University of Crete

Department of Biology

Fish Physiology

PhD Thesis

Molecular and endocrine regulation of the stress response during early developmental stages in European sea bass (*Dicentrarchus labrax*)

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Ευχαριστίες

Θα ήθελα να ευχαριστήσω τον καθηγητή Μιχάλη Παυλίδη για όλη την πολύτιμη υποστήριξη που μου παρείχε, πάντα με την καλή και χιουμοριστική του διάθεση.

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Ένα μεγάλο ευχαριστώ στην οικογένεια μου και τους φίλους μου που ομορφαίνουν τη ζωή μου και με στηρίζουν πάντα.

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ABSTRACT

The aims of this dissertation were to study the ontogeny of the endocrine stress response, to characterize the molecular programming of the Hypothalamus-Pituitary-Interrenal (HPI) axis, and to determine the impact of long term chronic mild stress applied early in life on the performance of fish at subsequent stages of development, 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 and α-MSH whole body concentrations and the expression of genes involved in corticosteroid biosynthesis, degradation and both cortisol and α-MSH signaling were determined. Expression of genes involved into the corticoid response regulation (*gr1*, *gr2*, *mr*, *crf*) combined with histological data indicated that, although a cortisol stress response is evident for the first time around first feeding, a pattern becomes progressively established in larvae at flexion until the formation of all fins. Moreover, mRNA transcript levels of *11β-hydroxylase* and *11β-hsd2* which are involved in cortisol synthesis and deactivation/metabolism, respectively, showed a strong correlation with the whole body cortisol concentrations. An α-MSH stress response, an additional to cortisol pathway regulating stress in teleosts, is evident for the first time in the early development of European sea bass at the stage of mouth opening showing a specific pattern characterized by elevated levels that becomes established around the formation of all fins. mRNA transcript levels of *pomc* and *mc2r* were altered after the acute stress application in a consistent elevated pattern especially as development proceeds, at the stages of flexion and after the formation of all fins, showing at the

same time a similar pattern with the whole body α-MSH concentrations. The acute stress application had no effect on the expression of *mc1r* but in the case of *mc4r* resulted in an increased transcription even as early as at the stage of mouth opening.

In fish, stress research is focused on the effects of acute or chronic severe noxius stimuli of physical, chemical and husbandry nature applied in juveniles or adult individuals, and there is no information on the effects of early exposure to long term chronic mild stressors on the development and performance of fish at subsequent phases of the life-cycle, but also no valid chronic low intensity stress protocol exists for fish at early development. To this end an unpredictable chronic low intensity stress (UCLIS) protocol was developed and evaluated for the first time in early development of E. sea bass. UCLIS protocol was based on the unpredictability, variety, frequency and moderate intensity of the applied stressors, providing a relatively realistic model of everyday aquaculture husbandry practices. The UCLIS application lasted for 14 consecutive days, starting at three different phases of early ontogeny (first feeding, flexion and development of all fins). Evaluation of the UCLIS protocol was performed through the determination of water-born cortisol concentrations of the larvae rearing tanks at regular intervals, recording of mortality and measurements of growth performance. In addition, its effects on subsequent developmental phases were evaluated by measurement of growth characteristics and by the determination of plasma cortisol in juvenile fish, prior and 1h after the application of an acute stressor. Our data show that European sea bass larvae are sensitive to mild husbandry stimuli with consequences even at subsequent phases of the life-cycle, with the stages of first feeding and all fins being the most critical, pointing out the necessity to reconsider common rearing practices. In particular, UCLIS application resulted in higher water

cortisol release rates in all groups where the stress was applied compared to the controls, proving to be a reliable non-invasive indicator of stress even during early ontogeny. Performance of fish in terms of growth rate was also affected by application of the stress protocol, as larvae that had been exposed to UCLIS at the beginning of first feeding and the formation of all fins displayed worst performance than fish exposed to UCLIS at flexion and controls. Early life stress did not affect plasma cortisol levels of juveniles exposed to additional acute stressors. However, fish were very sensitive to common handling practices and in addition, significant higher plasma cortisol concentrations were found in juveniles exposed to UCLIS at the stages of first feeding and the formation of all fins, compared to the other two groups, in accordance with the differences observed in growth rates.

Concluding, the data obtained from this study provide a better insight into the onset and regulation of the stress response in early development of E. sea bass and show for the first time that early life stress, in the form of common husbandry practices, has an impact both on larvae performance and also on later stages of the life-cycle in this species, as life history affected the growth performance and the stress response in juvenile fish.

ΠΕΡΙΛΗΨΗ

Σκοπός αυτής της διατριβής ήταν η μελέτη της οντογένεσης της ενδοκρινικής απόκρισης στην καταπόνηση (stress), ο χαρακτηρισμός των μοριακών μηχανισμών που εμπλέκονται στην ρύθμιση του άξονα υποθαλάμου-υπόφυσης-επινεφρίδιων (HPI) και ο προσδιορισμός της επίδρασης του χρόνιου ήπιου στρες κατά τα πρώτα αναπτυξιακά στάδια της ζωής στην ανάπτυξη και την επίδοση των ψαριών σε μετέπειτα αναπτυξιακά στάδια, χρησιμοποιώντας ως μοντέλο ένα μεσογειακό θαλάσσιο τελεόστεο ιχθύ, το ευρωπαϊκό λαβράκι (*Dicentrarchus labrax*).

Έμβρυα, προ-νύμφες και νύμφες λαβρακιού σε συγκεκριμένα αναπτυξιακά στάδια εκτέθηκαν σε οξύ στρες και προσδιορίστηκαν τα χρονικά πρότυπα των συγκεντρώσεων της κορτιζόλης και της α-MSH του σώματος, καθώς επίσης και η έκφραση γονιδίων που εμπλέκονται στη βιοσύνθεση και την αποικοδόμηση των κορτικοστεροειδών καθώς και στη σηματοδότηση τόσο της κορτιζόλης όσο και της α-MSH. Η έκφραση των γονιδίων που συμμετέχουν στην ρύθμιση της κορτικοειδούς απόκρισης (*gr1, gr2, mr, crf*) σε συνδυασμό με ιστολογικά δεδομένα, υποδεικνύουν ότι παρόλο που η απόκριση της κορτιζόλης στο στρες είναι εμφανής για πρώτη φορά στο στάδιο του πρώτου ταΐσματος, το πρότυπο της απόκρισης εγκαθιδρύεται σταδιακά στις νύμφες που βρίσκονται στις φάσεις της κάμψης της νωτοχορδής και μέχρι το σχηματισμό όλων των πτερυγίων. Η απόκριση της α-MSH στο στρες, ένα πρόσθετο μονοπάτι ρύθμισης του στρες πέραν της κορτιζόλης, είναι εμφανής για πρώτη φορά στο στάδιο του ανοίγματος του στόματος, εμφανίζοντας ένα συγκεκριμένο πρότυπο που χαρακτηρίζεται από αυξημένα επίπεδα, το οποίο εγκαθιδρύεται σταδιακά γύρω στο σχηματισμό όλων των πτερυγίων. Τα επίπεδα των mRNA μεταγράφων των *pomc* και *mc2r* μεταβλήθηκαν από την εφαρμογή του οξέως στρες παρουσιάζοντας ένα συνεπές πρότυπο αυξημένων επιπέδων, ειδικά με την

πορεία της ανάπτυξης από την κάμψη της νωτοχορδής μέχρι το σχηματισμό όλων των πτερυγίων, εμφανίζοντας επίσης παρόμοιο πρότυπο με αυτό της μεταβολής των συγκεντρώσεων της α-MSH του σώματος. Το οξύ στρες δεν είχε καμία επίδραση στα επίπεδα έκφρασης του *mc1r,* ενώ στην περίπτωση του *mc4r* είχε ως αποτέλεσμα την αύξηση των επιπέδων μεταγραφής ήδη από το στάδιο του ανοίγματος του στόματος.

Στα ψάρια, η έρευνα του στρες είναι επικεντρωμένη στη μελέτη της επίδρασης οξέως ή χρόνιου επιβλαβούς ερεθίσματος σωματικής, χημικής ή διαχειριστικής φύσεως σε νεαρά ή ενήλικα άτομα, ενώ δεν υπάρχουν πληροφορίες για την επίδραση της πρώιμης έκθεσης σε ήπια χρόνια στρεσογόνα ερεθίσματα στην ανάπτυξη και επίδοση των ψαριών σε επόμενες φάσεις του κύκλου-ζωής, ενώ επίσης δεν επίσης δεν υπάρχει κάποιο έγκυρο πρωτόκολλο ήπιου χρόνιου στρες για την πρώιμη ανάπτυξη των ψαριών. Για το σκοπό αυτό αναπτύχθηκε και εκτιμήθηκε για πρώτη φορά στην πρώιμη ανάπτυξη του λαβρακιού, ένα πρωτόκολλο μη προβλέψιμου, χρόνιου και ήπιας έντασης στρες (UCLIS). Η εφαρμογή του UCLIS διήρκησε 14 συνεχόμενες ημέρες, ξεκινώντας σε τρεις διαφορετικές φάσεις της πρώιμης οντογένεσης (πρώτο τάισμα, κάμψη της νωτοχορδής και σχηματισμός όλων των πτερυγίων). Η αξιολόγηση του πρωτοκόλλου UCLIS βασίστηκε στον προσδιορισμό των συγκεντρώσεων της κορτιζόλης που απελευθερώνονταν στο νερό των νυμφικών δεξαμενών εκτροφής ανά τακτά χρονικά διαστήματα, στην καταγραφή της θνησιμότητας και της επίδοσης στην ανάπτυξη. Επιπρόσθετα, έγινε εκτίμηση της επίδραση του UCLIS σε μετέπειτα αναπτυξιακές φάσεις μέσω της καταγραφής των αναπτυξιακών χαρακτηριστικών και του προσδιορισμού της κορτιζόλης του πλάσματος σε νεαρά ψάρια, πριν και 30 λεπτά μετά την εφαρμογή ενός οξέως στρες. Τα δεδομένα δείχνουν ότι οι νύμφες του λαβρακιού είναι ευαίσθητες σε ήπια

διαχειριστικά ερεθίσματα με συνέπειες ακόμα και σε μετέπειτα φάσεις του κύκλου ζωής, με τα στάδια του πρώτου ταΐσματος και της ανάπτυξης όλων των πτερυγίων να είναι τα πιο κρίσιμα, επισημαίνοντας της αναγκαιότητα αναθεώρησης των συνηθισμένων πρακτικών εκτροφής. Συγκεκριμένα, η εφαρμογή του UCLIS είχε σαν αποτέλεσμα τον υψηλότερο ρυθμό απελευθέρωσης της κορτιζόλης στο νερό σε όλες ομάδες που εφαρμόστηκε το στρες σε σύγκριση με τις ομάδες αναφοράς, αποδεικνύοντας ότι είναι ένας αξιόπιστος μη-επεμβατικός δείκτης του στρες ακόμη και κατά την πρώιμη οντογένεση. Επίσης, η εφαρμογή του πρωτοκόλλου στρες επηρέασε και την επίδοση των ψαριών, υπό την έννοια της του ρυθμού ανάπτυξης, καθώς οι νύμφες που είχαν εκτεθεί στο UCLIS στην αρχή των σταδίων του πρώτου ταΐσματος και του σχηματισμού όλων των πτερυγίων επέδειξαν χειρότερη επίδοση από τα ψάρια που είχαν εκτεθεί στο UCLIS στο στάδιο της κάμψης της νωτοχορδής και από τα ψάρια που άνηκαν στην ομάδα αναφοράς. Το στρες στα πρώιμα στάδια της ζωής δεν επηρέασε τα επίπεδα της κορτιζόλης του πλάσματος σε νεαρά ψάρια όταν αυτά εκτέθηκαν σε οξύ στρες. Παρόλα αυτά, τα ψάρια ήταν πολύ ευαίσθητα σε κοινές διαχειριστικές πρακτικές και επιπλέον, στα νεαρά ψάρια που είχαν εκτεθεί στο UCLIS στα στάδια του πρώτου ταΐσματος και του σχηματισμού όλων των πτερυγίων βρέθηκαν σημαντικά υψηλότερες συγκεντρώσεις της κορτιζόλης του πλάσματος σε σύγκριση με τις άλλες δύο ομάδες, σε συμφωνία με τις παρατηρούμενες διαφορές στους ρυθμούς ανάπτυξης.

