position within the cell cycle (17). After yolk sac resorption (seven days after fertilization), the fry were stained again for ß-gal-like activity. As in tilapia, the activity appeared localized in the posterior-ventral region of the fry. Further characterization in adult organs confirmed that this ß-gal-like activity remained localized in the stomach and intestine.

These results indicated that the *E. coli* lacZ gene, when employed as a reporter gene in tilapia and carp, could interfere with the endogenous ß-gal-like

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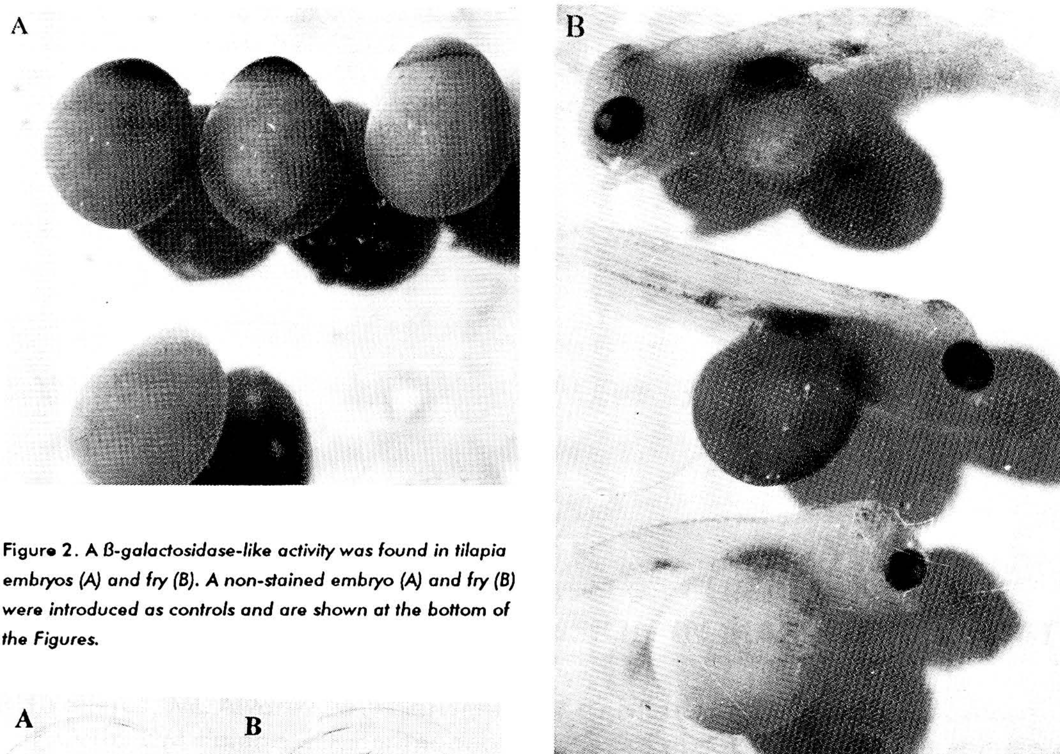

Figure 2. *A ß-galactosidase-like activity was found in tilapia embryos (A) and fry (B). A non-stained embryo (A) and fry (B) were introduced as controls and are shown at the bottom of the Figures.*

Figure 2. B.

