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Larval Development of Silver Sea Bream (*Sparus sarba*): Ontogeny of RNA-DNA Ratio, GH, IGF-I, and Na^+-K^+-ATPase

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Abstract: To ascertain some of the important biochemical and molecular events that take place during early larval development of silver sea bream (*Sparus sarba*), we undertook a study of changes in the morphology as well as the ontogeny of the RNA-DNA ratio, growth hormone (GH), insulin-like growth factor I (IGF-I) messenger RNA abundance, Na^+-K^+-ATPase subunit mRNA abundance, and Na^+-K^+-ATPase enzyme activity. Larvae samples were collected at 1 to 46 days posthatch (dph). At 7 dph the yolk sac was fully absorbed, and from 28 dph onward larvae underwent rapid developmental changes to the juvenile stage. The RNA-DNA ratio was highest at 1 dph, decreased to low levels between 7 and 21 dph, then increased by 28 dph, and then again by 46 dph. The ontogenetic profiles of GH, IGF-I, and Na^+-K^+-ATPase α1 and β1 subunits were studied using reverse transcriptase polymerase chain reaction, coupled with radioisotope hybridization of immobilized DNA. Growth hormone abundance reached a constant and high level from 35 dph onward, whereas the IGF-I level reached a peak at 35 dph and then significantly decreased. Both Na^+-K^+-ATPase α1 and β1 subunit mRNAs increased up to 35 dph, however, at 46 dph the α1 subunit remained high whereas the β1 subunit decreased. Na^+-K^+-ATPase activity was low in 1-dph larvae but increased rapidly as development progressed. The importance of these findings is discussed within the context of larval development.

Key words: fish, sea bream, larvae, growth hormone, IGF-I, Na^+-K^+-ATPase.

INTRODUCTION

Newly hatched fish larvae undergo a pronounced and coordinated series of molecular, biochemical, and morphologic changes as they grow and develop into juveniles. Some of the processes involved during larval development may be under hormonal control, and 2 hormones that could play a critical role during early larval development are growth hormone (GH) and insulin-like growth factor I (IGF-I). The growth-promoting action of GH has been proved as the administration of exogenous GH has been demonstrated to enhance the growth rate in fish (Foster et al., 1991; Flik et al., 1993; McLean et al., 1997; Ben-Atia et al., 1999), and the early appearance of GH in larval fish also suggests its important role in growth. Using in situ hybridization Ayson et al. (1994a) detected GH messenger

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RNA in the pituitary of newly hatched tilapia larvae, whereas studies using whole body RNA from gilthead sea bream larvae have shown the presence of GH transcript from 6 days posthatch (dph), using Northern blotting (Funkenstein et al., 1992) and from 3 dph using reverse transcriptase polymerase chain reaction (RT-PCR) (Funkenstein and Cohen, 1996). Also, GH transcript was detected in newly hatched rainbow trout larvae (Yang et al., 1999), and the presence of GH protein in the pituitary region or somatotrophs of newly hatched larvae has been reported (Cambré et al., 1990; Arakawa et al., 1992; Power and Canario, 1992).

Members of the IGF family also have been shown to have potent metabolic and growth-promoting actions (Froesch et al., 1985) as mediators of the somatotropic action of GH. In adult fish GH treatment has been demonstrated to increase tissue IGF-I mRNA levels (Cao et al., 1989; Duan et al., 1993) and serum immunoreactivity (Funkenstein et al., 1989). Although it remains to be established if the action of GH is direct or mediated through IGFs in newly hatched larvae, immunoreactivity to IGF-I in barramundi larvae has been reported (Richardson et al., 1995), and IGF-I transcripts have been found in embryos, eggs, and larvae of a number of fish species (Duguay et al., 1996; Funkenstein et al., 1996, 1997; Greene and Chen, 1997; Perot et al., 1999). Duguay et al. (1996) and Perot et al. (1999) have also described the expression profile of IGF-II mRNA during gilthead sea bream development.

During the early development of teleost larvae, the major osmoregulatory organs are not fully formed or functional and ionic balance appears to be achieved via mitochondria-rich (MR) cells located in the larval skin, particularly in the region of the embryonic yolk sac (Guggino, 1980; Hwang and Hirano, 1985; Hwang et al., 1994; Ayson et al., 1994b). Localized within larval MR cells is the sodium pump (Na^+-K^+-ATPase) (Hwang et al., 1999), a universal membrane-bound enzyme necessary for maintaining intracellular homeostasis by actively transporting Na^+ out of cells and K^+ into cells (Geering, 1990; Horisberger et al., 1991). The potential use of measuring Na^+-K^+-ATPase activity as an index of larval metabolism has yet to be appraised, but the metabolic cost of maintaining ionic balance during larval development can be high. Studies on sea urchin larvae have demonstrated that Na^+-K^+-ATPase activity could account for up to 40% of the metabolic rate (Leong and Manahan, 1997).

Na^+-K^+-ATPase is a heterodimeric enzyme, consisting of a catalytic α and glycosylated β subunit, which are coded for by separate genes, the ontogenetic profiles of which remain to be elucidated during teleost larval development. In the present study we investigated some changes in morphology during development of silver sea bream (*Sparus sarba*) larvae as well as measuring RNA-DNA ratio and dry weight as general indicators of growth. Molecular and biochemical studies in relation to fish larval development are few, and as such we also present the ontogenetic profiles of GH, IGF-I, Na^+-K^+-ATPase subunit mRNA, as well as Na^+-K^+-ATPase activity, during larval development of silver sea bream.

Materials and Methods

Larval Culture and Videograph Imaging

Sexually mature silver sea bream (*Sparus sarba*) of both sexes (males, 200–250 g; females, 350–400 g) were obtained from a captive broodstock and held in the Marine Science Laboratory at the Chinese University of Hong Kong. Female silver sea bream were induced to spawn by administration of human chorionic gonadotropin (0.1 μg/g) daily for 3 days. Eggs were stripped from females by applying gentle pressure on the abdomen and transferred to a container containing a small amount of seawater (33 ppt). Sperm were obtained from male silver sea bream and gently mixed with eggs for 30 minutes to allow for fertilization, after which time all eggs were transferred to a glass cylinder. The fertilized eggs were carefully removed from the surface of the liquid, leaving behind unfertilized eggs that had settled to the bottom.