Καταλήγοντας, τα δεδομένα που προέκυψαν από αυτή τη μελέτη παρέχουν μια βαθύτερη γνώση σχετικά με την οντογένεση και τη ρύθμιση της απόκρισης στο στρες στα πρώτα αναπτυξιακά στάδια στο λαβράκι και αποδεικνύεται για πρώτη φορά ότι το στρες, υπό τη μορφή κοινών διαχειριστικών πρακτικών, κατά τα πρώιμα στάδια

της ζωής έχει επίδραση τόσο στην αναπτυξιακή επίδοση των νυμφών όσο και σε μετέπειτα στάδια της ανάπτυξης σε αυτό το είδος, καθώς η «ιστορία» των ψαριών επηρέασε την ανάπτυξη και την απόκριση στην κορτιζόλη στο στάδιο των νεαρών ψαριών.

1. INTRODUCTION

1.1 European sea bass (Dicentrarchus labrax)

European sea bass is a fish species of high commercial interest in aquaculture and naturally is common in the Mediterranean Sea, the Black Sea and along the North Eastern Atlantic coasts, from Norway to Senegal.

Figure 1. *Dicentrarchus Labrax*

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Perciformes
Family	Moronidae
Genus	*Dicentrarchus*
Species	*D. labrax* (Linneaus, 1758)

The name *Dicentrarchus* derives from the presence of two dorsal fins. It has silver sides and a white belly. Juvenile fish maintain black spots on the back and sides (**Fig. 1**). It's a eurythermal and euryhaline species, tolerating temperatures between 2 and 32 °C and surviving in freshwater and high salinity waters. In natural conditions, this euryhaline species is reported to spend embryonic and larval phases in marine environments (salinity = 38) before the migration of juveniles to coastal zones, estuarine, and lagoons (Jennings and Pawson 1992; Varsamos et al. 2002), where salinity conditions may greatly fluctuate from hypersaline environments (up to 40) to freshwater (0.17). It inhabits coastal waters down to 100 m depth, but it is commonly found in estuarine areas and coastal lagoons which it enters during summer and then returns to the sea during the winter period. Both juveniles and adults often overwinter

in Mediterranean coastal lagoons. Young fish inhabit coastal waters, are social and form schools, also with other fish, but adult fish are less social. Young males become mature in nature at the age of 2- to 3 years old with the females one year later. Adult sea bass can reach an age of 30 years and a maximum weight and length of 15 kg and 1m, respectively (Kottelat and Freyhof, 2007). They are predators of small fish, shrimps and crabs and they change their feeding rhythms (diurnal and nocturnal) on a seasonal basis.

1.2 European sea bass farming

The European sea bass (*D. labrax*) is a fish of high commercial value and was the first marine non-salmonid species to be commercially cultured in Europe. At present is the most important commercial fish widely cultured in the Mediterranean region with Greece and Turkey being the biggest producers.

Sea bass has traditionally been cultured by extensive methods in seawater ponds and lagoons. Fish enter a lagoon, the entrance is closed and the trapped fish are fed naturally until they reach a marketable size. Intensification of sea bass production started in 1960s by France and Italy that competed to develop reliable mass-production techniques for juveniles, and, by the late 1970s, these techniques were well enough developed in most Mediterranean countries to provide hundreds of thousands of larvae.

Significant progress in sea bass farming has been achieved during the last 20 years as a result of accumulated scientific knowledge in the biology and husbandry of the fish. Sea bass production reached almost 120.000 tons in 2010 (FAO, 2010) and along with the production of sea bream makes up over 95 percent of total production in Greece. However, many problems still exist, regarding inappropriate growth rates,

susceptibility to diseases, and reproductive dysfunctions that are mostly linked with faulty management practices.

1.3 General developmental patterns, from zygote to exotrophic larvae

Following fertilization, the translucent ovum becomes a fertilized egg, a small spherical body of 1.3 mm in diameter. The structure is surrounded by an external envelope, the chorion, separated from the inner vitellin membrane by the peri-vitellin space resulting from the cortical reaction preceding the zygote formation. The zygote, 0 h post-fertilization (0 hpf), may be defined as a fine layer located over the yolk forming the blastoderm at the animal pole of the fertilized egg with the yolk exhibiting one or more floating oil globules. The hatching pre-larva is transparent showing, however, pigmented zones covering in particular the dorsal region; noticeable chord and myotomes suggest an immediate capacity of motion although the size of the yolk sac appears as a hydrodynamic disadvantage. This could in part explain the jerky movements of the new hatched individual probably related to the high loss resulting of predation in natural conditions. Newly hatched larva shows numerous chromatophores invading the optic capsules and covering the skin on the dorsal region and the walls of the yolk vesicle but the mouth is not yet open, indicating that the endotrophic period is not achieved. Besides this, the fact that the digestive tube is opened at its posterior end suggests the larvae are preparing to receive exogenous material. The jerky and uncontrolled swimming movements cease with the progressive development of dorsal and ventral fins (3–4 dph) at 15 ^{o}C (Cucchi et al., 2011). On the fourth day (4 dph), the yolk vesicle is practically restricted to the oil drop and jaw appears, but the mouth is not opened before the fifth (5 dph) day enabling the starting point of exotrophy. The fact that retinal pigmentation

is not observed until the development of exotrophic behavior suggests that eyes melanization occurs concomitantly with the requiring a rapid improvement of vision (Cucchi et al., 2011). The timing of pigmentation is probably adaptive, and could be due to the predation cost of possessing a highly reflective retina versus the necessity of a functional visual system before the starting of feeding activity. Before setting of eye pigmentation, the embryos are translucent, and thereof less vulnerable to visual predators (Blaxter, 1988).

1.4 Larval rearing

At the onset of gastrulation, the embryo shows well-defined cephalic and caudal regions while epiboly reaches 60% around 27 hours post fertilization (hpf) (**Fig. 2a**). At hatching which occurs within 92–93 hpf, E. sea bass pre-larvae are around 3.3-4.0 mm long, and lack functional eyes and a mouth (**Fig. 2b**). The yolk sac is almost half of the whole body length and the lipid droplets (1-4) generally fuse during embryonic development and to a single lipid droplet (0.40-0.45 mm). Within the following 4-6 days a mouth is developed and opens (**Fig. 2c**), complete yolk sac absorption is observed, eyes and larval body pigmented and pectoral fins are also developed. The first feeding stage begins 9-11 days later in order to let the swim bladder develop more properly. Around day 27-30 the flexion of the notochord is observed, where the notochord tip has reached its final position at approximately 45 degrees from the notochord axis (**Fig. 2d).** Caudal and anal fins develop by day 20 and dorsal and ventral fins from day 40 (Marino et al., 1993), where all fins have been established as in adults (**Fig. 2e**). Scales appear from day 70. The larval rearing period approximately lasts around 80 days, depending on water temperature and larval rearing system. Larval rearing consist of two phases; the first one (*exogenous phase*)

is based on feeding on live foods and the second phase (*weaning*) during which the fish are weaned off live feed onto commercial diets. At the final stages or at the end of the weaning phase, juveniles are much sturdier than post-larvae (Moretti et al., 1999) and can be moved from larval or weaning tanks to pre-ongrowing facilities. During this period sea bass juveniles are fed on artificial diets, usually graded 2-3 times for size and deformities evaluation until they reach a size of 1.0-2.5g. Juveniles produced for stocking sea cages are kept in flow-through tanks until they reach a size of 10-20g.

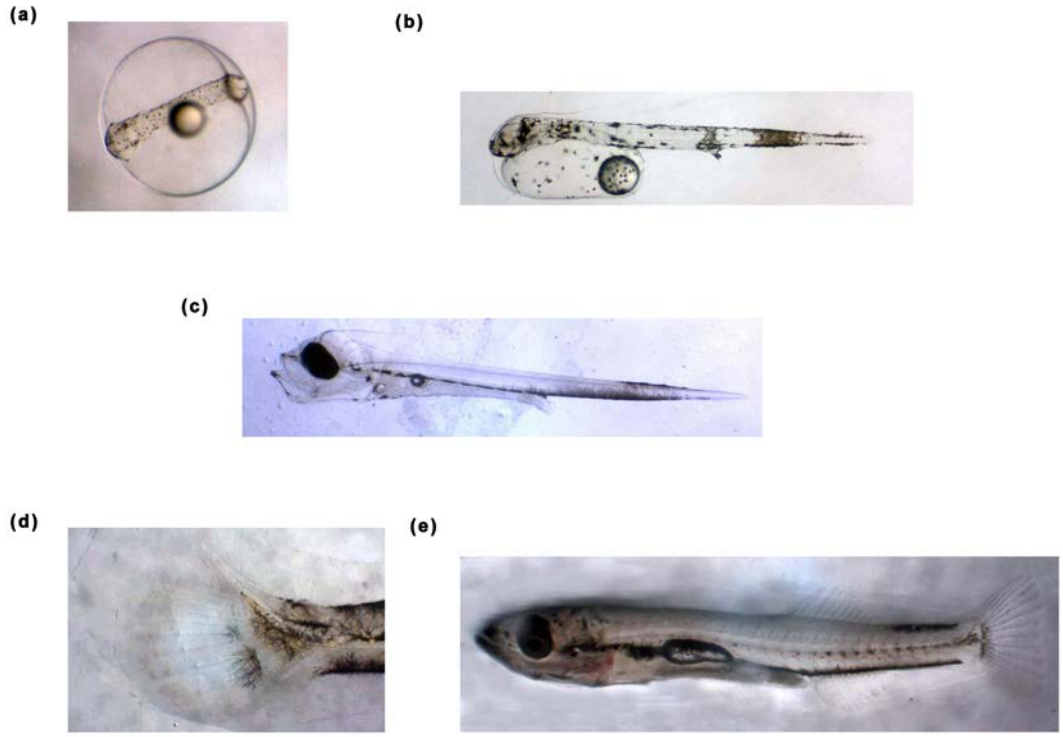

Figure 2. Early development of *Dicentrarchus labrax*. (a) Embryo; (b) pre-larvae at hatching (0 dph); (c) pre-larvae at mouth opening (6 dph); (d) the bending of the notochord at the stage of flexion (29 dph); (e) larvae at the stage where all fins have been developed as in adults (45 dph).

1.5 The Concept of Stress

Nearly from the introduction of the concept of stress by Cannon (1932) and Selye (1946), various attempts to better define the terminology of stress have been proposed. Generally, stress is regarded as a state during which an organism's homeostasis is threatened, and recovery from stress requires a complex set of adaptive mechanisms (Chrousos, 1998). Homeostasis is the stability of physiological systems that maintain life, such as pH, body temperature, glucose levels, and oxygen tension that are truly essential for life and are therefore maintained within a range optimal for the current life history stage. In common usage, stress usually refers to an event or succession of events that cause a response, often in the form of "distress" but also, in some cases, referring to a challenge that leads to a feeling of exhilaration, as in "good" stress. But, the term "stress" is full of ambiguities. It is often used to mean the event (stressor) or, sometimes, the response (stress response). Furthermore, it is frequently used in the negative sense of "distress," and sometimes it is used to describe a chronic state of imbalance in the response to stress.

In common with all vertebrates, fish respond to environmental challenges with a series of adaptive neuro-endocrine adjustments that are collectively termed the stress response. These in turn induce reversible metabolic and behavioral changes that make the fish better able to overcome or avoid the challenge and are undoubtedly beneficial, in the short-term at least. In contrast, prolonged activation of the stress response is damaging and leads to immunosuppression, reduced growth and reproductive dysfunction (Schreck, 1982; Barton and Iwama, 1991; Pickering, 1993; Sumpter, 1993; Wendelaar Bonga, 1997; McCormick et al., 1998). The first attempt to describe the short-term and long-term response to stress was done by the use of the General Adaptation Syndrome (GAS), as described by Selye in 1973. According to the GAS,

there are three distinctive stages in stress response that correspond to the *Alarm Reaction stage* when the body is being prepared to meet the threat or danger, *the Resistance stage* during which the body adapts to the stressors that it is exposed to and in order to reduce the effect of the stressor, and the *and the Recovery or Exhaustion stage* when the system's compensation mechanisms have successfully overcome the stressor effect but if the stressor continues beyond body's capacity, organism exhausts resources and becomes susceptible to disease and death.

