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Ontogenesis of the HPI axis and molecular regulation of the cortisol stress response during early development in *Dicentrarchus labrax*.

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The cortisol stress response and the molecular programming of the corticoid axis were characterized for the first time during early ontogeny in a Mediterranean marine teleost, the European sea bass (*Dicentrarchus labrax*). Sea bass embryos, pre-larvae and larvae at specific points of development were exposed to acute stressors and the temporal patterns of cortisol whole body concentrations and the expression of genes involved in corticosteroid biosynthesis, degradation and signaling were determined. Expression of genes (gr1, gr2, mr, crf) involved into the corticoid response regulation combined with histological data indicated that, although a cortisol stress response is evident for the first time around first feeding, a pattern becomes established in larvae at flexion until the formation of all fins. Moreover, mRNA transcript levels of 11β-hydroxylase and 11β-hsd2 showed a strong correlation with the whole body cortisol concentrations.

Concluding, our data reveal the presence of an adaptive mechanism in European sea bass at early ontogeny enabling to cope with external stressful stimuli and provide a better insight into the onset and regulation of the stress response in this species.

The teleostean hypothalamic–pituitary–interrenal (HPI) axis is a system comparable with the mammalian stress axis (hypothalamus-pituitary-adrenal; HPA), as a result of convergent evolution¹,² and it is of utmost importance in stress regulation as well as for the adaptation and/or acclimation of fish to their dynamic environment. Stress response includes the primary response, resulting in the rapid increase of circulating catecholamines and cortisol, the secondary leading to changes in several haematological and biochemical parameters, and the tertiary response that involves alterations at the whole animal and population level¹,³,⁴. The fish’s response to stressors may be of either an adaptive nature, allowing for homeostatic recovery, or a maladaptive nature having adverse effects on survival, growth, immune response, reproductive capabilities, behavior and general fitness⁵,⁶,⁷.

Exposure to stress can have a profound impact on the physiology and health of an organism later in life⁸,⁹. Studies carried out in mammals have shown that glucocorticoids play a key role in the programming of brain structures that can alter the responsiveness to stress⁹–¹¹. In fact, it has been shown that exposure to stressors during development results in permanent changes in stress coping phenotypes in mammals¹²,¹³, birds¹⁴, amphibians¹⁵, and fish¹⁶.

In teleostean fish, cortisol is the principal corticosteroid and plays an important role in a number of physiological processes including growth, immunoregulation, maintenance of energy balance, and reproduction²,¹⁶–¹⁸. During HPI axis activation corticotropin-releasing factor (CRF), produced in the hypothalamic preoptic area (POA), stimulates the pituitary gland corticotropes to secrete adrenocorticotropic hormone (ACTH), which regulates cortisol synthesis and secretion. In teleosts, cortisol plays also a vital role in the maintenance of hydromineral balance, as fish cannot synthesize aldosterone, and cortisol carries out this mineralocorticoid function¹⁹. Cortisol enters by passive diffusion into the cells where its action is mediated by the Glucocorticoid receptor(s) (GR) and the mineralocorticoid receptor (MR)²⁰, a class of ligand-activated transcription factors. During larval development, marine teleosts undergo dramatic changes in morphology, growth and
metabolism in order to accomplish their metamorphosis into juvenile fish. Throughout this period, cortisol regulates osmoregulatory function\textsuperscript{21,22} and is implicated in the metamorphosis from larvae to juveniles\textsuperscript{23,24}.

Studies conducted in European sea bass, \textit{Dicentrarchus labrax}, and other species showed the presence of maternal cortisol in embryos and that \textit{de novo} cortisol synthesis starts shortly after hatching but a significant elevation in whole body cortisol in response to a stressor becomes obvious days to weeks later, depending on the species\textsuperscript{25–30}.

However, our knowledge on the development of the hypothalamic–pituitary–interrenal (HPI) axis of European sea bass and its response to stressors during early ontogeny or the molecular mechanisms involved is scarce. To this end, we examined the temporal patterns of cortisol and genes related to the corticosteroid signaling (\textit{gr1, gr2, mr, crf}), corticosteroid synthesis (\textit{11\beta-hydroxylase}) and cortisol metabolism or inactivation (\textit{11\beta-hsd2}) at various stages during early ontogeny in order to assess the ontogenesis of the corticosteroid-signaling pathway. Moreover, we subjected European sea bass embryos, pre-larvae and larvae to an acute stressor in order to determine any differences in the timing or magnitude of the activation of the corticosteroid stress axis and the molecular response at each developmental point/stage.