The fertilized eggs were transferred to a 250-L fiberglass tank containing seawater and observed for hatching. First, hatched larvae were allowed to develop at 18° to 20°C with a photoperiod of 11 hours of light and 13 hours of dark and fed an alginate-based microparticulate diet (Prof. P.M. Collins, University of California, Santa Barbara), which was gradually replaced by a mixed diet of rotifers and brine shrimp as development progressed. Pools of larvae were collected at incremental times from 1 to 46 dph and stored at −80°C until analysis. For videograph imaging, larvae collected at 1, 7, 14, 21, 28, 35, and 46 dph were transferred to a beaker of seawater containing benzocaine (60 mg/L) using a plastic pipette. The larvae were immersed in this solution for 1 to 2 seconds and then placed onto a glass microscope slide. Larvae were observed at a magnification of ×40 using a microscope (Nikon, SE, Japan) with

a CCD color video camera (CS5110, Tokyo Electronic Industrial Company Ltd, Japan) attached to a TV monitor (VT-1418M, Sharp, Japan) for image display.

Dry Weight and RNA-DNA Ratio Measurements

For dry weight measurements pools of larvae were collected at 1, 7, 14, 21, 28, 35, and 46 dph and rinsed in distilled water. Rinsed larvae were then dried at 60°C for 48 hours, after which time they were weighed using a microbalance (C-31, Thermo Cahn Instruments, Madison, Wis.). For RNA-DNA ratio measurements, larval samples were homogenized in 1 ml of 0.01 M phosphate buffered saline (PBS, pH 7.2) using an Ultra Turrax T25 rotor stator homogenizer (IKA Labortehnik, Germany), centrifuged at 10,000 *g* for 10 minutes, and the clear supernatant was decanted and kept on ice. For RNA quantification 50 µl of supernatant from each sample was transferred into a sterile 1.5-ml Eppendorf tube containing 5 µl of ×10 DNase reaction buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, 20 mM $CaCl_2$, pH 7.5) and briefly vortexed, and then 1 µl (10 U) of DNaseI (Gibco-BRL, Rockville, Md.) was added. For DNA quantification 50 µl of supernatant from each sample was transferred into a sterile Eppendorf tube containing 1 µl (10 U) of RNase A (Sigma, St. Louis, Mo.). The contents of the tubes were mixed by briefly vortexing and then incubated at 37°C for 1 hour. After incubation, the volume in each tube was adjusted to 500 µl with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and then 500 µl of RiboGreen or PicoGreen reagent (Molecular Probes, Eugene, Ore.) was added to sample tubes for RNA or DNA quantification, respectively. The samples were excited at 480 nm, and the fluorescence emission intensity was measured at 520 nm using a fluorescence spectrophotometer (F-4500, Hitachi, Japan). Fluorescence emission intensity values of each sample were read from a standard curve of RNA or DNA (62.5 pg/ml to 2 ng/ml), prepared from standards supplied with RiboGreen and PicoGreen reagents.

RT-PCR of GH, IGF-I, and Na^+-K^+-ATPase Subunit Transcripts

To obtain sufficient RNA for experiments, total RNA was extracted from pools of 100, 15, and 10 larvae, which were collected at 1, 7, and 14 dph, respectively. Because more RNA was available as the larvae grew, single larvae were collected at 21, 28, 35, and 46 dph and used for RNA extraction. For each sampling time a total of 5 pools of larvae (1–14 dph) and 5 single larvae (21–46 dph) were taken and total RNA was extracted using an RNeasy mini kit (Qiagen GmbH, Hilden, Germany), treated with DNaseI, and then quantified spectrophotometrically. The number of larvae used for each RNA extraction (*N*) and the total RNA yield (RNA_{Total}) were recorded for later use in data calculation.

For first-strand complementary DNA synthesis, 1 µg of total RNA from each sample was added to a reaction mix (20 µl), containing 0.5 µg of oligo DT primer (Pharmacia LKB, Uppsala, Sweden), 2 µl dithiothreiotol (0.1 M), 1 µl dNTP mix (10 mM, Pharmacia LKB), 4 µl reaction buffer, and 1 µl of Superscript II reverse transcriptase (200 U/µl; Gibco-BRL). First-strand cDNA synthesis was allowed to proceed at 42°C for 1 hour, after which time the reaction was incubated at 70°C for 15 minutes and then stored at 4°C. PCR amplification of first-strand cDNA was performed with a series of oligonucleotide primers designed from previously cloned genes.

The primers used for GH amplification were based on sequence information from a GH gene of gilthead sea bream *Sparus aurata* (Funkenstein et al., 1991) and designed to amplify a 615-bp fragment, whereas those used for IGF-I amplification were the same as those used for gilthead sea bream larvae (Perot et al., 1999) and designed to amplify a 237-bp fragment. The primers used for Na^+-K^+-ATPase α1 and β1 subunit amplification were designed from previously cloned subunit cDNA fragments of silver sea bream branchial tissue (Deane et al., 1999) and designed to amplify fragments of 576 and 558 bp, respectively. As a normalization control for each RT-PCR, primers specific for 18S rRNA were used (Harasewych et al., 1997), which were designed to amplify a 540-bp fragment. All primers were synthesized by Genset (Singapore) and had the following sequences:

1GH	5′-ATGGACAGAGTGGTGCTCAT-3′
	5′-AGGGTGCAGTTGGCCTCTGGAGA-3′ (antisense)
IGF-I	5′-AGTGCGATGTGCTGTATC-3′ (sense)
	5′-CAGCTCACAGCTTTGGAAG-3′ (antisense)
Na^+-K^+-ATPase	5′-AAGGCTATCCCTAAGGGGGTGGG-3′ (sense)
α1 subunit	5′-CATGTCAGTTCCCAGGTCCATAC-3′ (antisense)
Na^+-K^+-ATPase	5′-TTCATCGGGACCATCCAAGCCAT-3′ (sense)
β1 subunit	5′-GCCGCCGATACCGTATACTTGAT-3′ (antisense)
18S rRNA	5′-GCCAAGTAGCATATGCTTGTCTC-3′ (sense)
	5′-AGACTTGCCTCCAATGGATCC-3′ (antisense)