The most recent attempt to redefine stress was the introduction of the concept of allostasis as proposed by McEwen and Wingfield (2003). Allostasis is the adaptive process of actively maintaining stability through change (Sterling & Eyer, 1988). Organisms in response to a stressor change their allostatic state by altering the activity levels of the primary mediators (e.g. glucocorticosteroids) in order to adjust to both predictable and unpredictable events (McEwen, 2003).

1.6 Welfare

Animal welfare is a relatively recent term and is by no means a straightforward concept. Although there is no universally accepted definition for the term of welfare it generally represents the physical and spiritual state of an animal in terms of health, happiness and longevity (Duncan & Fraser, 1997). The two major issues are the meaning or definition of animal welfare and how best to objectively measure it (Broom, 1991a,b; Dawkins, 1998; Mendl and Paul, 2004). FAWC guidelines on the ''Five Freedoms'' framework, freedom from hunger and thirst, discomfort, pain, injury, disease, fear and distress, as well as the freedom to express normal behavior, provide a logical framework with which to assess welfare issues (FAWC, 1996). However, recently both the concept of homeostasis and the "Five Freedoms"

guidelines as the basis for animal welfare study are a subject of controversy, as a new model based on the concept of allostasis regards the “Five Freedoms” framework as anthropocentric, utopist rather than realistic and opposed to natural procedures (Korte et al., 2007). According to this model, another drawback of the homeostasis based welfare model as proposed by Broom (Broom, 1988) is that welfare is seen as a continuum, ranging from very poor to very good in relation to the number of environmental challenges and it ignores the absence of environmental challenges which produces hypostimulation in the animal and consequently bad animal welfare (**Fig. 3**, Korte et al., 2007).

Physical health is the most universally accepted measure of welfare and is undoubtedly a necessary requirement for good welfare, but poor health can be both a cause and a result of poor welfare. However, for many, good animal welfare goes beyond just physical health and also involves a lack of mental suffering. This is a controversial issue, particularly when it comes to fish. Concepts of animal welfare have traditionally been applied to those which are considered to have the ability to experience pain, fear and suffering and as such have been associated with species with a higher level of cognition when compared to fish. However, there is scientific debate regarding the ability of fish to experience pain and fear. While some argue that fish lack essential brain regions or any functional equivalent, making it untenable that they can experience pain and fear (Rose, 2002), others suggest that there is anatomical, physiological, and behavioral evidence that make it conceivable that nociception in fish is experienced and that they have the potential to experience suffering in the form of pain and fear (Braithwaite & Huntingford, 2004; Chandroo et al., 2004a,b; Sneddon, 2002; Sneddon *et al*., 2003a,b). Fish are sophisticated animals, far removed from unfeeling creatures with a few seconds of memory, as is the popular

misconception. However, the scientific study of fish welfare is at an early stage compared with work on other vertebrates.

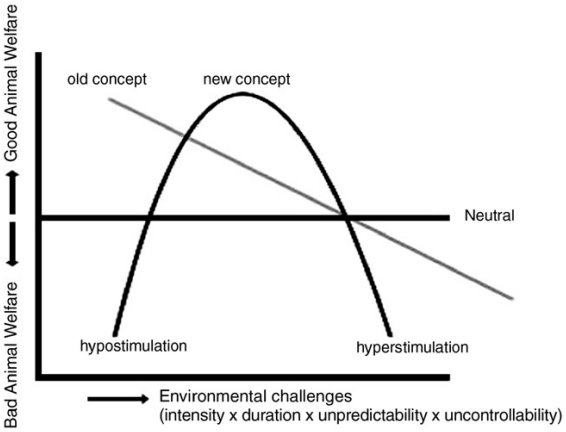

Figure 3. Animal welfare in relation to environmental challenges as shown by the out-dated concept based homeostasis and the new concept based on the inverted U-curve of (di)stress.

1.7 The stress response in fish

The 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 level (Wendelaar Bonga, 1997; Barton & Iwama, 1991; Barton, 2002). 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 fitness (Wendelaar Bonga, 1997; Barton & Iwama, 1991; Schreck, 1982; McCormic *et al.*, 1998).

1.7.1. HPI-axis activation

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 evolution (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999) and it is of utmost importance in stress regulation as well as for the adaptation and acclimation of fish to their dynamic environment. In fish, stress leads to the activation of the Hypothalamic-Sympathetic-Chromaffin cell axis and the HPI axis and the production of catecholamines and cortisol, respectively (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). As it is graphically illustrated in **Fig. 4**, the HPI axis stimulates the pituitary gland's corticotrope cells and the melanotrope cells to synthesize and secrete pro-opiomelanocortin (POMC)-derived peptides involved in the mediation and regulation of the stress response (Wendelaar Bonga 1997, Slominski *et al.* 2000). The *pomc* gene is mostly expressed in the pituitary gland and is translated into a single protein product, which is the precursor molecule of other neuropeptides, among which, adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (MSH) and β-endorfins (Smith & Funder 1988). During HPI axis activation corticotropin-releasing factor (CRF), produced in the hypothalamic preoptic area, stimulates the pituitary gland corticotropes to secrete adrenocorticotropic hormone (ACTH), which binds to melanocortin 2 receptor (MC2R) on the interrenal steroidogenic cells and activates cortisol synthesis and secretion, which then enters into the circulation and are distributed to target tissues (Barton, 2002; Wendelaar Bonga, 1997; Schiöth et al, 2005). Control of cortisol release is through negative feedback of the hormone at all levels of the HPI axis. 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

reproduction (Mommsen et al., 1999; De Jesus et al., 1991; De Jesus & Hirano, 1992; Vazzana et al., 2002). 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 function (Wendelaar Bonga, 1997; McCormick et al., 2008). Cortisol enters by passive diffusion into the cells where its action is mediated by the Glucocorticoid receptor(s) (GRs) and the mineralocorticoid receptor (MR) (Prunet et al., 2006), 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 (Ayson et al., 1995; Lin et al., 1999) and is implicated in the metamorphosis from larvae to juveniles (Kim & Brown, 1997; Deane & Woo, 2003). Apart from ACTH which is mediated in the control of cortisol release, another POMC-derived peptide, α-MSH, is also involved in the stress response in fish (Wendelaar Bonga 1997) and studies, carried out in salmonids have shown that ACTH and α-MSH cells are differentially activated.

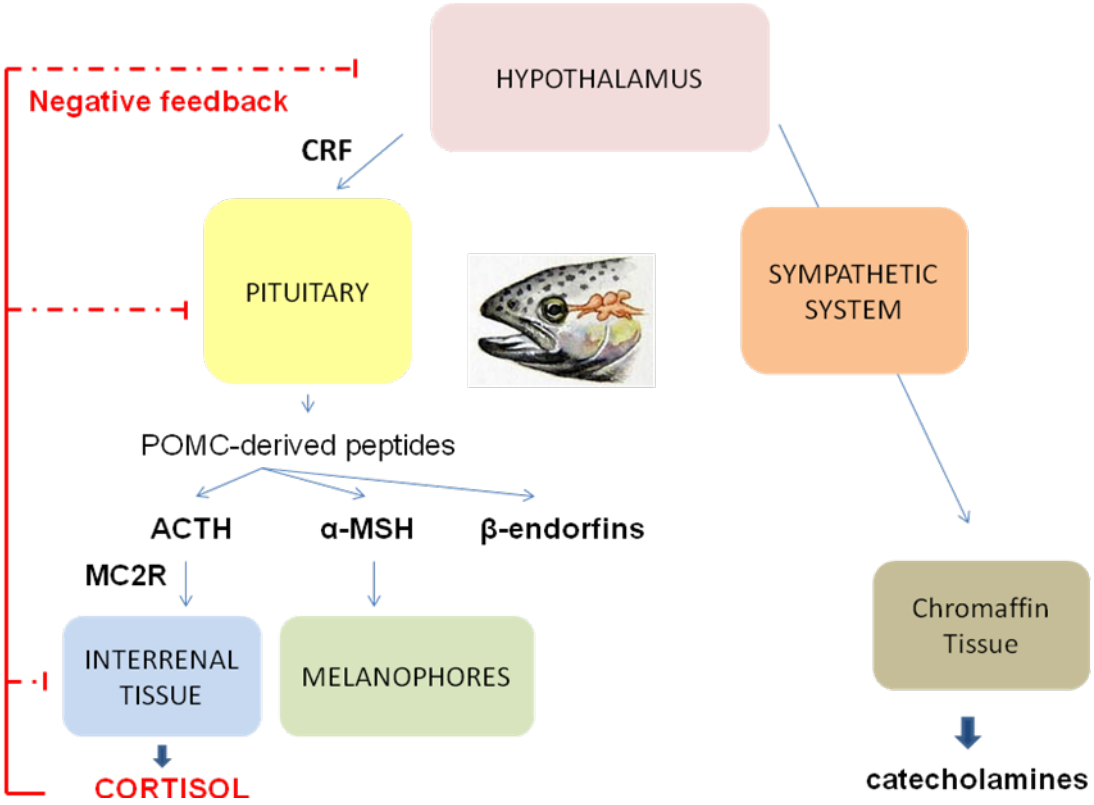

Figure 4. Diagram illustrating the stress response in fish. In fish, stress leads to the activation of the Hypothalamic-Sympathetic-Chromaffin cell (HSC) axis and the Hypothalamic-Pituitary-Interrenal (HPI) axis, resulting in the production of catecholamines and cortisol, respectively. During HPI axis activation, the hypothalamus secretes corticotropin-releasing factor (CRF), which stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH binds to melanocortin 2 receptor (MC2R) which in turn regulates cortisol synthesis and release into the circulation. Control of cortisol release is through negative feedback of the hormone at all levels of the HPI axis. ACTH is a pro-opiomelanocortin (POMC)-derived peptide, among which also α-melanocyte stimulating hormone (α-MSH) is involved in the mediation and regulation of the stress response.

1.7.2 Cortisol response

Cortisol, the main end product of the hypothalamus – pituitary – interrenal axis (HPI), is the most commonly measured indicator of stress in teleosts and usually provides a good reflection of the severity and duration of the stress response (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Fevolden et al., 2002). Cortisol in fish has been implicated in the regulation of a broad array of physiological functions that affect energy metabolism, growth, reproduction, hydromineral balance and the immune system (Wendelaar Bonga, 1997; Mommsen et al., 1999). Specifically, cortisol affects energy metabolism in fish by increasing liver glucose production and by stimulating proteolytic and lipolytic capacity (De Boeck et al., 2001; Aluru & Vijayan, 2007). Beyond its catabolic effects, cortisol suppresses somatic growth in fish through its actions on the growth hormone-insulin-like growth factor I axis (Kajimura et al., 2003; Peterson and Small, 2005) and its effects on the neuroendorine pathways that regulate food intake (Bernier, 2006). Cortisol impairs sexual maturation and reproduction via multiple effects including the suppression of plasma gonadotropin levels, the inhibition of gonadal steroidogenesis and a reduction in liver vitellogenin synthesis (Contreras-Sanchez et al., 1998; Pankhurst & Van der Kraak, 2000; Consten et al., 2002). In fish that lack aldosterone, cortisol can promote both ion uptake and ion secretion mechanisms as part of in its role in the regulation of hydromineral balance (McCormick, 2001).

In an acute stress response the elevated cortisol levels usually fall to resting values within a few hours. On the contrary, during chronic stress, cortisol concentrations can remain in high levels for days or even weeks (Pickering and Pottinger, 1989; Rotllant and Tort, 1997). However, in some cases of chronic stress cortisol levels can return to basal values due to the following factors such as control of cortisol release through

negative feedback of the hormone at the level of the hypothalamus and the pituitary, like inhibition of CRH and ACTH (Barton *et al*., 1987), the desensitization of the interrenal tissue to ACTH stimulation (Barton and Iwama, 1991; Rotllant *et al*., 2001), increased cortisol degradation rate (Vijayan and Leatherland, 1990) and the desensitization of cortisol target-tissues, by reduced abundance and/or affinity of its receptors (Pottinger, 1990; Pottinger *et al*., 1994, 2000).

