Stress-induced regulation of steroidogenic acute regulatory protein expression in head kidney of Gilthead sebream (*Sparus aurata*)

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Abstract

Steroidogenic acute regulatory protein (StAR) transfers cholesterol over the inner mitochondrial membrane. In mammals, StAR controls this rate-limiting step of steroidogenesis, but its expression and regulation has not been well explored in fish. The present work investigates StAR mRNA expression in the head kidney of the gilthead sebream (*Sparus aurata*) under different stressors. We have cloned the StAR cDNA (1461 bp) in sebream (accession number EF640987), which has an open reading frame of 861 nucleotides encoding a polypeptide of 286 aa, and displays high sequence identity with StAR of other fish and mammalian counterparts. Sebream StAR transcripts were found to be expressed exclusively in head kidneys and gonads. In fish under acute stress (chased with a net), plasma cortisol levels peaked within 1 h, were still high after 6 h, and decreased after 16 h, although no increases in head kidney StAR expression were observed at any time post-stressor. Fish under chronic high-density stress showed cortisol levels 90-fold higher than controls and StAR mRNA levels increased threefold. Lipopolysaccharide (LPS) injection increased head kidney StAR mRNA levels after 6 h, reached a maximum at 12 h, and decreased until 72 h. When the head kidney cells were incubated *in vitro* and treated with ACTH or LPS, ACTH induced an increase in StAR expression as expected, but LPS induced a reduction in StAR expression. In conclusion, StAR expression in sebream head kidneys is highly regulated by different stressors.

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Introduction

In teleost fish, the endocrine response to stress is composed of the adrenergic response and the hypothalamic–pituitary–interrenal (HPI) response (Wendelaar Bonga 1997). The HPI response leads to an increase in glucocorticoid levels, of which the major regulator of the synthesis of glucocorticoids (cortisol or corticosterone; Carsia *et al.* 1984, Netchitailo *et al.* 1984, Klingbeil 1985, Wendelaar Bonga 1997, Ariyoshi *et al.* 1998, Le Roy *et al.* 2000, Carsia & John-Alder 2003). In mammals, ACTH induces a rapid increase in corticosteroid synthesis and a longer term chronic cyclic AMP (cAMP)/protein kinase A (PKA)-based response that affects both the transcription and translation of several genes encoding for steroidogenic enzymes (reviewed by Sewer & Waterman 2003). In addition, in mammals a very rapid increase in both StAR mRNA (Fleury *et al.* 1996, Lehoux *et al.* 1998, Ivell *et al.* 2000, Le Roy *et al.* 2000) and StAR protein (Nishikawa *et al.* 1996; reviewed by Lehoux *et al.* 2003) occurs in response to ACTH. Moreover, plasma cortisol has been found to mirror levels of StAR mRNA (Ariyoshi *et al.* 1998, Le Roy *et al.* 2000) and StAR protein (Liu *et al.* 1996, Nishikawa *et al.* 1996). Transcription of the StAR gene (Clark *et al.* 1995) and activation of StAR protein (Arakane *et al.* 1996) in response to ACTH are mediated via the PKA pathway. In mammalian systems, therefore, StAR gene transcription (Liu *et al.* 1996), StAR protein levels (Sandhoff *et al.* 1998), and steroidogenesis (Sugawara *et al.* 2000) all increase in response...
to cAMP, via activation of the PKA intracellular signaling pathway. Current knowledge about StAR in vertebrates is based on mammalian studies, but the protein structure appears to be highly conserved across species (Bauer et al. 2000, Kusakabe et al. 2002, Goetz et al. 2004). StAR cDNA has been previously cloned from rainbow trout, and several regions of the protein showed a high degree of homology with human StAR (Kusakabe et al. 2002), especially in the C-terminal region, which contains hydrophobic sites for cholesterol binding (Tsujishita & Hurley 2000). Concerning fish studies, StAR transcripts have been detected in the steroidogenic tissues of rainbow trout, and the levels of StAR transcripts in the head kidney tissue, containing the steroidogenic interrenal cells, have been shown to increase in response to severe acute stress (Kusakabe et al. 2002, Geslin & Auperin 2004) or when ACTH was injected in eels (Li et al. 2003), suggesting that StAR is an important regulator of corticosteroidogenesis in fish. Despite these results for several fish species, there is relatively little information on StAR gene expression and regulation in perciform fish. As a result, we cloned, sequenced, and quantified StAR mRNA expression in adult gilthead seabream in basal conditions as well as in different stress situations. Moreover, we measured the StAR response to ACTH in an in vivo model of the head kidney interrenal cells. Finally, we have tested the effects of bacterial lipopolysaccharide (LPS) both in vivo and in vitro to check the response of StAR gene expression to an infectious process.