For each pair of specific primers, a PCR reaction (50 µl) containing 2 µl of first-strand cDNA, 0.2 µl of *Taq* DNA

polymerase (5 U/μl, Promega, Madison, Wis.), 5 μl of $MgCl_2$ (25 mM), 5 μl of reaction buffer, 0.5 μl of dNTP mix (10 mM), and 1 μl of each primer (50 pM) was prepared. PCR amplification was performed using a PTC-100 thermal cycler (MJ Research Inc, Waltham, Mass.) with cycle parameters of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension of 72°C for 4 minutes. In order to ensure amplification was within the linear range, 25 cycles of PCR were used. A single PCR product, of expected size, was obtained for each gene of interest, and these were subcloned into pCRscript plasmid vector (Stratagene, La Jolla, Calif.) and cycle sequenced using an ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer, Wellesley, Mass.). Reaction products were analyzed using an ABI 310 Genetic Analyzer (PerkinElmer) in order to confirm identity. No PCR products were detected from negative controls (reactions without reverse transcriptase added), and all PCR samples were stored at 4°C prior to further analysis.

Southern Blotting, Radioisotope Probing, and Semiquantification of mRNA Transcripts

For each PCR series an aliquot (10 μl) was taken from a representative sample of larvae collected at 1 to 46 dph and electrophoresed on a 1.4% wt/vol agarose gel (Bio-Rad), using a 100-bp DNA ladder (Pharmacia LKB) as a molecular size marker. Gels were then photographed under UV illumination and prepared for Southern transfer by immersion in 0.25 M of HCl for 15 minutes followed by immersion in a 0.5 M NaOH–0.15 M NaCl solution for a further 30 minutes. DNA was transferred to Hybond N+ membrane (Amersham, London, U.K.) using capillary blotting in 20× SSC (17.5% wt/vol NaCl; 8.8% wt/vol trisodium citrate) as described by Sambrook et al. (1989).

Following transfer membranes were rinsed in 6× SSC and the DNA was fixed to the membrane by UV cross-linking. For hybridization purified cDNA fragments of each gene were radiolabeled using a Rediprime random labeling kit (Amersham) and used for membrane hybridization in Rapid-Hyb buffer (Amersham) at 55°C for 16 hours. The membranes were then washed twice with a 2× SSC–0.1% SDS solution for 30 minutes, once in 0.1× SSC–0.1% SDS at 65°C for 15 minutes, air dried for 15 minutes, and then autoradiographed at −80°C using Hyperfilm (Amersham).

All samples were analyzed together in a single hybridization using DNA dot blots, which were prepared using a Bio-Dot microfiltration manifold (Bio-Rad). PCR amplification products (10 μl) from each larval sample were prepared and blotted according to instructions supplied with Hybond-N+ membrane and were hybridized and washed as described above. After washing the DNA dot blots were exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, Calif.) for 3 hours at room temperature, after which time the screens were scanned using the Storm PhosphorImaging system with ImageQuant software (Molecular Dynamics) for quantification of amplified fragment.

Na^+-K^+-ATPase Enzyme Assay

For larvae collected at 1, 7, and 14 dph, pools of 30, 15, and 5 larvae, respectively, were used; single larvae were used for remaining time points. Larvae were analyzed for Na^+-K^+-ATPase activity by homogenization in ice-cold SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% wt/vol sodium deoxycholate) and then centrifuged at 5000 *g* for 1 minute. An aliquot (50 μl) was used for enzyme assay at 25°C according to the method of McCormick (1993), and enzyme activity was calculated as nanomoles per minute per larva.

Data Calculation and Statistical Analysis

The abundance of each specific gene fragment was normalized to the corresponding 18S ribsomal RNA abundance for each sample to obtain the relative transcript abundance ($Transcript_{RA}$). For each sample the relative transcript abundance per larva ($Transcript_{RAL}$) at various larval collection time points was calculated using the following formula:

$$\frac{\text{Transcript}_{RAL} = \text{Transcript}_{RA}}{[N \div \text{RNA}_{Total}] \times \text{RNA}_{RT}}$$

where N = number of larvae used for each larval sample total RNA extraction; RNA_{Total} = Total RNA yield from pooled or single larval sample; and RNA_{RT} = amount of total RNA used in reverse transcriptase reaction.

The RNA-DNA ratios, dry weight values, calculated $Transcript_{RAL}$ for GH, IGF-I, and Na^+-K^+-ATPase subunits, and Na^+-K^+-ATPase activities for samples collected at each larval collection time were subjected to a 1-way analysis of