The development of non-invasive methods for cortisol measurement is of prime importance for the evaluation of the cortisol stress response. Studies in adult European sea bass have shown that there is a strong positive correlation between cortisol release rate into the water and plasma cortisol concentrations and that cortisol release rate into the water (ng g^{-1} h^{-1}) can be used as a reliable non-invasive method for the assessment of the stress response (Fanouraki et al., 2008). European sea bass is known to be susceptible to husbandry stressors and characterized by high blood cortisol levels (Rotllant et al., 2003, 2006, Fanouraki et al., 2011) compared to other commercially important aquaculture species such as the gilthead sea bream (Rotllant et al., 2000, 2006; Tort et al., 2001, Fanouraki et al. 2011, Szisch et al., 2005), rainbow trout (Barton et al., 1980; Ruane et al., 1999; Ellis et al, 2004), and red sea bream, *Pagrus major* (Biswas et al., 2006). Studies in adult European sea bass have shown that there is a strong positive correlation between cortisol release rate into the water and plasma cortisol concentrations and that cortisol release rate into the water (ng g^{-1} h^{-1}) can be used as a reliable non-invasive method for the assessment of the stress response (Fanouraki et al., 2008). This non-invasive method of cortisol measurement has many advantages as there is no need for anesthesia, blood sampling and manipulation of the fish, it can be carried out in small sized animals which would be difficult to take blood samples from, the results are not affected by any stress

induced by the sampling procedure and repeated water samplings can be carried out from the same population without having an effect on the experimental fish (Scott *et al*., 2001; Ellis *et al*., 2004; Scott and Ellis, 2007). However, the non-invasive approach requires a thorough appreciation of potential problems associated with sampling, processing, validation and interpretation of results. It requires, for example, the measurement of release rates, and not just simple measurement of a steroid water concentration and factors such as the total biomass and the water renewal rate can have a significant impact on the results so it is of utmost importance that these parameters must be controlled when designing experiments and interpreting the results.

1.7.3 Corticosteroid Receptors

During HPI activation, cortisol enters into the cells by passive diffusion where its action is mediated by the Glucocorticoid receptor(s) (GRs) and the mineralocorticoid receptor (MR) (Prunet et al., 2006), a class of ligand-activated transcription factors. In teleosts, GR and its ligand cortisol are best known for their roles in the stress response (Wendelaar Bonga, 1997; Charmandari et al., 2005) and, specifically, modulate aspects of intermediary metabolism, growth, behavior, and immune function (Wendelaar Bonga, 1997; Mommsen et al., 1999). Upon cortisol binding, GR forms a dimer and moves into the nucleus where it recognizes glucocorticoid response elements (GREs) within promoters of target genes leading to the transcriptional activation or suppression of the respective genes. Although most vertebrates possess one GR, the majority of teleosts that have been examined to date like rainbow trout (*Oncorhynchus mykiss*) (Bury et al., 2003) and a cichlid (*Haplochromis burtoni*) (Greenwood et al., 2003) and two species of puffer fish, *Tetraodon nigroviridis* and

Takifugu rubripes, have two GRs (Stolte et al., 2006). Two GRs are thought to have arisen from the whole genome duplication that occurred in ray-finned fish 350 million years ago (Meyer & Van de Peer, 2005; Vandepoele et al., 2004). After a genome duplication event, the majority of the new paralogs undergo nonfunctionalization, where one of the two duplicated genes is lost (Woods et al., 2005). However, in some instances, both genes are retained. However, studies in some other fish species like *Danio rerio* and *Sparus aurata* (Gilthead sea bream) have shown that only one GR is expressed (Alsop and Vijayan 2009; Acerete et al. 2007). In E. sea bass, two different GR genes (NCBI Gene Bank accession no. AY549305 and AY619996) have also been cloned and sequenced (Terova et al., 2005; Vizzini et al., 2007). These genes encode different GR isoforms (GR-1 and GR-2) with high amino acid sequence identity, but no significant sequence similarity at the transcriptional activation domain (Di Bella et al., 2008).

The roles of MR and its ligand are less clear. In mammals, the mineralocorticoid system controls salt and water balance via MR and its ligand aldosterone (Pascual-Le Tallec & Lombes, 2005). Aldosterone is an evolutionary more recent steroid (Bridgham et al. 2006), believed to be absent in teleostean fishes (Balment & Henderson 1987). In fish, cortisol is intimately involved in the regulation of water and mineral balance (Gilmour 2005) but also the poorly studied DOC could, via a MR, act as a mineralocorticoid in fishes

1.7.4 Cortisol biosynthesis and degradation

Steroids, including corticosteroids, are produced using the precursor cholesterol through several enzymatic reactions (Mommsen et al., 1999). The first step is the transfer of the hydrophobic cholesterol across the mitochondrial membrane by the

steroidogenic acute regulatory protein (StAR; Clark et al., 1994). Steroid biosynthesis begins with the conversion of cholesterol to pregnenolone by cytochrome P450 enzyme (CYP11A1) in the inner mitochondrial membrane (Alsop & Vijayan, 2009). Then pregnenolone undergoes several isomerisations and hydroxylations by different steroidogenic enzymes including cytochrome P450 17α-hydroxylase (CYP17A1), 3β-hydroxysteroid dehydrogenase (HSD3B2) and cytochrome P450 21 hydroxylase (CYP21A2) to produce 11-deoxycortisol. The conversion of 11-deoxycortisol to cortisol is catalyzed by 11β-hydroxylase (CYP11C1) (Alsop & Vijayan, 2009; Mommsen et al., 1999), whereas the circulating cortisol is converted to inactive cortisone by the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-hsd2; Draper & Stewart, 2005) (**Figure 5**).

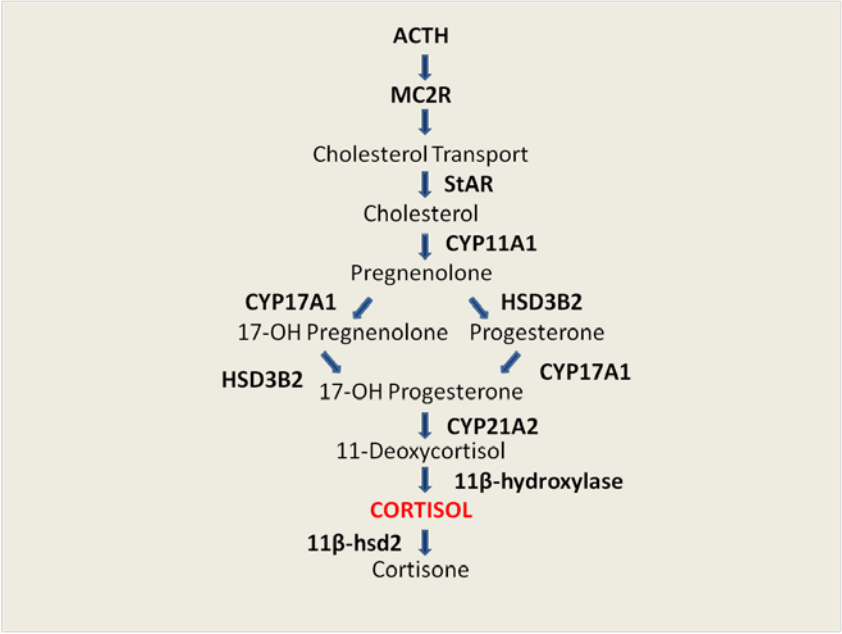

Figure 5. Diagram illustrating the cascade of reactions leading to cortisol biosynthesis and inactivation. Cortisol biosynthesis involves a cascade of reactions in which the final step is carried out by 11β-hydroxylase which catalyses the conversion of 11-deoxycortisol to cortisol, whereas the circulating cortisol is converted to inactive cortisone by the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-hsd2).

1.7.5 α-MSH in the stress response

Colour changes in fish are often related to stress. Alpha-melanophore-stimulating hormone (α-MSH) is pivotal in the regulation of skin pigmentation. It was the first hormone identified as a colour changing agent in poikilothermic species (Bagnara and Hadley, 1973). α-MSH stimulates melanin synthesis and melanosome movement within the melanophores (Castrucci et al., 1997), In teleosts, α-MSH is derived from the precursor hormone proopiomelanocortin (POMC) and is produced in the pars intermedia of the pituitary gland (Lamers et al., 1991; Steveson et al., 1996). In European sea bass (*Dicentrarhus labrax*) a single form of a functional *pomc* gene has been cloned and characterized (Varsamos, 2003). The melanocortins exert their physiological role by binding to a family of specific G protein-coupled receptors (GPCR) that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R-MC5R), although in teleost fish the number of receptors diverges (Cerdá-Reverter et al., 2011). The melanocortin 2 receptor (MC2R) is specifically activated by ACTH, while the other MCRs can be activated by the MSHs as well as ACTH (Schiöth et al., 2005). The interaction of α-MSH and melanocortin 1 receptor (MC1R) plays a key point in the control of the pigmentation and mutations of MC1R are responsible for reduced melanization, whereas the expression of melanocortin 4 receptor (MC4R) is thought to play a role in the regulation of the energy balance in fish through the modulation of feeding behavior (Cerdá-Reverter et al., 2003a, 2003b, 2011; Song and Cone, 2007).

Studies in gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) have shown that air exposure induced an increase in α-MSH levels (Arends *et al.* 1999; Sumpter *et al.* 1986). Moreover, studies, carried out in salmonids have shown that ACTH and α-MSH cells are differentially activated. In these studies, HPI

axis activation by handling and confinement led to elevated plasma concentrations of ACTH only, but when these stressors were combined with a thermal shock also α-MSH was increased (Sumpter et al., 1985, 1986). Similarly, the latter is supported by other studies in tilapia (*Oreochromis mossambicus*), where although long-term netting had no affect on ACTH concentrations, plasma cortisol levels were elevated, but when netting was combined with confinement both cortisol and ACTH were increased (Balm et al., 1994). Taken together, these results suggest a functional role for α-MSH during stress, but also suggest that α-MSH is not corticotropic.

1.8 Early life stress

Fish reared under intensive aquaculture conditions are exposed to various stressors which may affect the physiological stress response and can lead on negative consequences on performance, health status and welfare (Schreck, 1981; Barton and Iwama, 1991; Sumpter, 1993; Pickering, 1993; Wendelaar Bonga, 1997). Under chronic stress situations, fish usually develop irreversible changes in structural, physiological, metabolic and behavioral stress indicators and finally severe impairment in important physiological functions, health status and welfare (Ashley, 2007; Conte, 2004; Di Marco et al., 2008; Ellis et al., 2002; Sammouth et al., 2009; Terova et al., 2005; Tort et al., 2011).

Exposure to stress can have a profound impact on the physiology and health of an organism later in life (Kapoor *et al.*, 2006; Weinstock, 2008). 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 stress (Korosi & Baram, 2010; Seckl *et al.*, 2007; Szyf *et al.*, 2007). In fact, it has been shown that exposure to stressors during development results in permanent changes in stress coping

phenotypes in mammals (Korosi & Baram, 2010; Meaney *et al.*, 2007), birds (Love & Williams, 2008), amphibians (Hu *et al.*, 2008), and fish (Auperin & Geslin, 2008). Studies in primates and humans have shown that stress during early development has a profound impact on offspring physiology and behavior (Arling & Harlow, 1967; Carlson & Earls, 1997; Glaser, 2000; Harlow et al., 1965; Trickett & McBride-Chang, 1995). In rodents, the response to stress, but also the social interactions later in life appear to be affected when infants are separated by the mother completely or for extended periods of time (Kalinichev et al., 2002; Lehmann et al., 1999; Lovic et al., 2001). Conversely, offspring that experienced short periods of mother-infant separation showed not only a decreased stress response but also enhanced learning and memory (Meaney et al., 1991; Meaney et al., 1985). Moreover, gene expression, physiology, behavior, response to stress, and performance on cognitive ability has been observed to be altered depending on the level of maternal licking and grooming of pups during the first week postpartum (Caldji et al., 1998; Francis et al., 1999; Liu et al., 2000; Liu et al., 1997; Mrdalj, 2013).

In fish, stress research is focused on the effects of acute or chronic severe noxius stimuli of physical, chemical and husbandry nature applied in juveniles or adult individuals, and there is no information on the effects of early exposure to long term chronic mild stressors on the development and performance of fish at subsequent phases of the life-cycle. In addition, although there are two chronic mild stress protocols developed recently for adult European sea bass (Kollias, personal communication) and zebrafish (Piato, 2011) no valid chronic low intensity stress protocol exists for fish at early development.