Materials and Methods

Animals

Sexually immature gilthead seabream (Sparus aurata), with an average weight of 80 g, were obtained from a fish farm (Masnou, Barcelona, Spain) and maintained in a semi-closed seawater flow circuit with water at a temperature of 17 °C and a salinity of 37% under a 12 h light:12 h darkness cycle and a density of 7 kg/m³. Blood was sampled from the caudal vein using heparinized syringes in <1 min/individual and <5 min/treatment group. Plasma was obtained by centrifugation and stored at –80°C until analysis by RIA. To collect the seabream tissues, adult fish were killed by an excess of anesthesia (2-phenoxymethanol, 1:1000 v/v; Sigma–Aldrich, #P1126), which is the standard method to kill fish, and the tissues were removed and immediately frozen in liquid nitrogen until analysis.

Cloning and sequencing of gilthead seabream StAR

A cDNA clone with high homology to StAR was identified in a collection of expressed sequence tags obtained from a gilthead seabream cDNA library made in a ZAP Express III (Stratagene, Cedar Creek, TX, USA) with RNA pooled from several relevant immune tissues. This library was enriched for the sequences up-regulated after immune stimulation, and processed as described by our group (Castellana et al. personal communication). Two clones corresponding to the seabream StAR cDNA were identified, and each one was sequenced a minimum of four times in both directions using BigDye Terminator (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 automated sequencer (Applied Biosystems). The full-length sequence of the StAR cDNA was obtained by overlapping the two sets of partial sequences from each clone. The sequence was confirmed by performing RT-PCR to amplify the open reading frame (ORF).

Sequence analysis

Sequence data were compiled from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), and sequences were analyzed for similarity with other known sequences using BLAST within the ExPASy Proteomics Tools server. Alignments were performed using the Bioedit Sequence Alignment Editor (Hall 1999), which uses the CLUSTAL W algorithm (Thompson et al. 1994). The assembled full-length cDNA sequence was entered in GenBank with accession number EF640987. The pI and molecular mass of seabream StAR protein were predicted using the ExPASy Proteomics Server of the Swiss Institute of Bioinformatics (SIB; http://www.expasy.ch/tools/; Gasteiger et al. 2003).

Analysis of StAR tissue expression

For tissue expression analysis, total RNA was isolated from a pool of tissues using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) following the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed with Superscript III reverse transcriptase (Invitrogen SA), according to the manufacturer's protocol. The PCRs were performed using Taq DNA polymerase (Biotools, Madrid, Spain) with 2 mM MgCl₂, 800 nM primer final concentration, and 1 μl reverse-transcribed tissue RNA. The cycling conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 59 °C for 50 s, and 72 °C for 50 s. The final step was an extension at 72 °C for 10 min. The reactions were run on 1% agarose gel and stained with ethidium bromide. Parallel RT-PCRs were carried out with specific primers against a conserved region of 18S (primers 18S forward and 18S reverse) as a control (Table 1).