**Results**

**Ontogeny of the HPI axis in European sea bass.** Histology data revealed that the brain of sea bass was evident at hatching (Figure 1a) while a morphological differentiation of the pituitary was possible at day 5 post hatching (dph) (Figures 1b, 1d). At this day the first thyroid follicles appear and they increase in number and size as fish grow (Figure 1f). Pituitary gland is clearly visible and differentiated at 30 dph when neurohypophysis and adrenohypophysis are distinct (Figure 1e, 1f). Interrenal and chromaffin tissues are both located in head kidney adjacent to the cardinal vein. The kidney was present at 3 dph and it was characterized by the presence of kidney tubules with a distinct morphology (Figure 1c). However, morphological differentiation of the interrenal and chromaffin tissues was difficult at early stages of development using routine histology, and only at 28 dph, i.e. around flexion the respective endocrine cells were simultaneously evident.

**Temporal patterns of cortisol content and gene expression at early ontogeny.** European sea bass embryos had low basal cortisol content (2 ± 0.7 ng g\textsuperscript{−1}) that had declined at hatching (0.6 ± 0.3 ng g\textsuperscript{−1}) and subsequently slightly increased at mouth opening (1.5 ± 0.2 ng g\textsuperscript{−1}), but the differences were not statistically significant. The first peak (\textit{P}, 0.001) was observed at first feeding (6.8 ± 1.3 ng g\textsuperscript{−1}), after which whole-body cortisol mean concentrations dropped gradually from flexion (4.1 ± 1.2 ng g\textsuperscript{−1}; \textit{P} < 0.001) onwards to the formation of all fins (1.9 ± 0.5 ng g\textsuperscript{−1}) (Figure 2a). All genes assessed in the current study were expressed in all developmental stages examined. Transcripts of \textit{gr1} (Figure 2b) showed a gradual increase throughout early development, with lowest mRNA abundance recorded in embryos

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**Figure 1** | Histological analysis (a) Sea bass larvae at day 1 post hatching (dph). The brain covers the majority of the head area. Bar: 400 μm. (b) A sea bass larva at 5 dph with the pituitary morphologically differentiated (red arrow). Bar: 400 μm. (c) Kidney of a sea bass at 3 dph showing the distinct morphology of the kidney tubules (arrows). Bar: 100 μm. (d) Higher magnification of picture (b) with the hypothalamus and the pituitary (arrow). Bar: 100 μm. (e) Brain of a 30 dph sea bass with fully differentiated pituitary. Arrow: Adenohypophysis, Arrowhead: Neurohypophysis. Bar: 100 μm. (f) Sea bass head at 30 dph. Red arrow points to the developed pituitary, black arrows point to thyroid follicles. Bar: 100 μm.
Figure 2 | Temporal patterns of cortisol content and gene expression at early ontogeny. Changes in resting (0 h) whole body cortisol levels and mRNA transcript levels of gr1, gr2, mr, cfr, 11β-hsd2 and 11β-hydroxylase during the different developmental points/stages (embryos-EM, hatch-HAT, mouth opening-MO, first feeding-FF, flexion-FLX, formation of all fins-FINS). Values are means ± standard error (n=3). Means with different letters differ significantly from one another (P < 0.05).
11β-hydroxylase expression pattern showed statistically significant temporal differences at all points/stages of development. In particular, at hatching there was a significant increase ($P < 0.001$) at 24 h (i.e. hatching 100% completed) post-stress, while at mouth opening and first feeding there was a peak at 1 h and 2 h post-stress respectively. In flexion, 11β-hydroxylase abundance remained at high levels from 0 h till 1 h post stress after which there was a statistically significant decrease ($P < 0.05$) at 2 h. In all fins, the pattern of changes resembled that of cortisol, with low transcripts at 0 h, 1.6 to 1.4-fold upregulation at 0.5 and 1 h followed by a sharp down-regulation at 2 h (Figure 6a). 11β-hsd2 levels did not show significant changes around hatching and mouth opening. However, in first feeding there was a gradual decrease in mRNA transcripts from high values at 0 h to a minimum at 2 h post-stress. In flexion, 11β-hsd2 abundance was constant except for a sharp increase at 24 h. However, if we exclude the values at 24 h, there was a statistically significant increase at 1 h post stress, which is no longer “masked” by the high values at 24 h. As in the case of 11β-hydroxylase, at the formation of all fins the pattern of 11β-hsd2 changes are similar to that of cortisol, with peak values at 0.5 and 1 h post-stress followed by a drop to the basal values at 2 h and 24 h (Figure 6b).