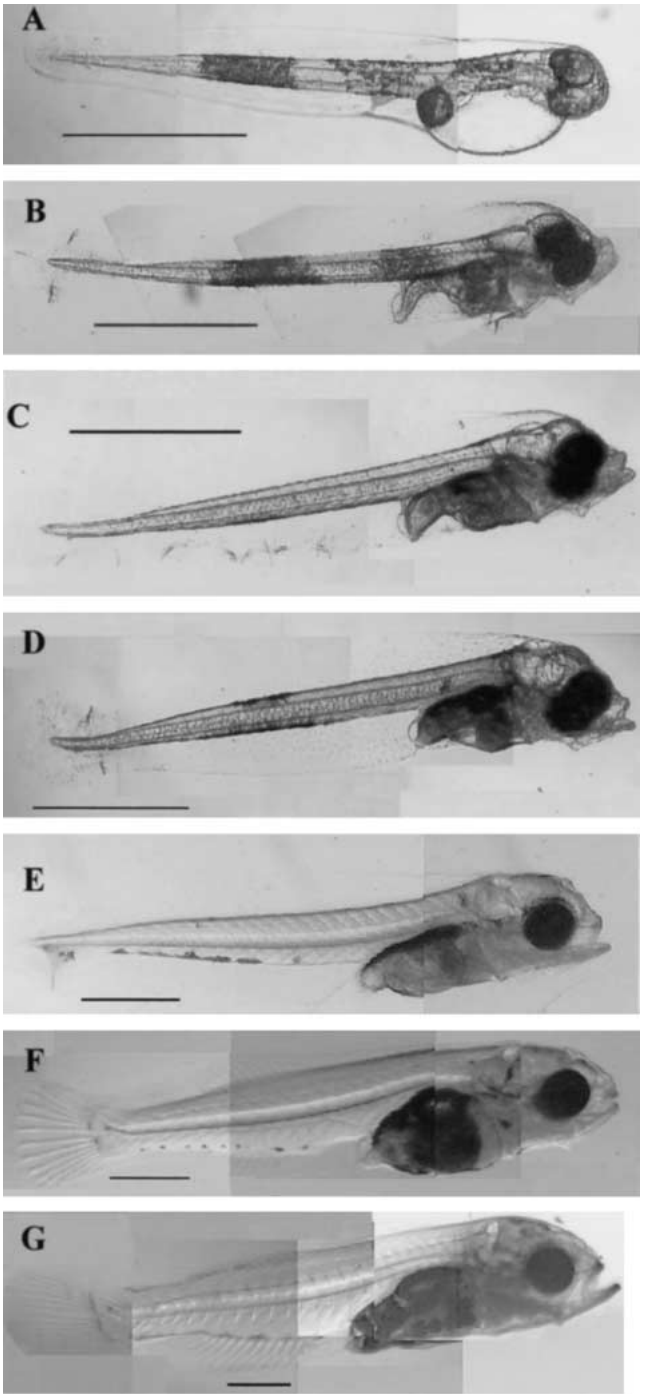

Figure 1. Morphologic characteristics of silver sea bream larvae at varying stages of development. Representative larvae from 1 dph (**A**), 7 dph (**B**), 14 dph (**C**), 21 dph (**D**), 28 dph (**E**), 35 dph (**F**), and 46 dph (**G**) were observed using videograph microscopy.
Scale bars = 1 mm

variance. In order to delineate significance among groups, a Student-Newman-Kuels test was used (Jandel Scientific). Significant differences were accepted if $P < 0.05$, and all data were expressed as mean ± SEM.

Results and Discussion

Morphologic Changes During Larval Development, RNA-DNA Ratio, and Dry Weight

Silver sea bream larvae samples were collected at intermittent times during development, and using microscopic videograph techniques we observed some key features in larval morphology during the first 46 dph (Figure 1). Analysis of the 1-dph group shows larvae that were morphologically similar to each other, being elongate with attached yolk sacs but without functional mouth, fins, and the appearance of heavily pigmented eyes. The development of mouth was complete by 3 dph, and all larvae were fed a microparticulate diet immediately. Samples taken at 7 dph are characterized by complete absorption of the yolk sac and a functional mouth, representative of the change from lecithotrophy to planktitrophy. Observations on 7-dph larvae also show the early development of fin analgen and components of the gut region. Flexion occurred at around 14 dph, and these larvae display caudal fin development, upturning of the notochord, and greater mobility. Larvae from 28 dph onward undergo rapid developmental changes to the juvenile stage characterized by a well-developed gut and fully formed fins, musculature, swimbladder, and lateral line system.

The ratio of RNA to DNA in whole larvae provides a useful indicator of nutrient status and growth rate (Buckley, 1984; Martin and Wright, 1987), and it is clear from the results obtained in this study that the RNA-DNA ratio in silver sea bream larvae displayed an ontogenetic pattern during development (Figure 2, A). The highest RNA-DNA ratio was found in larvae at 1 dph (50.32 ± 3.63) and appears to be typical of yolk sac larvae in general, as studies on red drum larvae (Westerman and Holt, 1994; Brightman et al., 1997) and Pacific herring larvae (Robinson and Ware, 1988) have also demonstrated this phenomenon. This high RNA-DNA ratio may be indicative of a high amount of maternal RNA deposited in the eggs prior to hatching. As the RNA-DNA ratio essentially provides an index of protein-synthesizing capacity in whole larvae, the high values observed in 1-dph silver sea bream larvae suggest a period of intense transcriptional "translational activity", and Westerman and Holt (1994) suggested that increased RNA-DNA ratios during early ontogeny are correlated with rapid cell division and increasing cell size. The RNA-DNA ratio decreased by 7.6-fold from 1 dph, reaching the lowest levels

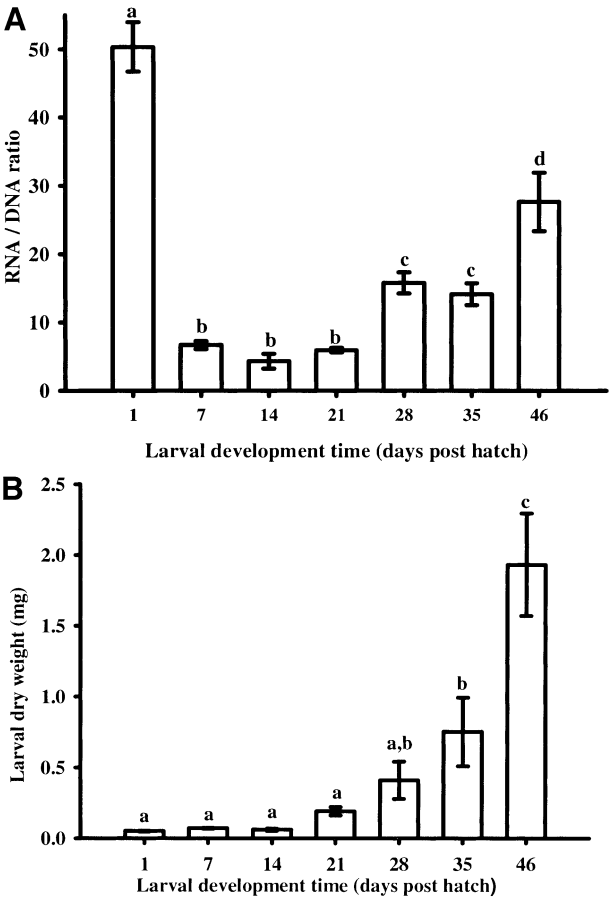

Figure 2. Growth indices during silver sea bream larval development. **A:** RNA-DNA ratio of silver sea bream larvae with values expressed as mean ± SEM (n = 5–7). **B:** Dry weight measurements of silver sea bream larvae with values expressed as mean ± SEM (n = 8–10). For both RNA-DNA ratio and dry weight measurements, mean values with different letters are significantly different from each other ($P < 0.05$).