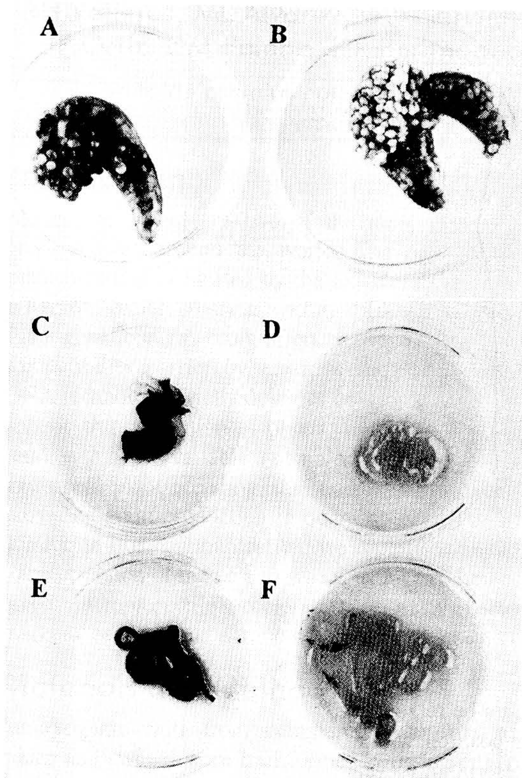

Figure 3. *A ß-galactosidase-like activity was localized in adult tilapia stomach and intestine. The organs were extracted from adult tilapia and stained (A, C and E) or not (B, D and F) for ß-galactosidase-like activity. A and B) gonads, C and D) stomach, E and F) intestine. The analysis for each organ was repeated at least three times giving similar results.*

activity if assayed early in development. However, the adult expression pattern of the ß-gal-like activity in tilapia and common carp allows the use of the *E. coli* lacZ gene in mass transfer experiments if the assay for its activity is to be done after yolk sac resorption. In this case, the background activity will be restricted to the posterior-ventral region of the fry, allowing the transgene expression to be assayed in other tissues.

However, in zebrafish embryos, the *E. coli* lacZ gene was successfully employed showing that the Rous sarcoma virus (RSV) regulatory region was 17 times stronger than the 5' regulatory sequences derived from the human cytomegalovirus (CMV); while in *in vitro* fish cells, the CMV promoter was more active than the RSV (4, 6, 18). These results suggested the hypothesis that different regulatory requirements exist in cells and embryos and that chimeric constructs designed for transgenic experiments should be assayed in transient expression experiments in fish embryos (4). This hypothesis was recently corroborated in transgenic tilapia lines generated with transgenes containing different regulatory regions (11), showing that RSV enhancer promoter sequences were more active than those derived from the CMV (Hernández, *et al.*, manuscript in preparation).

Endogenous background activities have been reported for ß-galactosidase (19) and neomycin (20) in

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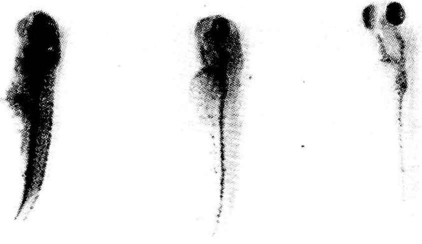

Figure 4. *Different patterns of ß-galactosidase-like activity was found in four day old common carp fry. A non-stained control is shown on the right.*

other fish species. This confirms the need to have several options for reporter genes.

For this reason we tested the HBsAg and CAT genes (4, 7). In tilapia and common carp, high frequency of positive embryos (40-50 %) were obtained in several experiments. The CAT expression ranged between 5 and 50 % conversion of chloramphenicol to its acetylated forms. The expression of the HBsAg was observed in approximately 50 % of the injected tilapia embryos with a mean value ±SD of 0.74 ± 0.16 ng/mL of the homogenate (7). The combination of these reporter genes may be useful when several constructs have to be tested for their strength, employing one of the genes as a reporter and the second as an internal standard to normalize the intrinsic variations of the system (amount of plasmid injected, efficiency of injection) (4, 7). In fact, in zebrafish embryos, the combination of CAT and lacZ reporter genes has been employed in our laboratory to assay different chimeric genes for transgenic fish experiments (4).

Recent results with the luciferase gene in medaka *(Oryzias latipes)* and with the GFP gene in zebrafish embryos indicate that it can be used as a potential source for monitoring gene expression in live transgenic fish (9, 21). We have also shown the GFP expression in zebrafish embryos 30 h after injection and experiments are under way to assay this reporter gene in tilapia and common carp.