Experimental procedure

We designed an acute stress, chronic stress, and experimental infection in gilthead seabream. For the acute stress experiment, fish were chased with a net in the tank for 5 min, and sampled 1, 6, and 16 h after the stress (n = 8 for each time point). Fish were killed by an excess of anesthesia (2-phenoxymethanol), and tissues and blood were obtained as described earlier. Undisturbed animals not subjected to stress were used as a control (n = 8).

For the chronic stress experiments, fish (n = 16) were kept for 15 days at a high density (50 kg/m³) in the tank. After that period, fish were killed by an excess of anesthesia, tissues were quickly removed and immediately frozen and blood obtained as indicated above. Undisturbed animals were used as controls (n = 16).
controls (analysis by RIA. Animals injected with saline were used as medium with or without LPS (10^16 h). Each time condition was replicated six times on a six-well culture plate. After incubation, medium was removed, pooled for each condition, and frozen immediately for cortisol determination, and total RNA was isolated with TRI reagent following the manufacturer’s instructions and pooled.

To simulate stress by infection, a concentration of 8 mg/kg of bacterial LPS was injected intraperitoneally. Fish (n=5) were killed by an excess of anesthesia (2-phenoxyethanol) at different times (6, 12, 24, 72 h) and tissues were quickly removed and immediately frozen. Blood was sampled from the caudal vein using heparinized syringes; plasma was obtained by centrifugation and stored at −80°C until analysis by RIA. Animals injected with saline were used as controls (n=5).

Preparation of head kidney cells and in vitro assays
Gilthead seabream head kidney cells were isolated and cultured by adapting the protocol previously described for trout macrophages (Mackenzie et al. 2003). Head kidneys were dissected from seabream killed by overanesthetization (2-phenoxyethanol) at 17°C, 5% CO2 with Dulbecco’s Modified Eagle Medium (Life Technologies, #11971-025) containing high glucose, 10% heat-inactivated fetal calf serum (Gibco, #16140-071), and penicillin (100 U/ml)/streptomycin (100 µg/ml; Life Technologies).

Following isolation, cells were left undisturbed for 3 h to stabilize the cortisol levels as described previously (Rotllant et al. 2001). After this period, the head kidney cells were incubated with medium with or without ACTH (150 ng/ml) for 1, 2, 6, and 18 h. In order to check the specificity of ACTH effects on StAR gene stimulation, in another experiment we incubated the head kidney preparations with ACTH and ACTH plus 1 µM corticotropin-inhibiting peptide (CIP; Sigma–Aldrich, #A1527), an ACTH antagonist that binds to ACTH receptor without activating its signal transduction, and therefore blocking ACTH effects.

In another set of experiments, cells were incubated in medium with or without LPS (10 µg/ml) for 1, 2, 6, and 16 h. Each time condition was replicated six times on a six-well culture plate. After incubation, medium was removed, pooled for each condition, and frozen immediately for cortisol determination, and total RNA was isolated with TRI reagent following the manufacturer’s instructions and pooled.

Quantitative real-time PCR
The cDNAs from the seabream tissue and head kidney preparations were used for quantitative PCR analysis using SYBR Green PCR Supermix (Bio–Rad). Results were evaluated with the ICYCLER IQ real-time detection system software (Bio–Rad). The sequences of the primers used in gene expression analysis are presented in Table 1. The total volume (20 µl) of every reaction contained 500 nM of each amplification primer, 10 µl 2×SYBR Green PCR Mix, and 5 µl of a 1:100 dilution of cDNA (1:1000 for 18S determination). Products were amplified in an ICycler IQ Real Time PCR Detection System (Bio–Rad Laboratories). Controls lacking cDNA and controls containing RNA were included. The real-time analysis consisted of 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s and 60°C for 30 s, 1 cycle of 95°C for 1 min, 1 cycle of 55°C for 1 min, and a melting curve of 81 cycles (from 55°C to 95°C). All samples were run in triplicate and fluorescence was measured at the end of every extension step. Threshold cycle (Ct) values for each sample were expressed as ‘fold differences’, calculated relative to untreated controls and normalized to endogenous control 18S rRNA.