2. OBJECTIVES

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 its molecular regulation is scarce and there are no data generally in fish species about α-MSH synthesis during early ontogeny, the response to stress via this pathway and the molecular mechanisms involved. Moreover, in fish, stress research is focused on the effects of acute or chronic severe noxius stimuli of physical, chemical and husbandry nature applied in juveniles or adult individuals, and there is no information on the effects of early exposure to long term chronic mild stressors on the development and performance of fish at subsequent phases of the life-cycle.

The objectives of this dissertation were

1) **To investigate the ontogenesis of the HPI axis and the molecular regulation of the cortisol and α-MSH stress response during early development (Papers I and II)**

2) **To investigate the effects of exposure to long term chronic mild stressors during early development on larvae and juvenile performance and the cortisol stress response in relation to the early life history (Paper III)**

3. MATERIALS & METHODS

An overview of the methods is presented here. For more details, see each individual article.

3.1 *Ethical Approval*

All experiments were performed in accordance with relevant guidelines and regulations. The laboratories of the Hellenic Center 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 (3Rs: replacement, 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.

3.2 Animals and husbandry conditions

Batches of fertilized eggs were obtained from a private fish farm and transferred to the installations of the Institute of Marine Biology, Biotechnology and Aquaculture, HCMR (Heraklion, Crete). Larval rearing was performed applying the pseudogreen-water technique (Papandroulakis et al., 2002), which comprises two phases: (1) the initial phase (lasted 45 days, until formation of all fins), in 500-l cylindroconical tanks, where both hatching and rearing took place starting with an initial density of 100 eggs l^{-1} and (2) the pre-weaning phase (days 45–60 post-fertilization) in 2000-l cylindro-conical tanks. Larvae were held during the whole experimental period undermean (±SD) water temperature of 18 (±1.6) °C, dissolved oxygen levels of 7.2 ±

0.8 mg l^{-1}, salinity of 36 and pH of 7.9 ± 0.3. Larvae rearing period lasted for 60 days and then fish were moved into weaning tanks (volume: 10 m^3 each) and kept for about five months under similar husbandry conditions.

3.3 Development of an unpredictable chronic low intensity stress protocol (UCLIS)

A chronic low intensity stress protocol was designed and applied for a period of 14 days starting at the beginning of three different early development phases: first feeding (at 9 dph), flexion (at 29 dph) and formation all fins (at 45 dph). The stress protocol consisted of optical, mechanical and social mild stimuli. Two different types of stressors were applied randomly on a daily basis, so that fish were kept under a mild unpredictable chronic stress that minimized the potential for habituation. The evaluation of the protocol was based on hormonal (water-born cortisol in larvae rearing tanks and plasma cortisol in juveniles) and performance (survival and growth) data during its application period (larvae phase) as well as at subsequent stage of development (juvenile fish).

3.4 Histological analysis

Sea bass larvae were killed with an overdose of anesthetic (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 mm 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.

3.5 Cortisol measurements

3.5.1 Whole body cortisol

Samples were homogenized according to Stouthart et al., and cortisol was measured in duplicate using a RIA in a 96-well plate according to Gorissen et al. All wells except the 'non-specifics' received 100 ml cortisol antibody (Cortisol Antibody[xm210] monoclonal and IgG purified (Abcam) and were incubated overnight at 4 ^{o}C. Subsequently, the plates were washed three times with 200 ml/well wash buffer and 100 ml blocking buffer was added to each well in order to block the non-specific sites. Plates were covered and incubated for one hour at 37 ^{o}C. After the incubation, 10 ml of standard (4 pg–2048 pg cortisol/10 ml) or 10 ml of undiluted homogenate was added to designated wells and 10 ml assay buffer was added to the non-specifics and B0. All wells received 90 ml (333 Bq) of 3H-hydrocortisone (PerkinElmer, USA) solution and plates were incubated at room temperature for 4 hours. The plates were then washed three times with wash buffer and after the final wash step, all wells received 200 ml of 'Optiphase hisafe-3 scintillation liquid' (PerkinElmer, USA). Beta-emission was quantified by a 3 min count per well using a Microbeta Plus (Wallac/PerkinElmer, USA).

3.5.2 Plasma cortisol

Free cortisol concentrations were measured using commercial DRG cortisol enzyme immunoassay kits (DRG Diagnostics, Frauenbergstrasse, Germany). All samples were run in duplicate.

3.5.3 Water cortisol

Water samples (1 l) were peristaltically pumped at circa 10 ml/min through a pre-filter (0.45 μm poresize: AcroCap™, Gelman Sciences, Ann Arbor, MI, USA) and then through an activated solid phase extraction cartridge (Sep-pak® Plus C18, Waters, UK). Cartridges were then stored frozen until assayed. Free corticosteroids were subsequently eluted with 4 ml ethyl acetate. Ethyl acetate was evaporated at 45 °C under nitrogen gas and the residue was re-dissolved in 1 ml of PBS buffer. Free cortisol concentrations were measured using commercial DRG cortisol enzyme immunoassay kits (DRG Diagnostics, Frauenbergstrasse, Germany).

The amount of hormone (H) in ng released over a given time interval (t) in h was calculated according to Ellis et al. (2004), and the hormone release rate (ng g^{-1} h^{-1}) was subsequently calculated from the differences in the amount of cortisol between sampling points, fish biomass and time, as also described in Fanouraki et al. (2008). Since there is no validated way to measure the exact number of live larvae within the rearing tanks, fish biomass was calculated based on the (a) approximate initial number of incubated eggs, (b) hatching rate, (c) mortality rate, and (d) the mean larvae weight, as estimated by daily measurements of a given sub-sample. The daily number of individuals in each tank was then estimated considering an exponential mortality rate while the estimation of the relevant biomass was made using the above parameters.

3.6 α-MSH radioimmunoassay

Whole-body α-MSH concentrations were measured *via* radioimmunoassay. α-MSH was labeled with ^{125}I using the iodogen method (Salacinski et al., 1981). Labeled α-MSH was purified by solid phase extraction (C8 Bakerbond column, J.T. Baker, Center Valley, PA, USA). The antiserum shows 100% cross reactivity with des-,

mono- and di-acetyl- α-MSH (van Zoest et al., 1989), and was used in a final concentration of 1:22,500. The second antibody to precipitate immunocomplexes was a sheep-anti-rabbit anti-body (Fitzgerald, Acton, MA, USA) and was used at a final dilution of 1:15.

3.7 RNA purification and cDNA 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 a Nanodrop® ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany), and its integrity was tested by electrophoresis in 1% agarose gels. Reverse transcription (RT) was carried out using QuantiTect Reverse transcription kit (Qiagen) using 1 µg of total RNA, according to the manufacturer's instructions.

3.8 Real-time quantitative PCR (qPCR)

Relative gene expression was determined with quantitative polymerase chain reaction (qPCR) assays using the *KAPA SYBR® FAST* qPCR Kit (Kapa Biosystems), according to the manufacturer's instructions. The resulting fluorescence was detected with CFX Connect Thermal Cycler (Bio-Rad). Levels of mRNA expression were normalized based on the reference genes *18S* and *elf1a*. A standard curve was constructed for each gene, using 4 serial dilutions (1:5) of a pool of all cDNA samples by graphing the negative log of the dilution factor against the relative cycle threshold value. To be considered suitable for analysis, each primer pair was required to have a

linear standard curve with an r^2 value above 0.98 and primer amplification efficiency between 90% and 100%. geNORM analysis (Vandesompele et al., 2002) was performed in order to validate the reference genes that served as internal control (M values < 0.5).

3.9 Statistical analysis

All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). Data are presented as means ± standard deviation (SD). Statistical comparisons of cortisol, α-MSH concentration and gene expression of unstressed specimens (0h) between the different developmental points/stages and statistical comparisons of temporal patterns of cortisol, α-MSH concentrations and gene expression between the different time points following exposure to a stressor at each developmental point/stage were made using one-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 $P < 0.05$.

For comparison of the growth rates between the different conditions during the larval phase, multiple regression analysis was used. This method was applied for the comparison of both the total length (TL) and the wet weight (WW), following an ln-transform for the second. Statistical comparisons of (i) temporal patterns of water cortisol release rates between the different groups within each respective developmental phase that the stress protocol was applied, (ii) total length and body weight at two and five months after the end of the UCLIS trial, and (iii) plasma cortisol levels between minimum handling, common handling and acute stressed fish were made using one-way ANOVA to assess differences among groups and Tukey's

or Dunn's post-hoc tests to assess the level of significance. The significance level used was $P < 0.05$.

4. RESULTS AND DISCUSSION

4.1. Ontogenesis of the HPI axis and molecular regulation of the cortisol stress response during early development in Dicentrarchus labrax. (Paper I)

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*. The temporal patterns of cortisol and genes related to the corticosteroid signaling (*gr1*, *gr2*, *mr*, *crf*), corticosteroid synthesis (*11β-hydroxylase*) and cortisol metabolism or inactivation (*11β-hsd2*) at various stages during early ontogeny were examined in order to assess the ontogenesis of the corticosteroid-signaling pathway. Moreover, E. sea bass embryos, pre-larvae and larvae were exposed 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.

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 simultaneously evident. 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 (De Jesus et al., 1991), tilapia (Hwang and Wu, 1993), rainbow

trout (Barry et al., 1995*a*), Asian sea bass (Sampath-Kumar et al., 1995), common carp (Stouthart et al., 1998), gilthead sea bream (Szisch et a, 2005), Atlantic salmon (Nechaev et al., 2006), zebrafish (Alsop and Vijayan, 2008), and in previous work in E. sea bass (Pavlidis et al., 2011).

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 *gr*1, 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 (Di Bella et al., 2008). In addition we show, for the first time, the expression profile of *mr*, *crf*, *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 (Alsop and Vijayan, 2008).

Crf 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 steroidogenic 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 (Alsop and Vijayan, 2008).

The acute stress challenge tests didn't result in a cortisol response in embryos, hatch and mouth opening stages, apart from a maximum at 24h 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-hypophysial-interrenal axis is observed at first feeding, where a peak in whole body cortisol levels was observed at 0.5h post stress, followed by a protracted decrease until at 24h when it reached resting levels. These results, in accordance with the molecular data, imply 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 2h. 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 28days 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) (Fishelson, 1999). 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 bass (Pavlidis et al., 2011), rainbow trout (Barry et al., 1995*a*), the yellow perch (Jentoft et al., 2002) and the zebrafish (Alsop and Vijayan, 2008). However, this is the first time that the exact pattern of cortisol response following exposure to acute husbandry 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 *crf*; 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 *crf* produced in the hypothalamic preoptic area (POA), stimulates the pituitary corticotropes to secrete adrenocorticotropic hormone (ACTH) (Alderman et al., 2008; Huising et al., 2004), which in turn stimulates synthesis and secretion of cortisol into the circulation (Bernier et al., 2009). *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 2h 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 0.5h post stress followed by a down-regulation at 2h. The down-regulation of *gr1* and *gr2* is in accordance with data from other studies carried out in sea bass exposed to very high stocking densities (Terova et al., 2005), in coho salmon

(Shrimpton, 1996), in Atlantic salmon (McCormic et al., 1998), in common carp (Stolte et al., 2008), in the hippocampus of rats exposed to increasing corticosteroid levels (Hugin-Flores et al., 2004), in mouse pups with high corticosteroid levels due to 24-h maternal deprivation (Schmidt et al., 2003). The statistically significant increase of the expression levels of both *gr1* and *gr2* observed at 24h 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 0.5h to 2h 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. *Crf* expression pattern at all fins follows an increase at 1h and a gradual decrease until 24h post stress, which is in accordance with the pattern observed for cortisol at this stage, where the peak of cortisol levels is at 2h post stress. In flexion, there is a statistically significant increase at 2h that remains at high values still at 24h, indicating that at this stage the *crf* 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 larvae (Ayson et al., 1995; Deane and Woo, 2003; Szisch et al., 2005) and it is also implicated in neural development and in the induction metamorphosis (De Jesus et al., 1991; Lam 1995). The inability of the stress response system to respond to the stress-elevated cortisol levels via the *crf* and the *gr*s 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 zebrafish 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* signaling (Alsop and Vijayan, 2008). Studies in mice showed that mice lacking a functional *gr* survive until birth but die shortly thereafter due to impaired lung development (Cole et al., 1995) and that there is no abnormal embryonic development detected in a mutant zebrafish line that does not develop corticotropic pituitary cells (Dickmeis et al., 2007). Other studies demonstrated that knocking down maternal *gr* leads to developmental defects in mesoderm formation in zebrafish (Pikulkaew et al., 2011) and that *gr* signaling is essential for zebrafish muscle development (Nesan et al., 2012). 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 *gr*s, 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-deoxycortisol 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 fish (Fernandino et al., 2013). 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 stressor (Aluru and Vijayan, 2006). 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 2h 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 1h post stress, just prior to the cortisol peaks (at 2h), and at 2h the transcript levels drop again to the resting values.