**Discussion**

The temporal changes of whole-body cortisol levels during the early developmental stages of teleosts show that the initial maternal deposit of cortisol is depleted during embryogenesis and reaches a minimum around the time of hatch and then the larva begins to synthesize cortisol *de novo*, a pattern which is observed in a similar way across a number of species. These results are in agreement with results obtained in this study as well as in studies in Japanese flounder, tilapia, rainbow trout, Asian sea bass, common carp, gilthead sea bream, Atlantic salmon, zebrafish, and in previous work in E. sea bass.

Gr1 is present in the embryos with very low transcript levels but soon after hatch its expression follows a continuous elevation during development. Gr2 abundance in embryos is higher than gr1, and its expression pattern is characterized by an initial elevation in hatch and a second in first feeding followed by a relatively steady expression, thereafter. Previous immunohistochemical and *in situ* hybridization data also verify the presence of gr2 mRNA transcripts and of the glucocorticoid receptor early in development with increasing expression towards the juvenile stage. In addition we show, for the first time, the expression profile of mr, cfr, 11β-hydroxylase and 11β-hsd2 during the early stages of development. Mr is present in embryos in very low copies and its expression increases as development proceeds, following a pattern similar to that obtained for mr expression during embryogenesis in zebrafish.

Cfr expression is detected in embryos in very small quantities; however, there is a peak in mRNA transcripts at hatch that decline in mouth opening and then gradually increase from first feeding to the formation of all fins, indicating a maturation of the HPI axis. The expression of 11β-hydroxylase is upregulated immediately before the rise in larval cortisol levels which occurs in first feeding, pointing to the activation of the steriodogenic pathway around that time. 11β-hsd2 mRNA transcripts follow a transient pattern as they appear at high levels in embryos, drop in hatch, reach a maximum in first feeding and then drop again at the following developmental stages. The high amounts in embryos may be associated with the maternal cortisol deposit that needs to be metabolized and the second peak which appears in first feeding coincides with the first peak of cortisol during early development of sea bass, and may reflect the immediate response of the corticoid system to the sudden accumulation of cortisol. Apart from embryos where the mRNA abundance of 11β-hsd2 and 11β-hydroxylase is high, the expression patterns of these genes are quite similar to the respective patterns obtained from zebrafish.
The acute stress challenge tests didn’t result in a cortisol response in embryos, hatch and mouth opening stages, apart from a maximum at 24 h post stress. However, further research is needed to clarify whether this maximum is a result of a delayed stress response or reflects differences in the developmental point/stage. Histological data showed that the first appearance of a distinct hypothalamo-hypophysal-interrenal axis is observed at first feeding, where a peak in whole body cortisol levels was observed at 0.5 h post stress, followed by a protracted decrease until at 24 h when it reached resting levels. These results, in accordance with the molecular data, imply