Cortisol measurements
For cortisol measurements, the samples were kept frozen at −20°C until assays could be performed. Cortisol levels were measured both in plasma samples from adult fish and the culture media of head kidney cells by an RIA method (RIA) according to Rotllant et al. (2001). The antibody used for the assay was purchased from Biolink, SL (Costa Mesa, CA, USA) in a final dilution of 1:6000. Antibody cross-reactivity with cortisol is 100%. The radioactivity was quantified using a liquid scintillation counter.

Statistical analysis
For gene expression quantification by real-time PCR on in vitro assays, total RNA was pooled from six different wells for each treatment and analyzed in triplicate. Statistical significance was analyzed by one-way ANOVA followed by Tukey’s post hoc test, using the software package SPSS (Chicago, IL, USA) for Windows. Differences were considered significant when P<0.05. All cortisol measurements were performed

Table 1 Sequences of primers used in gene expression analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence</th>
<th>Direction</th>
<th>Size of amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>StAR</td>
<td>5’-AGTAACCTGGATCGCAAGCTCC-3’</td>
<td>Forward</td>
<td>440</td>
</tr>
<tr>
<td>StAR</td>
<td>5’-CCACCACATTACCCAGAGTCT-3’</td>
<td>Reverse</td>
<td>440</td>
</tr>
<tr>
<td>StAR real time</td>
<td>5’-GCTGGATCCAAAAGCAATCT-3’</td>
<td>Reverse</td>
<td>175</td>
</tr>
<tr>
<td>StAR real time</td>
<td>5’-CTTGCTCTTTGACTACTGT-3’</td>
<td>Forward</td>
<td>175</td>
</tr>
<tr>
<td>r18S</td>
<td>5’-CGAGAATAACGTCTGTG-3’</td>
<td>Forward</td>
<td>211</td>
</tr>
<tr>
<td>r18S</td>
<td>5’-GGGCAGGACTTAAATCAA-3’</td>
<td>Reverse</td>
<td>211</td>
</tr>
</tbody>
</table>

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using medium of six wells per experiment on in vitro experiments and eight animals per condition on in vivo assays, and its statistical significance was analyzed by one-way ANOVA followed by Fisher's LSD post hoc test. Differences were considered significant when \( P < 0.05 \).

**Results**

**StAR cloning and sequencing**

A gilthead seabream (\( S. \) *aurata*) cDNA library made in \( \lambda \) ZAP Express III (Stratagene) from several relevant immune sources was used to clone the steroidogenic acute regulatory protein, StAR (GenBank accession number EF640987). The 1461 bp StAR full cDNA contains a 123 bp 5' UTR, an 861 bp ORF and a 477 bp 3' UTR.

**Deduced amino acid sequence analysis**

The \( S. \) *aurata* StAR gene encodes a 286 amino acid protein, which has a predicted molecular mass of 32 kDa and a pI of 9. Amino acid analysis was performed to determine the relatedness of gilthead seabream StAR to StARs from other fish (Fig. 1). At the amino acid level, seabream StAR shows the highest sequence identity to StAR protein from black bass (94%), whereas identity with rainbow trout (87%) is lower. On the other hand, seabream StAR shows about 69% identity with other vertebrate StARs, such as birds and amphibians, and a 66% sequence identity with human StAR.

**StAR expression and regulation by stress**

Primer sequences used in the PCR studies are shown in Table 1. Using RT-PCR, we found the StAR gene to be expressed in head kidneys and gonads. The basal expression of StAR was higher in head kidney than in gonads, and the rest of tissues showed no StAR gene expression (Fig. 2). In addition, we found no expression in muscle samples (data not shown).