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.

4.2. Development and regulation of the α-MSH stress response during early ontogeny in European sea bass, Dicentrarhus labrax. (Paper II)

The α-MSH ontogenetic pattern, its response to stress during early ontogeny and the molecular mechanisms involved were characterized for the first time during early ontogeny in a Mediterranean marine teleost, the European sea bass (*Dicentrarchus labrax*). To this end, sea bass embryos, pre-larvae and larvae at specific points of development were exposed to acute stressors and the temporal patterns of whole body α-MSH and the expression profile of *pomc, mc1r, mc2r* and *mc4r* genes and their response to a stressor were determined.

During ontogenesis, the temporal changes of whole-body α-MSH levels of sea bass showed a gradual increase from low levels during the first stages to maximum values at the stages of flexion and development of all fins. There are very few data available on α-MSH during early ontogeny apart from a study carried out during the early developmental stages in scyliorhinid dogfish (*Scyliorhinus torazame*) that showed a gradual increase of the MSH-producing cells in the adenohypophysis (Chiba and Oka, 2002). The observed increase in whole-body α-MSH concentrations at the advanced stages of early development may reflect the involvement of α-MSH in the formation of melanophores and the coloring of the body which consists with the period around the formation of the fins, as α-MSH is also involved in the control of skin pigmentation and melanophore formation (Eberle, 1988; Fujii and Oshima 1986, 1994; Lu et al, 1998). Expression of *pomc* increases at the stage of flexion and its peak is in line with the first statistically significant elevation of α-MSH levels. *Mc2r*

abundance remains at low levels until the stage of first feeding where it reaches a maximum and then decreases gradually at the later stages of development. Previous studies of our group have shown that sea bass larvae begin to synthesize cortisol around the stage of first feeding (Pavlidis et al., 2011; Tsalafouta et al, 2014), which coincides with the expression profile observed for *mc2r*. Similar results have been obtained in zebrafish, where the expression of *mc2r* is upregulated immediately before the rise in whole-body larvae cortisol concentrations (Alsop and Vijayan, 2008). *Mc1r* expression was not altered depending on the developmental stage, whereas expression levels of *mc4r* appeared low from the embryo stage until mouth opening showing an increase at the stage of first feeding and remained at similar levels thereafter till the stage of the full formation of all fins.

The acute stress challenge tests did not have any effect on α-MSH levels in embryos and hatch stages. The first α-MSH response is observed at the stage of mouth opening with peak values at 2h post stress. Similarly, at the stage of first feeding α-MSH concentrations start to rise at 0.5h post stress to reach maximum values at 2h. At the stage of flexion the response is characterized by elevated α-MSH levels at 2h to reach a peak at 24h after application of the stressor, indicating a prolonged response compared to the cortisol stress response where peak values were reported to occur at 2h post stress (Tsalafouta et al, 2014). As development proceeds, the pattern of the α-MSH response to stress seems to become established until the stage of all fins where the magnitude of the response is higher, showing a gradual increase of α-MSH levels to peak values at 2h which fall to resting values at 24h post stress. These results are supported by other studies in adult gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*), which have shown that the application of acute stress led to an increase of α-MSH levels (Arends *et al.* 1999; Sumpter *et al.* 1986).

α-MSH is a POMC-derived peptide, so in order to reveal the molecular mechanisms related to the onset of the α-MSH stress response, qPCR experiments were carried out in order to analyze transcript levels of *pomc*. Expression levels of *pomc* after the acute stress application appear to be altered for the first time at the stage of mouth opening, where its abundance increases and shows maximum values at 1h post stress. This pattern continues in the later stages of development, where mRNA expression of *pomc* is upregulated along with α-MSH levels, indicating a strong relation between *pomc* mRNA expression and α-MSH production. At the stages of first feeding and flexion, peak values for *pomc* are observed at 0.5h to return afterwards to resting levels, whereas at the stage of all fins the pattern of *pomc* stress response is characterized by maximum values at 2h post stress that gradually drop to resting levels at 24h. These results are in accordance with the results obtained from a study in adult channel catfish, where an up-regulation of *pomc* mRNA was observed after water was drained at the fish eye-level, i.e. crowding stress (Karsi et al., 2005).

The melanocortins exert their physiological role by binding to melanocortin receptors (MC1R-MC5R). MC2R is specifically activated by ACTH, while the other MCRs can be activated by the MSHs as well as ACTH (Schiöth et al., 2005). In the present study, the acute stress application had no statistically significant effect on *mc2r* mRNA levels in embryos and pre-larvae at hatching and larvae at mouth opening. However, at first feeding a down-regulation of the receptor was observed with minimum values at 1h and 2h post stress, which at 24h return to control (0h) levels. This down-regulation of *mc2r* after stress at first feeding had no effect on the up-regulation of cortisol which showed peak values at 0.5h post stress (Tsalafouta et al., 2014). During stages of flexion and all fins a different pattern was observed, where transcript levels of *mc2r* were up-regulated after application of the stressor, with peak

values at 0.5h and 1h post stress, respectively, showing similar patterns with the observed cortisol patterns obtained under the same conditions (Tsalafouta et al., 2014). The different pattern obtained for *mc2r* at the stage of first feeding might reflect the differences in the magnitude and duration of the cortisol response which is evident as development proceeds. The observed up-regulation of *mc2r* after stress at the stages of flexion and development of all fins is in accordance with the data obtained in a study conducted in rainbow trout where application of an acute stressor led to increased levels of *mc2r* transcripts (Aluru and Vijayan, 2008). Up-regulation of *mc2r* after acute stress is further supported by a study by Tokarz and colleagues (2013) in zebrafish, where the expression level of *mc2r* increased significantly until about 30 min after the stressor and subsequently decreased to the mRNA levels of unstressed fish (Tokarz et al., 2013). Both *mc1r* and *mc4r* recognize α-MSH and are involved in the control of the pigmentation and the modulation of food intake, respectively (Cerdá-Reverter et al., 2003a, 2003b, 2011; Song and Cone, 2007). *Mc1r* expression was not altered following application of the stressor at any of the stages examined, whereas *mc4r* expression appeared to be affected by stress even as early as at the stage of mouth opening showing peak values at 1h post stress, which coincides with the first α-MSH response to stress observed. In first feeding, the acute stress had no effect on the levels of *mc4r*. However, the elevated pattern of *mc4r* expression appeared again at the stage of flexion with peak values at 0.5h post stress and the same pattern remained until the stage of the full formation of all the fins where transcript levels peaked at 1h post stress. The inability to respond to the stress applied at the stage of first feeding via *mc4r*, might reflect the role of *mc4r* in the modulation of the feeding behavior in E. sea bass even at early development as the survival of the

larvae at this crucial step of the transition from the autotrophic state to exogenous feeding is of critical importance.

In summary, we characterized for the first time in a Mediterranean marine teleost, the European sea bass (*Dicentrarchus labrax*), the temporal pattern of whole body α-MSH and the expression profile of *pomc*, *mc1r*, *mc2r* and *mc4r* genes during ealy ontogeny. Additionally, sea bass embryos, pre-larvae and larvae were exposed to acute stressors and the temporal patterns of whole body α-MSH and the expression of *pomc*, *mc1r*, *mc2r* and *mc4r* genes prior to and 0.5h, 1h, 2h, and 24h after application of the stressor, were determined. Overall, these data, combined with data on the cortisol response during early ontogeny (Pavlidis et al., 2011; Tsalafouta et al., 2014) give us for the first time a more thorough view on the two mechanisms involved in the stress response in E. sea bass with similar patterns observed for α-MSH and cortisol. α-MSH is a truly pleiotropic hormone, with, among others, effects on skin coloration, feed intake and metabolism. To what extent α-MSH contributes to each of these processes separately in early development remains to be determined, but our results indicate that the α-MSH stress response is paramount in early development of early vertebrates on earth, the teleostean fishes.

4.3. Early life stress and effects at subsequent stages of development in European sea bass, D. Labrax (Paper III)

To investigate the effects of exposure to long term chronic mild stressors during early development on larvae and juvenile performance and cortisol stress response, an unpredictable chronic low intensity stress (UCLIS) protocol was developed for the first time and evaluated in European sea bass, *Dicentrarchus labrax*. The UCLIS consisted of two different stressors applied randomly on a daily basis for a period of

14 days starting at the beginning of three selected developmental stages (first feeding, flexion, all fins). The stress protocol consisted of optical mechanical and social mild stimuli and the stressors were applied in a randomized order, so the fish were kept under a mild unpredictable chronic stress that minimized the potential for habituation. The strength of the UCLIS protocol developed is the unpredictability, variety, frequency and the moderate intensity of the applied stressors together, providing a relatively realistic model of everyday aquaculture husbandry practices. Evaluation was performed through the determination of water-born cortisol concentrations of the larvae rearing tanks at regular intervals, recording of mortality and measurements of growth performance. In addition, its effects on subsequent developmental phases were evaluated by measurement of growth characteristics and by the determination of plasma cortisol in juvenile fish, prior and 1h after the application of an acute stressor.

The UCLIS application resulted in higher water cortisol release rates (1.5 to 4.3 fold) throughout the experimental period in the rearing tanks of stressed larvae compared to the controls, in all developmental phases applied. Thus, our data show for the first time that water born cortisol shows good correlation between parameters indicative of stressed status of the larvae in the holding tank and may be used as a reliable indicator of stress not only in juvenile and adult fish but also during early ontogeny.

During larvae rearing and based on the growth rate, in terms of total length and wet weight, first feeding and flexion stages appeared to be more sensitive to the stimuli applied. However, at about two months after the end of the stress period, the first sampling at the juvenile stage, showed that the UCLIS did also have an impact when it was applied on larvae that were at the stage of all fins. Similarly, during the second sampling, *i.e.* approximately five months after the end of the stress period, comparison of growth between the different groups clearly showed that fish exposed

to UCLIS at the beginning of first feeding and the formation of all fins displayed worst performance than fish exposed to UCLIS at flexion and controls. Thus, and based on survival and growth data of both larvae and juvenile fish, the most critical period for exposure to noxius stimuli appeared to be that of first feeding and all fins. These results are in accordance with another study of our group in which histological, hormonal (cortisol) and molecular data showed that the first appearance of a distinct and functional hypothalamo-hypophysial-interrenal axis is observed at first feeding but the stress response becomes fully functional and mature until only at the stage of all fins (Tsalafouta et al., 2014). We may speculate that the differential effects of the UCLIS depending on the developmental stage applied may be due to the critical importance of various factors during the transition of fish to exogenous feeding at the stage of first feeding and the full functionality of the stress axis at the stage of the development of all fins. In addition, our data show for the first time that not only juveniles and adults but also European sea bass larvae are sensitive to mild husbandry stimuli with consequences even at subsequent stages of development.

Based on personal observations showing a high sensitivity of European sea bass to ordinary handling practices, we tested two different sampling methods; a common handling practice which includes decrease of the water level of the rearing tank, crowding and netting, and a minimum handling practice by which the fish were immediately caught with a net. Our data, based on plasma cortisol, clearly verified that sea bass juveniles are very sensitive to common handling practices and that this may be one explanation for the huge variability in basal cortisol concentrations reported for this species up to date (Planas et al., 1990; Cerdá-Reverter et al., 1998; Marino et al., 2001; Vazzana et al., 2002; Rotllant et al., 2003, 2006; Peruzzi et al., 2005; Maricchiolo et al., 2008; d'Orbcastel et al., 2010; Fanouraki et al., 2011;

Filiciotto et al., 2012; Fatira et al., 2014) and emphasizes the need for the development of new handling procedure for sea bass in order to minimize stress during sampling. Furthermore, whether early life stress has an impact also on the sensitivity to stress at subsequent stages of development, as indicated by the differences observed in cortisol concentrations based on early life history in fish caught by common handling practice, remains to be thoroughly investigated.