(6.64–5.91) between 7 and 21 dph when the yolk sac was completely utilized, reflecting a diminished protein-synthesizing capability as larvae enter a period when they need to initiate exogenous feeding. Studies on a number of fish have demonstrated a positive correlation between RNA-DNA ratio and short-term growth rate (Wilder and Stanley, 1983; Miglavs and Jobling, 1989; Ferguson and Danzman, 1990; Bastrup et al., 1992), and it appears that the increased RNA-DNA ratio in silver sea bream larvae from 28 dph onward correlated well with increased growth, as determined by dry weight measurements, which from 21 dph increased steadily by 1.8 to 3.2 fold, reaching a value of 1.93 ± 0.42 mg dry weight upon cessation of larval sample collection (Figure 2, B).

Ontogeny of GH and IGF-I mRNA Abundance

To investigate the ontogeny of GH and IGF-I transcripts during silver sea bream larval development, we used RT-PCR coupled with radioisotope hybridization and found a single cDNA product of 615 bp for GH (Figure 3, A) and 237 bp for IGF-I (Figure 3, B). The amplified cDNA products of both GH and IGF-I transcripts were detected at all larval collection times using 1 μg of total RNA from pooled larvae (1–14 dph) or single larvae (21–46 dph). Similarly a single GH transcript has been detected during early development of rainbow trout (Yang et al., 1999) and gilthead sea bream (Funkenstein and Cohen, 1996), and a single IGF-I transcript has been detected during early development of gilthead sea bream (Perot et al., 1999). In this study the Southern blots of transcript signals can only be used qualitatively to show the presence of transcripts at each larval collection time but cannot be used for semiquantitative determination of specific transcripts. RT-PCR uses a standard amount of total RNA, and data presented solely as relative mRNA abundance for developing larvae would not be sufficient to allow for firm conclusions since transcript abundance relative to whole body RNA cannot be accounted for. As such we chose to express our data as relative mRNA abundance per larva using the calculation described in "Materials and Methods" in order to provide a clearer picture of transcript expression profiles.

In this study we detected GH mRNA at 1 dph (Figure 4, A), which is indicative of the presence of GH mRNA from a maternal origin, or early GH transcription, or both. The abundance of silver sea bream larval GH increased progressively reaching a constant amount at 35 to 46 dph, indicative of a high rate of GH transcription or mRNA turnover, possibly as a result of a fully formed and functional pituitary gland. The early detection of IGF-I mRNA in newly hatched larvae has been previously described for gilthead sea bream (Perot et al., 1999) and rainbow trout (Greene and Chen, 1997), and similarly in this study we detected IGF-I transcript in silver sea bream larvae at 1 dph (Figure 4, B). The 5-fold increase in IGF-I mRNA abundance between 28 dph and 35 dph indicates a state of high IGF-I transcription, possibly linked to the numerous processes involved during developmental changes to the juvenile stage. Indeed a potent role of IGF-I on growth, differentiation, and morphogenesis has been described in a number of vertebrate systems (Froesch et al., 1985; Duan, 1997).