Under acute stress (5 min chase with a net in the tank), plasma cortisol reached a maximum peak 1 h post-stressor, and levels were about 160 times higher than those in control, undisturbed animals (229.46 ± 43.18 nM versus 1.36 ± 0.64 nM respectively). Cortisol remained at very high levels 6 h after the acute stress (262.86 ± 105.31 nM) and after 16 h they had largely decreased, although they were still significantly higher than controls (27.48 ± 7.26 nM). We found no increase in StAR levels (measured by real-time quantitative PCR) after 1, 6, or 16 h of acute stress (Fig. 3).

Under chronic stress (15 days in overcrowded conditions), we found that stressed fish showed a significant increase (about 90 times) in plasma cortisol levels compared with control animals (129.33 ± 24.66 nM versus 1.48 ± 0.72 nM respectively). StAR mRNA levels in head kidneys from stressed animals were significantly higher (about 2.5-fold) than those of undisturbed animals (Fig. 4).

In fish injected with bacterial LPS at a dose of 8 mg/kg to simulate an acute stress by infection, real-time quantitative PCR showed that StAR mRNA levels in head kidneys were slightly increased 6 h after the injection (1.36-fold), and reached maximum levels at 12 h (4.46-fold). After that, StAR mRNA levels decreased progressively, although after 72 h, they were still clearly higher than the levels of saline-injected animals (3.31-fold; Fig. 5). Plasma cortisol levels followed the same pattern already described by our group in control and LPS-injected seabream (Acerete et al. 2007), peaking at 6 h post-injection and decreasing progressively with time (at 12 and 24 h) to the levels of control animals after 72 h.

**StAR expression and regulation on in vitro head kidney preparations**

When the seabream head kidney cells were incubated with 150 ng/ml ACTH, cortisol levels in the incubation media dramatically rose after 1 h (5553% of control). After 6 h, cortisol levels were lower, although still higher than control levels (628% of control; Fig. 6B). The StAR mRNA levels measured by quantitative real-time PCR increased in ACTH-stimulated cells at 1 h (about threefold the control levels). However, after 6 h of incubation, StAR mRNA levels were lower compared with controls (Fig. 6A).

In addition, CIP, the ACTH receptor antagonist, in a concentration of 1 \( \mu \)M, blocked effectively the ACTH-induced stimulation of StAR gene expression when co-incubated with ACTH, but it did not affect the StAR basal expression in the head kidney preparations when incubated alone (Fig. 7).

On the other hand, when the head kidney preparations were incubated with bacterial LPS (10 \( \mu \)g/ml), cortisol levels in the incubation media decreased at all times tested; after 1 h of incubation, cortisol levels decreased to 20.37% of control levels in LPS-incubated cells, and a similar situation was found after 16 h (reduction to 48.49% of control; Fig. 8B). The expression levels of StAR decreased although not significantly after 1 h of incubation, whereas after 16 h there was a significant reduction compared with controls (79% reduction; Fig. 8A).

**Discussion**

In this study, the complete sequence of a StAR cDNA (with a length of 1461 bp) has been obtained from a seabream cDNA library made from several relevant immune tissues. The \( S. \) *aurata* StAR protein is composed of 286 amino acid residues, a size that is identical or similar in length to those of teleosts and mammals identified so far. The seabream peptide displays a high identity to that of other fish and mammalian StAR proteins. When compared with the StAR protein of other vertebrate species, the putative phosphorylation sites by PKA (RRSS; reviewed by Strauss et al. 1999) are conserved in the seabream StAR protein. In the N-terminal 25 residues, the seabream StAR protein displays 100% identity to that of
Figure 1 Alignment of the deduced amino acid sequence of seabream StAR with those of other vertebrates analyzed by Clustal W multiple alignment. The sequences were obtained from the GenBank database: gilthead seabream (EF640987), rainbow trout (BAB18779), Atlantic cod (AAP44111), Japanese eel (BAC66210), zebrafish (AAH75967), black bass (AAZ92554), chicken (AAG28594), rat (AAH88859), and human (AAB88174). Amino acid numbers are shown on the right. Amino acid residues that are identical in all StAR proteins are shown with gray background. Gaps in the amino acid sequences are indicated with a dash (—). The N-terminal 25 residues are indicated by a solid horizontal line. Two potential sites of protein kinase A-mediated serine phosphorylation, which may be important to the regulation of StAR activity, are boxed (Arakane et al. 1997). Amino acid residues that disrupt StAR function when mutated are indicated by asterisks (Stocco 2002). The single letter code is used for the amino acids.