In conclusion, European sea bass, which is known to be very susceptible to husbandry stressors and characterized by high blood cortisol levels (Roche and Bogé, 1996; Fanouraki et al., 2011) compared to other species, proved to be sensitive to mild husbandry stressors applied during the larval period, as well as to common sampling practices at the juvenile phase. Common husbandry practices during early development appeared to have an impact not only when applied, but also at subsequent developmental phases, providing the necessity to reconsider common rearing practices. Water cortisol proved to be a promising non-invasive stress indicator during larvae rearing, but a more thorough validation needs to be carried out. This is the first time that an unpredictable chronic low intensity (UCLIS) protocol was developed and applied in the early development of fish. The UCLIS protocol should be evaluated in other fish species, to provide a useful tool to investigate the neuro-endocrine mechanisms implicated in the stress response. It is worth to notice that the offspring of pelagic spawners in contrast to mammals, rodents and many other fish species receive no parental care, providing in this way a very good model to study genomic-environmental stress interactions in early development.

5. CONCLUSIONS

- The first peak in whole-body cortisol concentrations after the acute stress application was observed at first feeding 0.5h post stress, implying that European sea bass individuals are capable of responding to external noxious stimuli as early as at first feeding

- Histological data showed that the first appearance of a distinct hypothalamo-hypophysial-interrenal axis is observed at first feeding, where the first peak in whole body cortisol levels was observed

- Molecular data on *gr1*, *gr2*, *mr* and *crf* provide a better insight into the onset and molecular regulation of the stress response during early development in E. sea bass and in combination with the cortisol patterns observed indicate that the stress response becomes fully functional and mature until only around the stage of all fins

- Expression data of *11β-hydroxylase* and *11β-hsd2* showed a strong correlation of the transcript levels of these genes with the whole body cortisol concentrations, verifying 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

- An α-MSH stress response characterized by elevated levels is evident for the first time around the stage of mouth opening showing a specific pattern that becomes established around the formation of all fins

- mRNA transcript levels of *pomc* and *mc2r* were altered after the acute stress application in a consistent elevated pattern especially as development proceeds, at the stages of flexion and after the formation of all fins, showing at the same time a strong correlation with the whole body αMSH concentrations. *Mc1r* expression was not altered following application of the stressor at any of the stages examined, whereas *mc4r* expression appeared to be affected by stress even as early as at the stage of mouth opening showing peak values at 1h post stress, which coincides with the first α-MSH response to stress observed.

- An unpredictable chronic low intensity protocol (UCLIS) was developed and applied for the first time in the early development of fish and its effects on performance were evaluated both during its application period and at subsequent stages of development.

- The effects of UCLIS on fish performance were evaluated both during its application period and at subsequent stages of development..

- Mild husbandry stimuli during early development appeared to have an impact on European sea bass larvae, with consequences even at subsequent stages of

development and with first feeding and all fins being the most critical phases, providing the necessity to reconsider common rearing practices.

- Based on plasma cortisol, juveniles proved to be very sensitive to common handling practices and this may be one explanation for the huge variability in basal cortisol concentrations reported for this species up to date

- Whether early life stress has an impact also on the sensitivity to stress at subsequent stages of development, as indicated by the differences observed in cortisol concentrations based on early life history in fish caught by common handling practice, remains to be thoroughly investigated.

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PAPER I

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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 evolution[1,2] 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 level[1,3,4]. 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 fitness[1,3,5,6].

Exposure to stress can have a profound impact on the physiology and health of an organism later in life[7,8]. 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 stress[9–11]. In fact, it has been shown that exposure to stressors during development results in permanent changes in stress coping phenotypes in mammals[9,12], birds[13], amphibians[14], and fish[15].

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 reproduction[2,16–18]. 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 function[1,19]. 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[21,22] and is implicated in the metamorphosis from larvae to juveniles[23,24].

Studies conducted in European sea bass, *Dicentrarchus labrax*, and other species showed the presence of maternal cortisol in embryos and that *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[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 (*gr1*, *gr2*, *mr*, *crf*), corticosteroid synthesis (*11β-hydroxylase*) and cortisol metabolism or inactivation (*11β-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 adenohypophysis 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^{-1}) that had declined at hatching (0.6 ± 0.3 ng g^{-1}) and subsequently slightly increased at mouth opening (1.5 ± 0.2 ng g^{-1}), but the differences were not statistically significant. The first peak ($P < 0.001$) was observed at first feeding (6.8 ± 1.3 ng g^{-1}), after which whole-body cortisol mean concentrations dropped gradually from flexion (4.1 ± 1.2 ng g^{-1}; $P < 0.05$) onwards to the formation of all fins (1.9 ± 0.5 ng g^{-1}) (Figure 2a). All genes assessed in the current study were expressed in all developmental stages examined. Transcripts of *gr1* (Figure 2b) showed a gradual increase throughout early development, with lowest mRNA abundance recorded in embryos

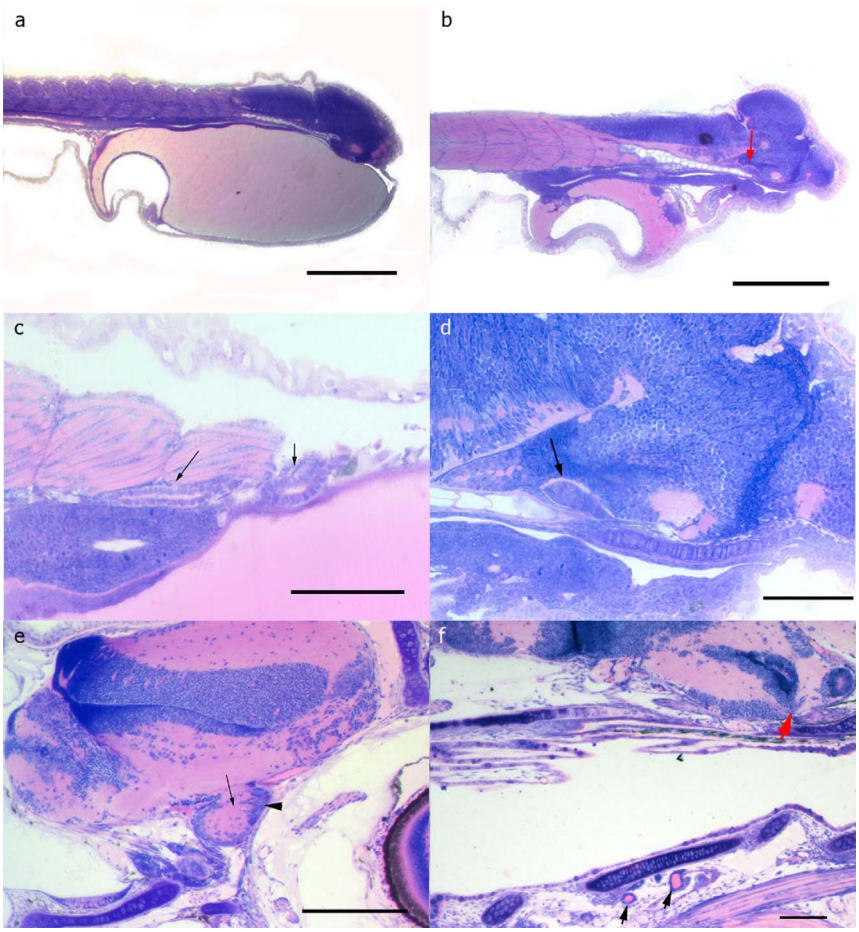

Figure 1 | Histological analysis (a) Sea bass larvae at day 1 post hatching (dph). The brain covers the majority of the head area. Bar: 400 μm. (b) A sea bass larva at 5 dph with the pituitary morphologically differentiated (red arrow). Bar: 400 μm, (c) Kidney of a sea bass at 3 dph showing the distinct 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.

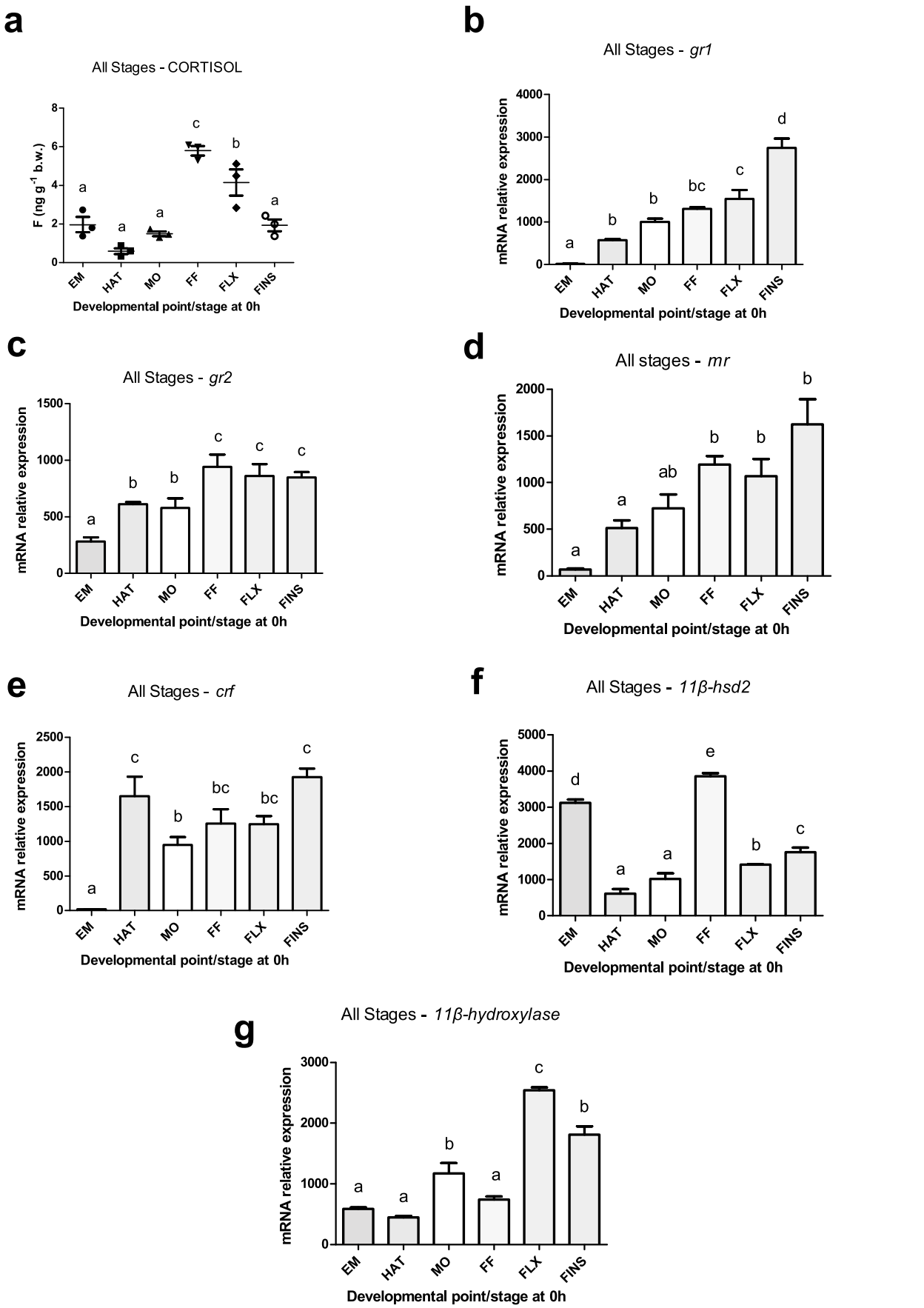

Figure 2 | Temporal patterns of cortisol content and gene expression at early ontogeny. Changes in resting (0 h) whole body cortisol levels and mRNA transcript levels of *gr1*, *gr2*, *mr*, *crf*, *11β-hsd2* and *11β-hydroxylase* during the different developmental points/stages (embryos-EM, hatch-HAT, 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 ($P < 0.05$).

and highest at the formation of all fins. Expression of *gr2* (Figure 2c) was higher in embryos than *gr1* and showed a statistically significant increase ($P < 0.05$) at hatching and mouth opening, followed by a second increase at first feeding until the formation of all fins ($P < 0.001$). The mRNA abundance of *mr* (Figure 2d) showed a similar pattern to *gr1* with minimum levels in embryos, a slight increase at hatching and mouth opening and a statistically significant increase ($P < 0.001$) at first feeding until the formation of all fins. Expression of *crf* (Figure 2e) was detected in low levels in embryos, and then a bimodal pattern was observed with a statistically significant higher level of transcripts at hatching ($P < 0.001$) and the formation of all fins than at mouth opening ($P < 0.05$). *11β-hydroxylase* mRNA (Figure 2f) showed low levels in embryos, hatching and first feeding, a statistically significant increase ($P < 0.001$) in mouth opening and the formation of all fins and a peak at flexion. Finally, transcript levels of *11β-hsd2* (Figure 2g) showed a bimodal pattern of changes with high level of transcript in embryos and at first feeding and lowest at the other examined points or stages of development.