Figure 3 | Ontogeny of the cortisol stress response. The cortisol response prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor during early ontogeny. Values are means ± standard error (n=3). Means with different letters differ significantly from one another (P < 0.05).
Figure 4 | Expression of $gr1$ and $gr2$ after application of a stressor during early ontogeny. (a) Expression profile of $gr1$ prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. (b) Expression profile of $gr2$ prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. Values are means ± standard error (n = 3 pools of ca. 30 mg for embryos, hatched eggs and larvae samples, apart from juveniles where pools of 1–2 fish were used). Means with different letters differ significantly from one another ($P < 0.05$).
Figure 5 | Expression of *mr* and *crf* after application of a stressor during early ontogeny. (a) Expression profile of *mr* prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. (b) Expression profile of *crf* prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. Values are means ± standard error (n=3). Means with different letters differ significantly from one another (P < 0.05).
Figure 6 | Expression of \(11\beta\)-hydroxylase and \(11\beta\)-hsd2 after application of a stressor during early ontogeny. (a) Expression profile of \(11\beta\)-hydroxylase prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. (b) Expression profile of \(11\beta\)-hsd2 prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. Values are means ± standard error (n=3). Means with different letters differ significantly from one another (\(P < 0.05\)).
that as early as at first feeding sea bass individuals are capable of responding to external noxious stimuli. In addition the first peak observed in whole-body cortisol concentrations at first feeding reflects the essential role of cortisol to carbohydrate and protein metabolism towards transition to exogenous feeding. As development proceeds, the magnitude and duration of the response is higher and a pattern seems to be established from flexion until the formation all fins where cortisol values reach a maximum at 2 h. This is further supported by the histological data showing that while the kidney tubules with a distinct morphology were present at hatching, a clear morphological differentiation of the interrenal and chromaffin tissues was possible only at 28 days post hatch (i.e. at flexion) using routine histology. Similar results were found in cichlid fishes where the head kidney from 12 to 30 days after fertilization is functionally mixed, with the nephron and developing hemopoietic and endocrine (chromaffin and interrenal tissue)62. These results indicate that even at the stage of first feeding fish are capable of a stress-induced stimulation of cortisol and that the HPI axis becomes gradually established until the development of all fins. This is in accordance with previous work conducted in European sea bass46, rainbow trout63, the yellow perch64 and the zebrafish65. However, this is the first time that the exact pattern of cortisol response following exposure to acute hus-bandry stressors is revealed at early developmental stages.

With the aim to shed light on the molecular mechanisms related to the onset of the cortisol stress response, we carried out qPCR experiments in order to measure the mRNA transcript levels of genes related to the HPI axis, gr1 and gr2, mr and cf; and genes related to the biosynthesis and degradation of cortisol, 11β-hsd2 and 11β-hydroxylase. During HPI axis activation, gr1, gr2 and mr are the mediators of the transcriptional effects of circulating cortisol on target tissues and cf is produced in the hypothalamic preoptic area (POA), stimulates the pituitary corticotropes to secrete adrenocorticotropic hormone (ACTH)66,67, which in turn stimulates synthesis and secretion of cortisol into the circulation68. Gr1 expression levels after application of an acute stressor were not altered in any developmental stage apart from all fins, where a down-regulation was observed at 2 h post stress. The same is the case of the mRNA levels of gr2, as the only response to the stress was detected at the stage of all fins, where an increase was observed at 0.5 h post stress followed by a down-regulation at 2 h. The down-regulation of gr1 and gr2 is in accordance with data from other studies conducted in sea bass exposed to very high stocking densities71, in coho salmon72, in Atlantic salmon72, in common carp73, in the hippocampus of rats exposed to increasing corticosteroid levels74, in mouse pups with high corticosteroid levels due to 24-h maternal deprivation75. The statistically significant increase of the expression levels of both gr1 and gr2 observed at 24 h post stress in first feeding may be related, as with the case of cortisol, either to a delayed stress response or to the role of cortisol in metabolism and neural development for the passage of fish to exogenous feeding. The mRNA abundance of mr was not altered at any of the developmental stages examined apart from first feeding where the expression levels decreased from 0.5 h to 2 h post stress. However, as this is not repeated in the later stages and especially at all fins where the HPI axis is expected to be more mature, this alteration may not reflect a response to stress but rather a suppression related to the needs of the developmental stage. Csf expression pattern at all fins follows an increase at 1 h and a gradual decrease until 24 h post stress, which is in accordance with the pattern observed for cortisol at this stage, where the peak of cortisol levels is at 2 h post stress. In flexion, there is a statistically significant increase at 2 h that remains at high values still at 24 h, indicating that at this stage the csf system is being established, but the prolonged expression and the delayed response compared to the pattern of cortisol at the same stage, reveals that it is not mature yet.