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tissues remains to be clarified. Different letters indicate significant differences ($P<0.05$).

We have found StAR mRNA to be expressed in the head kidney and gonads. This finding is in agreement with previous data in several fish species where StAR is mainly expressed in the steroidogenic tissues (Bauer et al. 2000, Li et al. 2003, Goetz et al. 2004), although it is in contraposition to the results obtained in other species, where StAR mRNA expression has been described in extravaginal and extra-adrenocortical tissues, such as the brain and heart of the freshwater stingray (Nunez et al. 2005); the kidney of Atlantic croaker (Nunez & Evans 2007); the intestine, pyloric ceca, spleen, and kidney of trout (Kusakabe et al. 2002); human fetal and adult kidney (Sugawara et al. 1995); and several areas of the human brain (King et al. 2002). Therefore, StAR tissue distribution can be slightly different according to the species, and its role in these other tissues remains to be clarified.

![Figure 2](image1.png)

**Figure 2** Pattern of tissue expression of StAR in gilthead seabream. Tissues from adult seabream were analyzed by RT-PCR as described in the text for StAR gene expression. H, heart; B, brain; L, liver; G, gills; Go, gonads; HK, head kidney; S, spleen. 18S was used to show equivalency in the amount of cDNA of the sample. Results are representative of three independent experiments.

![Figure 3](image2.png)

**Figure 3** The effects of acute stress on StAR expression in Sparus aurata. Fish were subjected to an acute stress as described in the text, and head kidneys were removed after 1, 6, and 16 h. Control fish remained undisturbed. StAR levels in head kidneys were analyzed by quantitative real-time PCR and normalized to 18S rRNA. Cortisol levels in plasma were also analyzed. $n=8$ for each condition. Different letters indicate significant differences ($P<0.05$).

![Figure 4](image3.png)

**Figure 4** Effects of chronic stress on StAR expression in Sparus aurata. Fish were maintained in a high-density confinement (50 kg/m$^3$) for 15 days. StAR levels in head kidneys were analyzed by quantitative real-time PCR and normalized to 18S rRNA. Cortisol levels in plasma were also analyzed. $n=16$ for each condition. Different letters indicate significant differences ($P<0.05$). We applied two different types of stressors (acute stress caused by chasing the fish with a net and a chronic stress by overcrowding) that caused a significant increase in the plasma cortisol levels of stressed fish compared with undisturbed fish. In the case of the acute stress, this increase was rapid and fish reached the highest cortisol levels between 1 and 6 h. After that period, cortisol levels decreased, although they were still higher than controls 16 h after the stressor. This kinetic profile is in accordance with the previously published data on salmonid fish (Pickering et al. 1982, Patino et al. 1987, Geslin & Auperin 2004) and white sucker (Bandeen & Leatherland 1997). StAR levels in our experiment did not increase significantly at any time post-stressor, a result also obtained by Geslin & Auperin (2004) in rainbow trout, who found no increase in StAR expression in the head kidney of fish chased with a net for 5 min. By contrast, these authors found that a stronger acute stress induced by anesthesia provoked a higher increase (30-fold) of cortisol levels in plasma and an increase in StAR mRNA levels in head kidney, a finding that led the authors to suggest that high levels of cortisol would promote the expression of StAR.