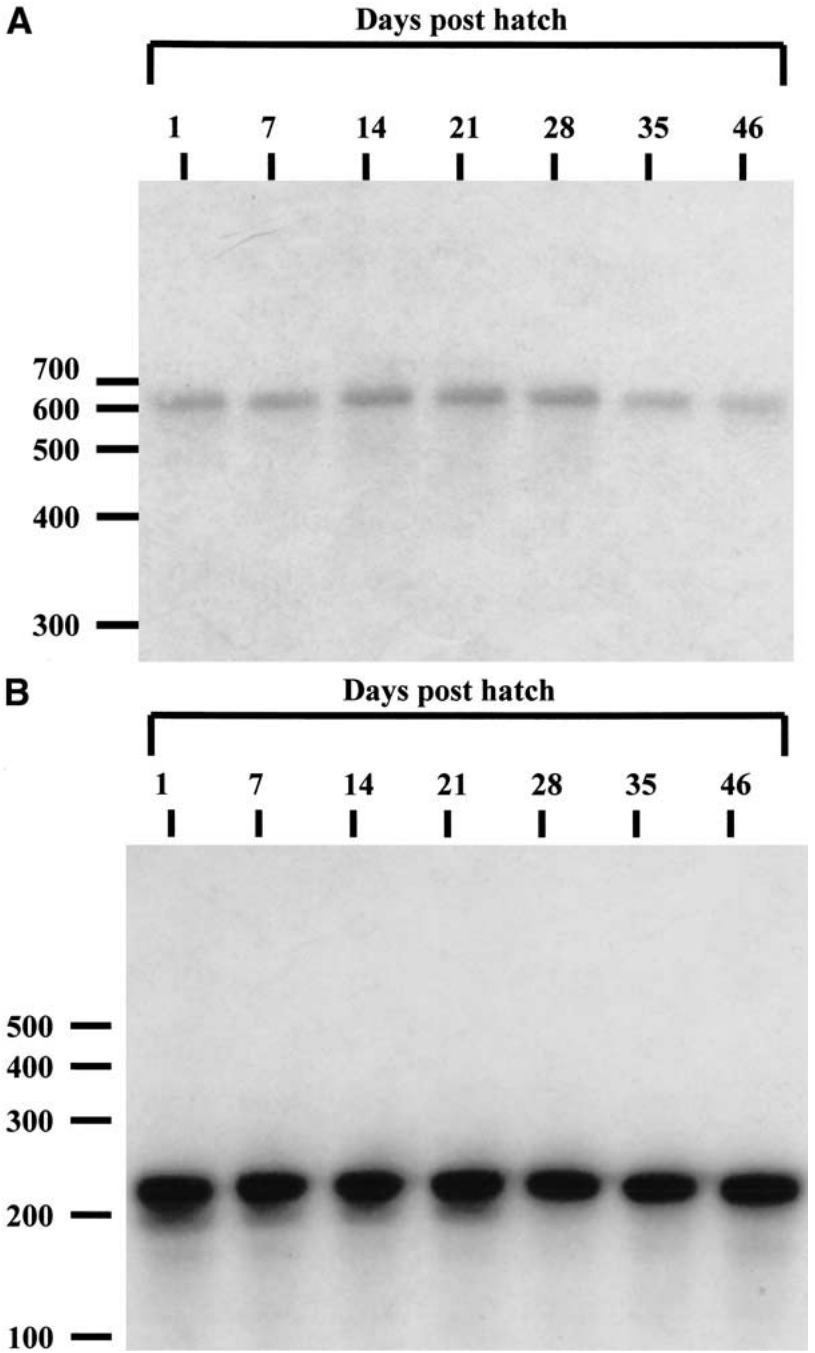

Figure 3. A representative Southern blot showing the presence of GH transcript (**A**) and IGF-I transcript (**B**) during silver sea bream larval development (1–46 dph). The larval development times from 1 to 46 dph are indicated above each lane, and the positions of molecular size markers are indicated in base pairs.

The ontogenetic changes in GH and IGF-I transcripts during silver sea bream larval development appeared to bear an allometric relationship to dry weight as they increased 33 and 70 fold, respectively, at maximum levels, whereas larval dry weight increased only 20 fold over 46 days. Presently, there is strong evidence for the role of IGF-I as a mediator for the somatogenic action of GH and a functional GH–IGF-I axis regulated via negative feedback in adult fish (Cao et al., 1989; Funkenstein et al., 1989; Pérez-Sánchez et al., 1992; Duan et al., 1993; Duan, 1997). Whether such an axis is functional in developing larvae is unknown, although Perot et al. (1999) suggested that the

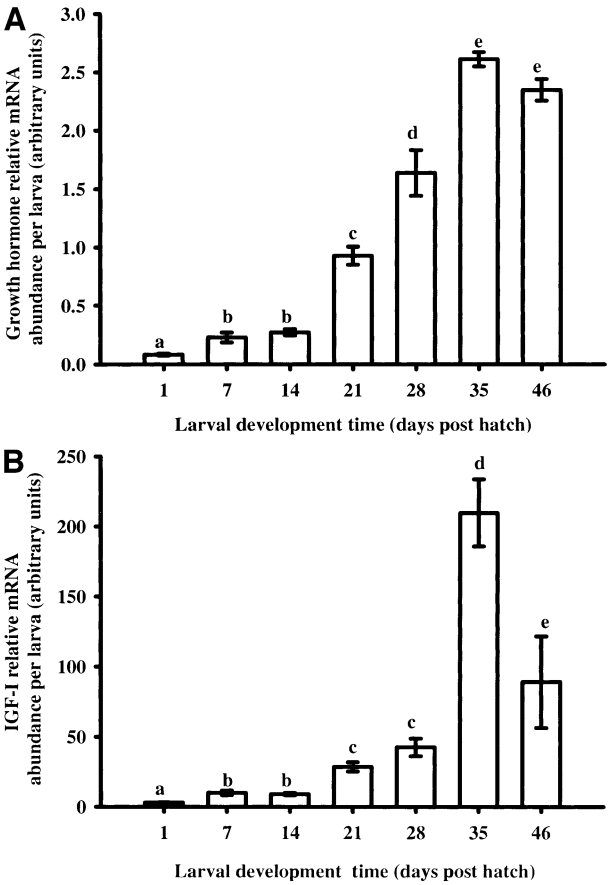

Figure 4. Ontogeny of GH and IGF-I transcripts in silver sea bream larvae from 1 to 46 dph using semiquantitative RT-PCR. The values for GH transcript (**A**) and IGF-I transcript (**B**) were normalized to the corresponding 18S rRNA from the same sample and then calculated per individual larva. All values are expressed as mean ± SEM ($n = 5$), and mean values with different letters are significantly different from each other ($P < 0.05$).

early expression of gilthead sea bream IGF-I may be GH-independent as IGF-I mRNA was detected before the appearance of GH mRNA. From the data obtained in this study, we cannot confirm or refute the existence of a functional GH–IGF-I axis in silver sea bream larvae because our analyses are based solely on relative mRNA level. Further experiments focused on evaluating IGF-I levels following GH treatment and GH levels following IGF-I treatment, during larval development, are required before firm conclusions can be drawn.

Ontogeny of Na^+-K^+-ATPase

Fundamental to teleost ionic exchange mechanisms is Na^+-K^+-ATPase, a plasma membrane-bound enzyme that exists as a heterodimeric molecule composed of a catalytic α subunit and a glycosylated β subunit. In this study we aimed to investigate the ontogeny of Na^+-K^+-ATPase α1 and β1 subunit isoforms and relate this to functional enzyme activity during silver sea bream larval development. Following RT-PCR and radioisotope hybridization, we found single cDNA products of 576 and 558 bp for the catalytic α1 and glycosylated β1 subunits, respectively, and both transcripts were detected at all stages of larval development (Figure 5). Similarly using Northern blotting of total branchial RNA, we have detected single transcripts for both subunits in adult silver sea bream (Deane et al., 1999, 2000). Of greater interest were the expression profiles of these subunits during early larval development of silver sea bream, and it was found that the α1 subunit was low at 1 dph and increased as development progressed, reaching a high and constant amount between 35 and 46 dph (Figure 6, A). The β1 subunit followed a similar profile to that of α1 but decreased 2.5 fold between 35 and 46 dph (Figure 6, B). The translation of modulated larval Na^+-K^+-ATPase subunit mRNA into functional pumps was examined using the Na^+-K^+-ATPase-specific inhibitor ouabain, in enzyme assays, and it was found that activity increased steadily from 1 to 46 dph (Figure 7).