During fish ontogenesis cortisol is a critical hormone when changes occur at the metabolic demands of the larvae22-24,57 and it is also implicated in neural development and in the induction metamorphosis46-51. The inability of the stress response system to respond to the stress-elevated cortisol levels via the cf and the grs at these stages until only at all fins, might be of critical importance for the survival of the larvae and the normal progress of the development. There are very limited data available about the role of gr and mr in fish development. The major mineralocorticosteroid in mammals and non-mammalian vertebrates is aldosterone. However, in fish, deoxycorticosterone (DOC) is considered to be a mr ligand instead of aldosterone, as the latter is not detected in fish, but also cortisol is a high-affinity ligand for mr. A recent study carried out in zebrasfish showed that both GR and MR are present during embryogenesis and suggested that gr plays a more important role after hatching in zebrafish, whereas mr is suggested to be important at the earlier stages of development, and that after hatching a ligand other than cortisol, perhaps DOC, may be responsible for mr signaling59. Studies in mice showed that mice lacking a functional gr survive until birth but die shortly thereafter due to impaired lung development60 and that there is no abnormal embryonic development detected in a mutant zebrafish line that does not develop corticotrophic pituitary cells61. Other studies demonstrated that knocking down maternal gr leads to developmental defects in mesoderm formation in zebrafish62 and that gr signaling is essential for zebrafish muscle development63. In the present study, mr expression profiles during the stress response in first feeding seems to give mr a more important role at this stage than that of grs, which at the later stages of development is inverted. This is in accordance with the results obtained from the other studies mentioned above.

We tested the hypothesis that the molecular events related to the appearance of the cortisol synthesis pathway are tightly linked to the enzymes which take part in cortisol biosynthesis and degradation. Therefore, we quantified the temporal expression of 11β-hydroxylase that generates cortisol from 11-deoxycortisol64 and 11β-hsd2, an enzyme that converts the biologically active cortisol to the inactive cortisone. After the acute stress application the transcript levels of 11β-hydroxylase appear statistically significant altered at hatch and mouth opening but these changes may not reflect a stress response, but these changes could represent the role of this enzyme in gonad differentiation, as 11β-hydroxylase appears to be a key transducer in the mechanism of sex determination in fish65. However, in first feeding appears a strong -relation between 11β-hydroxylase transcripts and cortisol increase. This pattern continues also in the later stages of development, where mRNA expression of 11β-hydroxylase is upregulated along with cortisol levels. This is in accordance with data obtained for rainbow trout, where mRNA abundance of 11β-hydroxylase also increases in response to an acute stressor65. In first feeding, where the first response is observed, 11β-hsd2 mRNA transcripts are at high amounts in the larvae and gradually decline to a minimum at 2 h post stress; if compared with the cortisol pattern, it becomes clear that the mRNA levels of this enzyme are correlated with the amount of cortisol present. At the following developmental stages this becomes more obvious as the maximum mRNA levels of this gene are observed at 1 h post stress, just prior to the cortisol peaks (at 2 h), and at 2 h the transcript levels drop again to the resting values.

The cortisol stress response and the regulation of genes related to the corticoid axis in combination with histological analysis were studied for the first time during early ontogeny in European sea bass, Dicentrarchus labrax. Sea bass embryos, pre-larvae and larvae were exposed to acute stressors and the temporal patterns of cortisol whole body concentrations and the expression of genes involved in corticosteroid biosynthesis (11β-hydroxylase), degradation (11β-hsd2) and signaling (gr1, gr2, mr and cf) were determined. Histological data showed that although the kidney was present at hatching, morphological differentiation of the interrenal and chromaffin tissues was difficult at early stages of development using routine histology, and only around flexion the respective endocrine cells were simulta-
stressful stimuli and provide a better insight into the onset and regulation of the corticoid response. As development proceeds, a pattern with a higher magnitude and longer duration of the cortisol stress response was established from flexion until the formation of all fins. Expression data of genes related to the regulation of the corticoid response (gr1, gr2, mr and crf) indicated that, although a cortisol stress response is evident at first feeding, the HPI axis seems to be fully mature only at the stage of all fins. Moreover, the mRNA transcript levels of 11β-hydroxylase and 11β-hsd2 showed a strong correlation with the whole body cortisol concentrations. Overall, the data indicate that fish do respond to external noxious stimuli as early as at first feeding but the cortisol stress response becomes fully functional and mature until only at the stage of all fins. In addition, several changes occurred in early development (embryos, hatching and mouth opening) may resemble, apart from an adaptive to stress role of cortisol, its implication in other important aspects of development and metabolism. In conclusion, our data reveal the presence of an adaptive mechanism in European sea bass at early ontogeny enabling to cope with external stressful stimuli and provide a better insight into the onset and regulation of the stress response in this species.