![Figure 5](image4.png)

**Figure 5** Effects of LPS injection on head kidney StAR expression. A dose of 8 mg/kg LPS was injected as described in the text, and STAR expression levels in head kidneys were analyzed by quantitative real-time PCR. Expression levels of StAR were normalized to 18S rRNA. Different letters indicate significant differences ($P<0.05$).
after stress require an activation of StAR gene, although lower levels do not. However, in the present experiment, plasma cortisol levels in stressed fish increased 160-fold, which is a very high value (about five times higher than in trout; Geslin & Auperin 2004), yet the StAR mRNA levels did not increase significantly over controls. This suggests that, although seabream is highly responsive to acute stress (as shown by cortisol levels), the StAR expression in head kidney could be regulated differently and the rise in plasma cortisol levels may be due to a different mechanism rather than increased gene transcription. For example, it is well known in mammals that StAR protein could be regulated post-transcriptionally (Fleury et al. 1998), translationally (reviewed by Stocco 1999), and/or post-translationally via serine phosphorylation by a PKA, which also results in the activation of StAR protein (Lehoux et al. 1998, Miller & Strauss 1999, Artemenko et al. 2001).

When we exposed seabream to high density for 2 weeks, the cortisol levels in the plasma increased dramatically (threelfold), a result that agrees with those obtained by other authors for many other fish species, since a prolonged elevation of plasma cortisol levels is frequently used as a marker for exposure to a chronic stressor, with the magnitude of the cortisol response generally reflecting the severity of the stressor (reviewed by Wendelaar Bonga 1997). In addition, we found that StAR mRNA levels in the head kidney of chronically stressed animals were increased threefold over controls. Interestingly, when we analyzed StAR gene expression on an individual basis, we found

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**Figure 6** In vitro effects of ACTH on StAR expression in head kidney. Cell suspensions were performed as described in the Materials and Methods section and incubated with 150 ng/ml ACTH. StAR mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S rRNA (A). Cortisol levels of culture medium in response to ACTH are also represented (B). Different letters indicate significant differences (P<0.05).

**Figure 7** CIP (1 μM) effectively blocks the ACTH-stimulated increase in StAR expression without affecting the basal levels in head kidney in vitro preparations. Cell suspensions were performed as described in the Materials and Methods section and incubated with 150 ng/ml ACTH, ACTH plus 1 μM CIP, or 1 μM CIP alone. StAR mRNA levels were analyzed by RT-PCR (A) and quantitative real-time PCR (B) and normalized to 18S rRNA. Different letters indicate significant differences (P<0.05).

**Figure 8** In vitro effects of LPS on StAR expression in head kidney. Cell suspensions were performed as described in the Materials and Methods section and incubated with 10 μg/ml LPS. StAR mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S rRNA(A). Cortisol levels of culture medium in response to LPS are also represented (B). Different letters indicate significant differences (P<0.05).
that some of them had much higher StAR mRNA levels and cortisol plasma levels than others (data not shown), which may correlate with the already demonstrated fact that socially subordinated individuals in small groups of fish show higher circulating cortisol levels, and therefore more stress than others (Winberg & Lepage 1998, Hoglund et al. 2000). This may be an interesting implication of StAR as a marker of fish behavior and stress. Further studies will be undertaken in this area.