It has been established from studies on mammals that increased Na^+-K^+-ATPase activity is regulated by a coordinate increase of both α and β subunits (McDonough et al., 1988). However, when the expression profiles for silver sea bream α1 and β1 are compared with that of Na^+-K^+-ATPase activity, a clear correlation is not readily apparent. For example, the 80-fold increase in Na^+-K^+-ATPase activity between 1 and 28 dph was not paralleled by a similar fold increase in Na^+-K^+-ATPase subunit mRNA, which increased 10 fold (α1) and 14 fold (β1) over the same period. A plausible explanation for such a discrepancy is the possible involvement of different α- and β-subunit isoforms during larval development, a phenomenon that cannot be accounted for in the present study since our RT-PCR assay is specific for α1 and β1. It would appear from accumulating evidence, mainly from mammalian studies, that Na^+-K^+-ATPase pumps of the α1β1 type are ubiquitous, possibly playing a general housekeeping role, but other isozymes consisting of different combinations of subunits may be regulated in a tissue-specific manner (Blanco and Mercer, 1998). While no information exists as to the expression of different α and β isoforms in developing fish larvae, it has been shown that developmental changes occur in the relative levels of α isoforms in rat myocardium

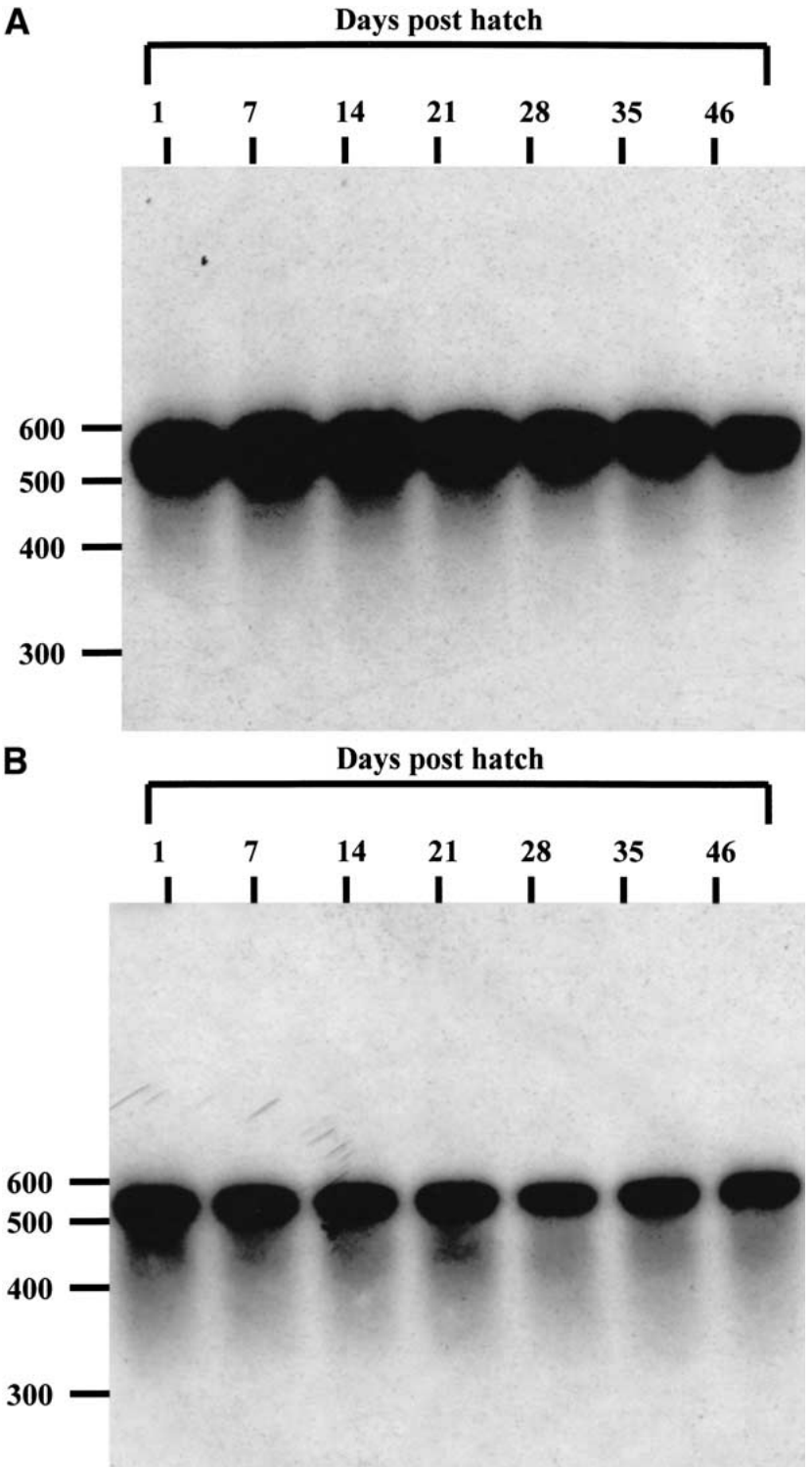

Figure 5. A representative Southern blot showing the presence of Na^+-K^+-ATPase α-subunit transcript (**A**) and Na^+-K^+-ATPase β-subunit transcript (**B**) during silver sea bream larval development (1–46 dph). The larval development times from 1 to 46 dph are indicated above each lane, and the positions of molecular size markers are indicated in base pairs.

(Lucchesi and Sweadner, 1991), and different functional roles also of α isoforms have been proved in mouse skeletal muscle (He et al., 2001). It appears that in order to correlate Na^+-K^+-ATPase subunit expression with Na^+-K^+-ATPase enzyme activity, cumulative values for all α and β isoforms during larval development are needed.