Methods

Animals and husbandry conditions. Batches of fertilized European sea bass eggs were obtained from a private fish farm (DIAS S.A.) and transferred to the installations of the Institute of Aquaculture, Hellenic Center for Marine Research (Heraklion, Crete). Larval rearing was performed applying the pseudosgreen-water technique, in 300 L cylinder-conical tanks, with an initial density of 100 eggs L\(^{-1}\) in which both hatching and rearing took place. Tanks were coupled to a biological filter and were enriched with proteins and PUFA (INVE Aquaculture S.A., Belgium) until 10 days post hatching (dph). During larval rearing and pre-weaning a sample of 10 larvae was taken daily (PROTON 2–3, INVE Aquaculture S.A., Belgium) using automatic feeders. On day 45 post hatch (dph). Larvae (flexion and formation of all fins) were exposed to high hatching (as above), chasing with a net for 20 sec, confinement (collection in beakers), and air exposure for 5 sec before being transferred to baskets within a 500 L tank. Samples for molecular and endocrine (cortisol) analysis were collected with a net above), chasing with a net for 20 sec, confinement (collection in beakers), and air exposure for 5 sec before being transferred to baskets within a 500 L tank. Different stressors were applied based on the tolerance of larvae at the particular developmental stages (Figure 7). In particular, embryos were exposed to transportation stress for 8 hours at a density of 50 g L\(^{-1}\) and then to netting and air exposure for 1 min until distribution to the incubation tanks. Pre-larvae (hatching, mouth opening, first feeding) were exposed to chasing with a net for 20 sec and high hatching (1,000–1,500 ml min\(^{-1}\) vs. 150–200 ml min\(^{-1}\)) for 90 sec. Larvae (flexion and formation of all fins) were exposed to high hatching (as above), chasing with a net for 20 sec, confinement (collection in beakers), and air exposure for 5 sec before being transferred to baskets within a 500 L tank. Samples for molecular and endocrine (cortisol) analysis were collected with a net at 0, 0.5 h, 1 h, 2 h and 24 h post-stress, flash frozen in liquid N\(_2\) and stored at −80 °C. All experiments were performed in accordance with relevant guidelines and regulations. The laboratories of the Hellenic Centre for Marine Research are certified and obtained the codes for breeding animals for scientific purposes (EL-91-BIO-04). Furthermore all procedures involving the handling and treatment of fish used during this study were approved by the HCMR Institutional Animal care and use committee following the Three Rs (Reduction, Refinement) guiding principles for more ethical use of animals in testing, in accordance to Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals.

Histological analysis. Sea bass larvae were killed with an overdose of anesthesia (ethylene glycol monophenyl ether, Merck, 807291) and fixed in buffered formalin. Samples were dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). Serial sections were obtained at a thickness of 3–5 μm on a microtome (Leica RM2245, Germany) using disposable blades. After drying, slides were stained with methylene blue/azure II/basic fuchsin and examined under a light microscope in order to record the first appearance of the tissues comprising the HPI axis and to describe the relevant organs/tissues.

### Table 1: Morphological characteristics of European sea bass larvae collected at various stages during early ontogeny

<table>
<thead>
<tr>
<th>Development</th>
<th>Description</th>
<th>DPH</th>
<th>Total Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos</td>
<td>70% of embryos in 50% epiboly stage</td>
<td>−2</td>
<td></td>
</tr>
<tr>
<td>Hatch</td>
<td>70% of embryos are hatched</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mouth opening</td>
<td>Mouth opens, complete yolk sac absorbion</td>
<td>6</td>
<td>4.71 ± 0.09</td>
</tr>
<tr>
<td>First feeding</td>
<td>First day of exogenous feeding</td>
<td>9</td>
<td>5.25 ± 0.19</td>
</tr>
<tr>
<td>Flexion</td>
<td>65% completed the notochord flexion</td>
<td>29</td>
<td>11.1 ± 0.44</td>
</tr>
<tr>
<td>Fins</td>
<td>All fins have been developed</td>
<td>44</td>
<td>15.48 ± 0.21</td>
</tr>
</tbody>
</table>
Whole body cortisol. Samples were homogenized according to Sosthna et al. 44. Cortisol was measured in duplicate using a RIA in a 96-well plate according to Gorissen et al. 45. All wells except the ‘non-specifics’ received 100 µl cortisol antibody (Cortisol Antibody [xm210] monoclonal and IgG purified (Abcam) and were incubated overnight at 4 °C. Subsequently, the plates were washed three times with 200 µl/well wash buffer and 100 µl blocking buffer was added to each well in order to block the non-specific sites. Plates were then washed three times with wash buffer and after the final wash step, all wells received 200 µl of ‘Optiphase hisafe-3 scintillation liquid’ (PerkinElmer, USA). Beta-emption was quantified by a 3 min count per well using a Microbeta Plus (Wallac/PerkinElmer, USA).