We have been able to demonstrate strong in vitro effects of ACTH on mRNA levels of StAR in seabream head kidney. In the time-course experiment with seabeam head kidney preparations, we found an important increase in StAR mRNA levels 1 h after treatment with 150 ng/ml ACTH, and no effect thereafter. As expected, cortisol levels were also elevated after 1 h and returned to normal levels after 18 h of incubation with ACTH (data not shown). ACTH stimulated the StAR gene expression in the seabeam head kidney preparations by binding to its specific membrane receptors, as demonstrated when 1 µM CIP, an antagonist of the ACTH receptor (Li et al. 1978), effectively blocked the ACTH-stimulated StAR expression in our in vitro model, without affecting the basal levels of StAR expression. The rapid increase in cortisol levels, which we observed after ACTH treatment, is in agreement with several previous studies conducted under various experimental conditions (Donaldson 1981, Nichols & Weisbart 1984, Girard et al. 1998, Pottinger & Carrick 2001, Hagen et al. 2006). It also agrees with the previous data found in the same species, S. aurata, in our laboratory (Rotllant et al. 2001, Ganga et al. 2006) showing that ACTH caused a rapid increase in cortisol levels in superfused head kidney tissue. In addition, our results on StAR expression correlate with data from mammals showing that acute in vivo or in vitro treatment with ACTH leads to a rapid and significant increase (between 1 and 3 h after the treatment) in StAR mRNA in the adrenals (Ariyoshi et al. 1998, Fleury et al. 1998, Lehoux et al. 1998, Ivell et al. 2000, Le Roy et al. 2000). The results are also similar to other fish species, such as eel, where Li et al. (2003) found that ACTH injection increased StAR mRNA levels 1-5 h post-injection, and in trout, where Aluru et al. (2005) reported a 170% increase in interrenal StAR transcripts in response to 0-5 IU/ml ACTH after 3 h of incubation. Interestingly, recently, Hagen et al. (2006) found no rapid stimulation of StAR mRNA levels in the rainbow trout head kidney interrenal cells. They reported only a late effect after 18 h, although they used the same ACTH concentration (150 ng/ml) which they found produced the strongest peak in cortisol production.

LPS injection is the most frequently used acute stress model to mimic the fish response to an immune stressor (Balm et al. 1995, Balm 1997, Mackenzie et al. 2005). When we injected seabeam with 8 mg/kg i.p. of bacterial endotoxin to induce infection, it provoked a marked rise in plasma cortisol levels similar to those previously described (Balm et al. 1995, Holland et al. 2002) and to the results previously reported (Acerete et al. 2007). This produced a cortisol peak after 6 h (threelfold the controls) and a progressive decrease in cortisol until 72 h, which are similar to the results reported in rats (Grinevich et al. 2001) where a single LPS injection stimulated plasma cortisol levels. LPS injection also increased StAR gene expression in our in vivo seabream head kidney model and, together, these results confirm the previously described important role of immunoendocrine interactions in the head kidney of fish (Schreck & Bradford 1990, Weyts et al. 1999).

In contrast with the stimulatory effects of LPS in vivo, we found inhibitory effects of LPS (10 µg/ml) on in vitro cortisol production in a head kidney preparation. This is in agreement with the previous studies where LPS was not able to increase cortisol production in head kidneys (Balm et al. 1995). These authors reported that LPS (50 µg/ml) decreased the ACTH-stimulated cortisol production without being toxic to the head kidney preparations, an inhibitory effect that we observed as well. In addition, LPS incubation caused a decrease in StAR mRNA levels in the head kidney preparations, coincident with the reduction in cortisol levels that we observed. Therefore, it appears that the effects of LPS treatment may differ significantly from in vitro or in vivo treatments. Whereas in vivo, an experimental infection invokes a systemic reaction in which not only immune responses are generated but also neural and endocrine signals, both from pituitary sources and interrenal tissue; LPS administered in vitro may interfere with the local cortisol production or secretion mechanisms. One may speculate that in vivo either pituitary signals prevail in the interrenal tissue in the presence of the LPS or that circulating immune signals such as cytokines resulting from an experimental infection do not significantly affect the function of the HPI axis. Therefore, more work is in progress regarding these effects.

In conclusion, we have isolated a cDNA from seabeam that encodes a protein very similar in form and function to StAR in other vertebrates. It is expressed in head kidney and gonads, and while fish under acute stress showed increased cortisol levels but no changes in StAR mRNA expression, a chronic stress provoked a marked increase in StAR expression and cortisol levels. Moreover, ACTH caused a rapid increase in cortisol and StAR mRNA levels in the head kidney cells in vitro, whereas LPS infection stimulated StAR expression in vitro but was inhibitory in vivo. All these data together show that seabeam StAR expression and cortisol production in interrenal cells can be highly affected by different stress conditions (acute/chronic as well as stress by infection) and provides new data on the important relationship between the immune and endocrine systems in fish.

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