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Note added in proof

When this manuscript was finished, a paper by Samsul Akam MD, Popplewell A and Maclean N (Transgenic Research 1996; 5:87-95) appeared showing the expression of a lacZ transgene in tilapia (Oreochomis niloticus) and the staining of the endogenous ß-gal activity described in our work. A review manuscript also appeared covering the use of other reporter transgenes in fish (Iyengar A, Müller F and Maclean N. Transgenic Research 1996; 5:147-166).

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AQUACULTURE OF RED DRUM (Sciaenops ocellatus) AND OTHER SCIAENIDS

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REPORTES

Introduction

Recent refinements in red drum (*Sciaenops ocellatus*) aquaculture have led to a rapid increase in commercial production of these species in southern regions of the United States (1). Major advances in the artificial control of reproduction and rearing of red drum larvae and juveniles in captivity are summarized here. In addition, the potential applications of these technologies to the culture of other sciaenid fishes are discussed.

Reproduction

Precocious maturation

The time for red drum to reach sexual maturity can be reduced by more than half by rearing them at summer temperatures (24-28 °C) and a constant photoperiod (12L:12D) and feeding them a high food ration (2, 3). Red drum, which normally mature at 4-5 years of age, can be induced to undergo sexual maturation and begin spawning before they are two years old by this technique.

Gonadal recrudescence

Exposure to condensed annual photoperiod and temperature cycles is the preferred method of inducing gonadal recrudescence in all the sciaenid species investigated so far. Environmental manipulation techniques to induce gonadal recrudescence and spawning of sciaenids in captivity were first developed for spotted seatrout (*Cynoscion nebulosus*) and red drum by Arnold and coworkers. Gonadal growth and spawning of spotted seatrout occurred under summer conditions (15L:9D, 26 °C) whereas autumn conditions (9L:15D, 23 °C) were required for red drum spawning (4). Condensed (4-6 months) annual cycles are now routinely used to induce gonadal recrudescence in red drum and orangemouth corvina (*Cynoscion xanthulus*) (5, 6).

Spawning

Special procedures are not usually required to induce spawning of red drum and spotted seatrout; once the gametes are fully developed, the broodstock are maintained at the temperature and photoperiod conditions at which they spawn naturally. Spawning can usually be induced by gradually lowering and then raising the water temperature if it does not occur naturally (2, 5). Once spawning begins, it will continue indefinitely if the fish are held under optimun environmental conditions for spawning up to 7.5 years for one group of red drum broodstock (2). Hormonal treatments are reliable methods of inducing spawning of orangemouth corvina and other sciaenid species that do not respond predictably to environmental manipulation (7). A single injection of a LHRH analog (20-100 µg/Kg bw) induces spawning of a variety of sciaenid fishes 30-36 h later (8). A hormonal method of spawning seatrout, in which LHRHa is administrated in the diet, has been developed to eliminate the stress associated with capture and handling (9). Oral administration of 0.5-1.0 mg LHRHa/Kg bw in the diet (dead shrimp) induced spawning in spotted seatrout 32-38 h later. Spotted seatrout can be induced to spawn repeatedly by oral administration of LHRHa at 20-30 day intervals, the interval required for a new cohort of oocytes to complete their growth. In contrast, oocyte growth and feeding are interrupted for two weeks after each injection of LHRHa resulting in fewer spawns and decreased egg production during the spawning season. Repeated oral administration of a LHRH analog is a valuable method, therefore, for large scale production of spotted seatrout eggs.

Rearing

Larval culture

Sciaenid larvae are easily grown on cultured *Brachionus plicatilis* and *Artemia* sp. Microencapsulated diets are readily accepted if they are of appropiate size and can be maintained in the water column, but larvae are unable to obtain sufficien nutrition without the addition of live prey (10). The alimentary canal is simple in first feeding red drum larvae but the digestive glands and measurable concentrations of digestive enzymes are present (11). Larvae can be weaned to dry micro-particulate diets at one week of age with high survial and excellent growth rates (12). Test diets containing 50 % protein and 18-20 % lipid with 2.5-3.5 n-3 HUFA produced the best growth (13). Succesful weaning of red drum larvae from live prey after only one week is unique and of great importance in the development of economical, year-round production of juveniles.

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