RNA purification and DNA synthesis. Samples of embryos, pre-larvae and larvae were let to thaw on ice, disrupted and homogenized using the TissueRuptor (Qiagen, Hilden, Germany) for 20 s in 600 µl RLT plus buffer (RNEasy Plus Mini Kit Qiagen, Valencia, USA). Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Valencia, USA). RNA yield and purity was determined by measuring the absorbance at 260 and 280 nm using the Nanodrop® ND-1000 UV–Vis spectrophotometer (Pegamarkers, Regensburg, Germany) with a purity ratio greater than 1.9% agarose gels. Reverse transcription (RT) was carried out using 1 µg RNA with QuantiTect Reverse transcription kit (Qiagen).

Primer design. Primers for Glucocorticoid Receptor 1 (gr1), Glucocorticoid Receptor 2 (gr2), Mineralocorticoid Receptor (mr), Corticotropin Releasing Factor (crf), eukaryotic Elongation Factor 1 (eEF1a), 40S Ribosomal Protein S30 (11rps30), 18S rRNA (18S) and ribosomal 18S RNA (18Sr) were obtained by a previous work of our group 46,47. Primer design for steroid 11β-hydroxylase (11β-hydroxylase) was based on the available sequence with accession number AP449173.2. Forward and reverse primers for 11β-Hydroxysteroid Dehydrogenase type II (11β-hsd2) were designed based on the conserved regions as revealed by multiple sequence alignments of other teleost fish.

In the case of 11β-hydroxylase, the forward primer (11β_Fwd) was a 21-mer with the sequence 5’-cagggaggtttcctttggaac- 3’ and the reverse primer (11β_Rev) an 19-mer primer with the sequence 5’-agagggcacagacctgtgagaa- 3’. For 11β-hsd2, the sequence of the forward primer (hsd_Fwd) was 5’-caccccgagccacgactc- 3’ and the reverse primer (hsd_Rev) had the sequence 5’-accacaagcccaccagcagc- 3’. The products of each primer pair were further checked with sequencing in order to confirm that they amplified the desired genes.

Quantitative real-time PCR (qPCR). The mRNA expression of genes encoding for gr1, gr2, mr, crf, 11β-hydroxylase and 11β-hsd2 was determined with quantitative polymerase chain reaction (qPCR) assays using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems). Reactions were cycled and the resulting fluorescence was detected with M Mini Thermal Cycler (Bio-Rad) under the following cycling parameters: 95°C for 3 min (HotStarTaq DNA Polymerase activation step), 94°C for 15 s (denaturation step), 60°C for 30 s (annealing step), 72°C for 20 s (extension step), 40 cycles (1 step). Levels of gr1, gr2, mr, crf, 11β-hydroxylase and 11β-hsd2 mRNA were normalized based on the reference genes eEF1a and Eflu. A relative standard curve was constructed for each gene, using 4 serial dilutions (1:5) of a pool of all cDNA samples. We also performed geNORM analysis 37 in order to validate which are the most suitable reference genes to serve as an internal control and we concluded to eEF1a and 18Sr.

Statistical analysis. All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). All data are presented as means ± standard error of the mean. Data were initially screened for normality and homogeneity. Statistical comparisons of temporal patterns of cortisol and gene expression of unstressed specimens (0 h) by reaction (0.5 h) were made using one-way ANOVA. Statistical comparisons of cortisol content and gene expression between the different time points following exposure to a stressor and the various developmental points/stages tested were made using two-way ANOVA. Holm–Sidak’s honestly significant difference test for multiple comparisons was used to determine significant differences among groups. The significant level used was P < 0.05.

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Author contributions
A.T. and M. P. wrote the main manuscript. A.T. and N.P. carried out larvae rearing and sampling. M.G. and G.F. made cortisol measurements. P.K. carried out histology experiments and prepared figure 1. A.T. prepared figures 2–7. All authors reviewed the manuscript.

Additional information
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