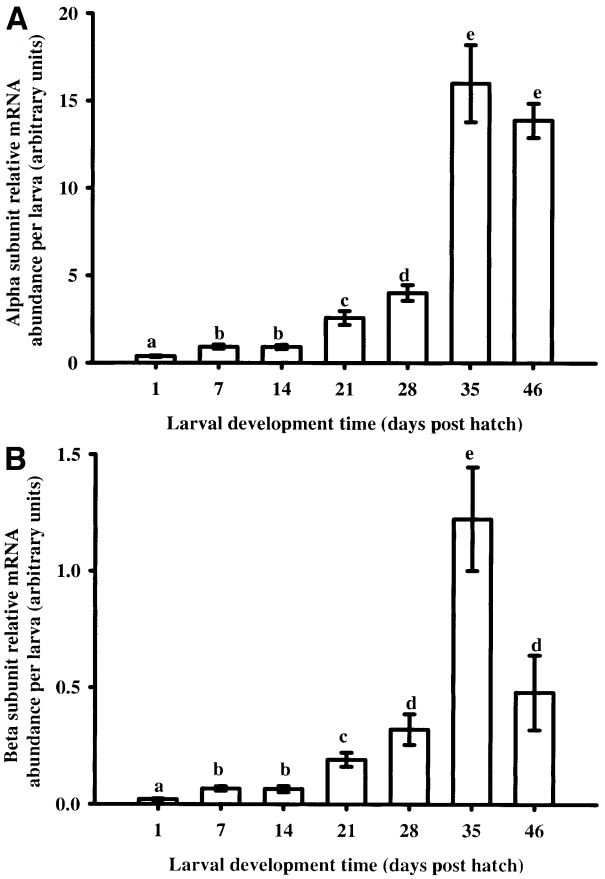

Figure 6. Ontogeny of Na^+-K^+-ATPase α- and β-subunit transcripts in silver sea bream larvae from 1 to 46 dph using semiquantitative RT-PCR. The values for Na^+-K^+-ATPase α-subunit transcript (**A**) and Na^+-K^+-ATPase β-subunit transcript (**B**) were normalized to the corresponding 18S rRNA from the same sample and then calculated per individual larva. All values are expressed as mean ± SEM ($n = 5$), and mean values with different letters are significantly different from each other ($P < 0.05$).

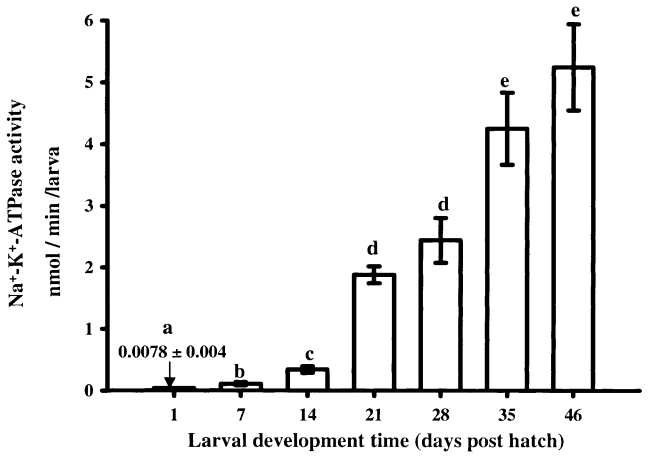

Figure 7. Ontogeny of Na^+-K^+-ATPase enzyme activity during silver sea bream larval development (1–46 dph). The Na^+-K^+-ATPase activity for the 1-dph group is indicated, and all values are expressed as mean ± SEM ($n = 5$). Mean values with different letters are significantly different from each other ($P < 0.05$).

The regulatory mechanisms that control the formation of stoichiometric α/β complexes in fish are unknown, but several studies on mammalian systems have provided evidence that the β subunit is necessary for stability, correct assembly, and proper insertion of the heterodimeric Na^+-K^+-ATPase molecule into the plasma membrane (Geering et al., 1989; McDonough et al., 1990; Noguchi et al., 1990). In addition, data from our previous study on branchial Na^+-K^+-ATPase in adult silver sea bream suggested that β-subunit expression may be a rate-limiting factor for increased Na^+-K^+-ATPase activity as cortisol administration elevated Na^+-K^+-ATPase α1-subunit mRNA but both β1-subunit and Na^+-K^+-ATPase activity remained unchanged (Deane et al., 2000). In the present study we found that β1 subunit decreased significantly at 46 dph, and this down-regulation may be indicative of changes in Na^+-K^+-ATPase activity within branchial tissue, but this effect would not be easily detectable because whole body Na^+-K^+-ATPase activity was measured, masking any possible tissue-specific changes.

Leong and Manahan (1997) suggested that Na^+-K^+-ATPase could serve as a useful index for measuring physiologic status during sea urchin development because of its major metabolic significance. Although Na^+-K^+-ATPase has not been appraised as a potential index for growth in fish larvae, several key metabolic enzymes including citrate synthase and lactate dehydrogenase have been used with varying degrees of success (for review, see Ferron and Leggett, 1997). At present we can only evaluate the potential of measuring Na^+-K^+-ATPase as an index of larval growth for silver sea bream by comparing relative changes with other parameters that we have reported in this study. It can be seen from the data obtained in this study that between 1 and 21 dph Na^+-K^+-ATPase enzyme activity increased approximately 60 fold, whereas over the same time period dry weight was not significantly altered, RNA-DNA ratio remained low, GH mRNA increased 11 fold, and IGF-I mRNA increased 9.5 fold. Therefore, the possibility that measurements of Na^+-K^+-ATPase activity could serve as a useful index for larval growth warrants further study; however, before such an index can be validated, more studies encompassing a wide variety of fish species are required.

Final Conclusions and Perspectives

We have demonstrated that silver sea bream larval development is characterized by a series of pronounced morphologic, biochemical, and molecular events. We have described the ontogenetic profiles of both GH and IGF-I, and an unresolved question that warrants further study is at which point (if any) during silver sea bream development is a functional GH–IGF-I axis established. We have also demonstrated for the first time the ontogeny of Na^+-K^+-ATPase $\alpha 1$ and $\beta 1$ subunit mRNA as well as Na^+-K^+-ATPase activity, and it does appear that measuring certain fundamental metabolic enzymes may provide for useful indices during fish larval development. The transition of Na^+-K^+-ATPase from the larval skin to key osmoregulatory organs such as gill and kidney during larval development is not well understood at present; further studies using immunolocalization for both Na^+-K^+-ATPase subunits are required in order to understand this key process.

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