Ontogeny of the cortisol stress response and molecular onset of genes related to the HPI axis. Figures 3 shows the cortisol response and Figures 4–6 the expression profile of the different stress-related genes prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor during early ontogeny (embryos, hatch, mouth opening, first feeding, flexion and formation of all fins). There was no statistically significant effect of stress on cortisol levels of embryos at 0.5 h, 1 h and 2 h post-stress, while there was a significant ($P < 0.001$) increase at 24 h after the application of the stressor, *i.e.* approximately 48 h following fertilization. An identical pattern of changes was observed after stress at hatching and mouth opening. A statistically significant ($P < 0.05$) effect of the stressor on whole-body cortisol concentrations soon after the application of the acute stressors was observed for the first time at first feeding, where whole body cortisol increased from the 0 h basal values (6.8 ± 1.3 ng g^{-1}) to a maximum at 0.5 h (10.8 ± 1.0 ng g^{-1}) and 1 h (9.6 ± 0.5 ng g^{-1}) to return to basal values at 24 h (3.5 ± 0.5 ng g^{-1}). At flexion, a prolonged peak was observed with basal cortisol values at 0 h (4.1 ± 1.2 ng g^{-1}) and peak values ($P < 0.05$) at 0.5 h (17.1 ± 1.7 ng g^{-1}), 1 h (19.9 ± 6.5 ng g^{-1}) and 2 h (23.4 ± 3.1 ng g^{-1}) post stress. In addition, the magnitude of the stress response was higher ($P < 0.001$) than the respective at first feeding. Finally, at the formation of all fins, cortisol content at 0 h (1.9 ± 0.5 ng g^{-1}) sharply increased at 0.5 h (17.1 ± 1.6 ng g^{-1}) to reach a maximum at 2 h (33.7 ± 2.7 ng g^{-1}) after stress and return to basal values at 24 h (3.3 ± 1.4 ng g^{-1}). The magnitude of the stress response was statistically significant higher ($P < 0.001$) than the respective in first feeding and flexion. Transcripts of *gr1* (Figure 4a) showed no statistically significant changes following exposure to the stressors at hatching and mouth opening. However, in first feeding and flexion there was a 1.9- and 1.8-fold upregulation ($P < 0.001$) at 24 h, respectively, while at the formation of all fins a significant 1.4-fold downregulation ($P < 0.05$) compared to controls was observed at 2 h post-stress. *A* similar pattern of expression was observed in *gr2* transcripts (Figure 4b), with stable mRNA abundance during hatching and mouth opening and an upregulation ($P < 0.05$) at 24 h at first feeding and flexion. In the formation of all fins, *gr2* reaches a maximum at 0.5 h after stress and then mRNA levels fall down to a minimum at 2 h. Transcript levels of *mr* (Figure 5a) showed no statistically significant changes between the different time points, apart at first feeding were a decrease is observed from 0.5 h till 2 h after stress. Exposure to stressors did not affect *crf* abundance around hatching, mouth opening and first feeding, however, in flexion there was an upregulation ($P < 0.001$) at 2 h and 24 h post-stress. In all fins, *crf* transcripts peaked, as whole-body cortisol concentrations, at 0.5 h post stress followed by a gradual decrease to the basal levels of control at 24 h (Figure 5b).

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 downregulation 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[16], tilapia[38], rainbow trout[25], Asian sea bass[39], common carp[33], gilthead sea bream[27], Atlantic salmon[40], zebrafish[28], and in previous work in E. sea bass[30].

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 *gr*1, 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[41]. In addition we show, for the first time, the expression profile of *mr*, *crf*, *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[28].

Crf 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 steroidogenic 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[28].

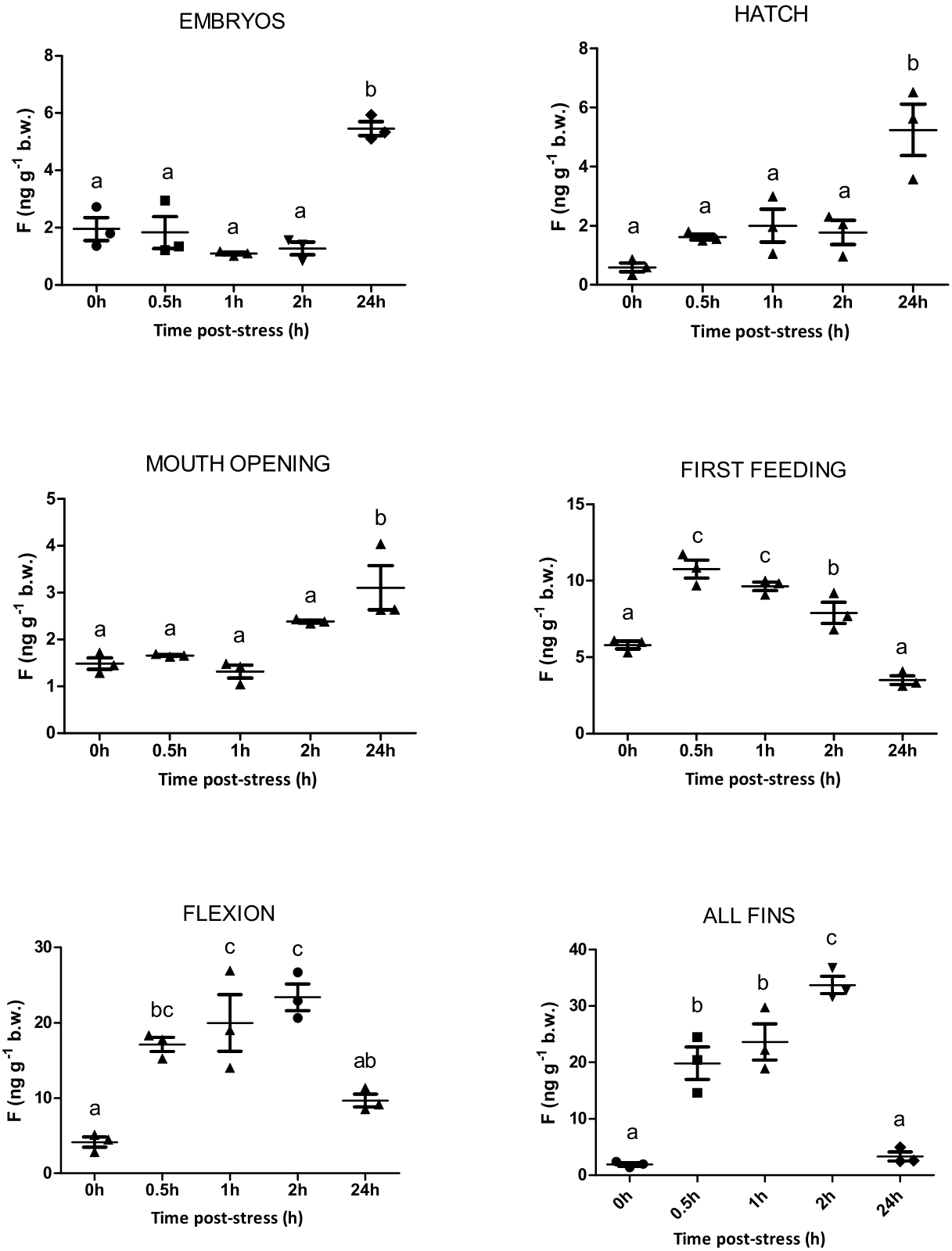

Figure 3 | Ontogeny of the cortisol stress response. The cortisol response prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor during early ontogeny. Values are means ± standard error (n=3). Means with different letters differ significantly from one another ($P < 0.05$).

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-hypophysial-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

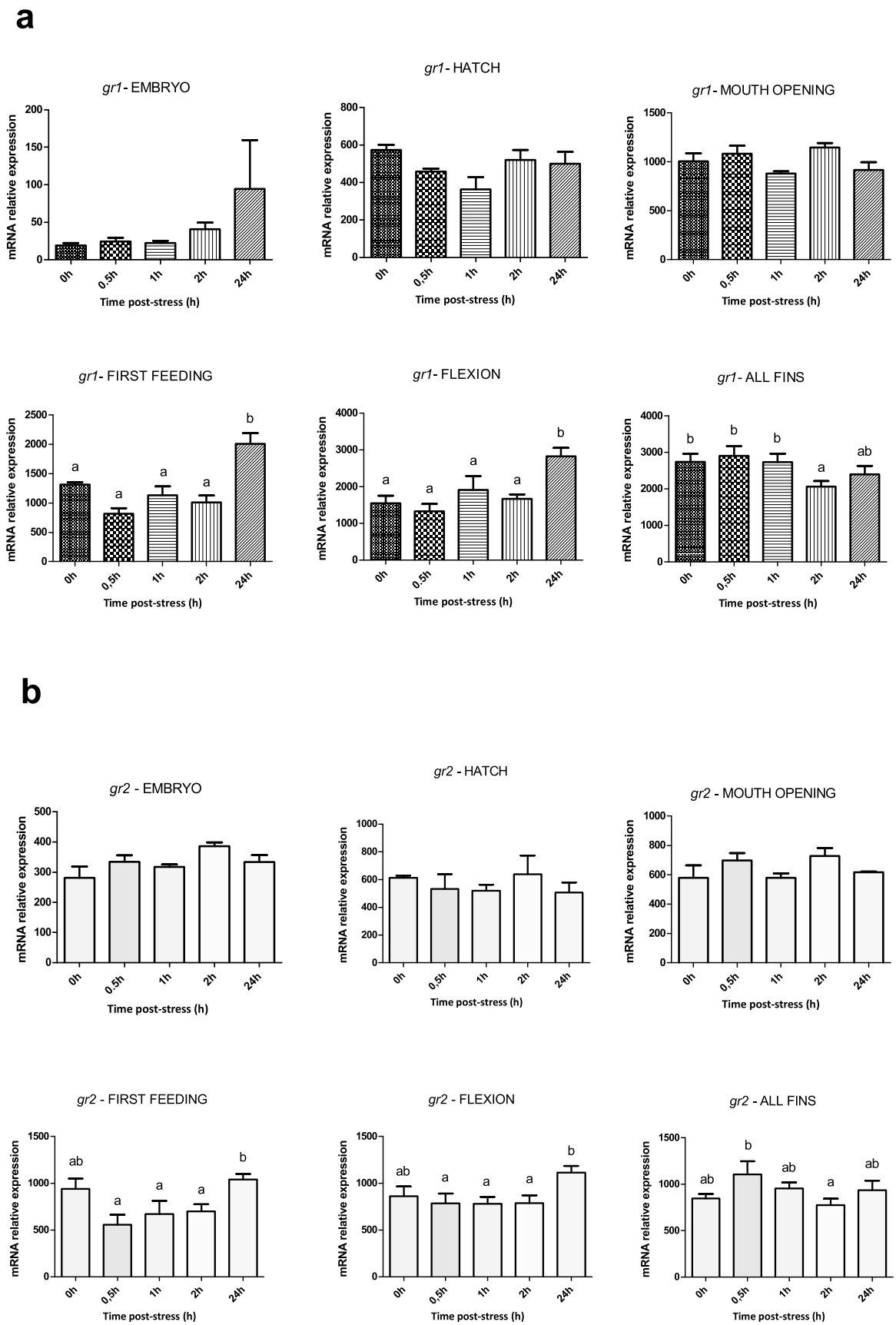

Figure 4 | Expression of *gr1* and *gr2* after application of a stressor during early ontogeny. (a) Expression profile of *gr1* prior to (0 h) and after (0.5 h, 1 h, 2 h and 24h) the application of the stressor at the different developmental points/stages. (b) Expression profile of *gr2* prior to (0 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 ($P < 0.05$).

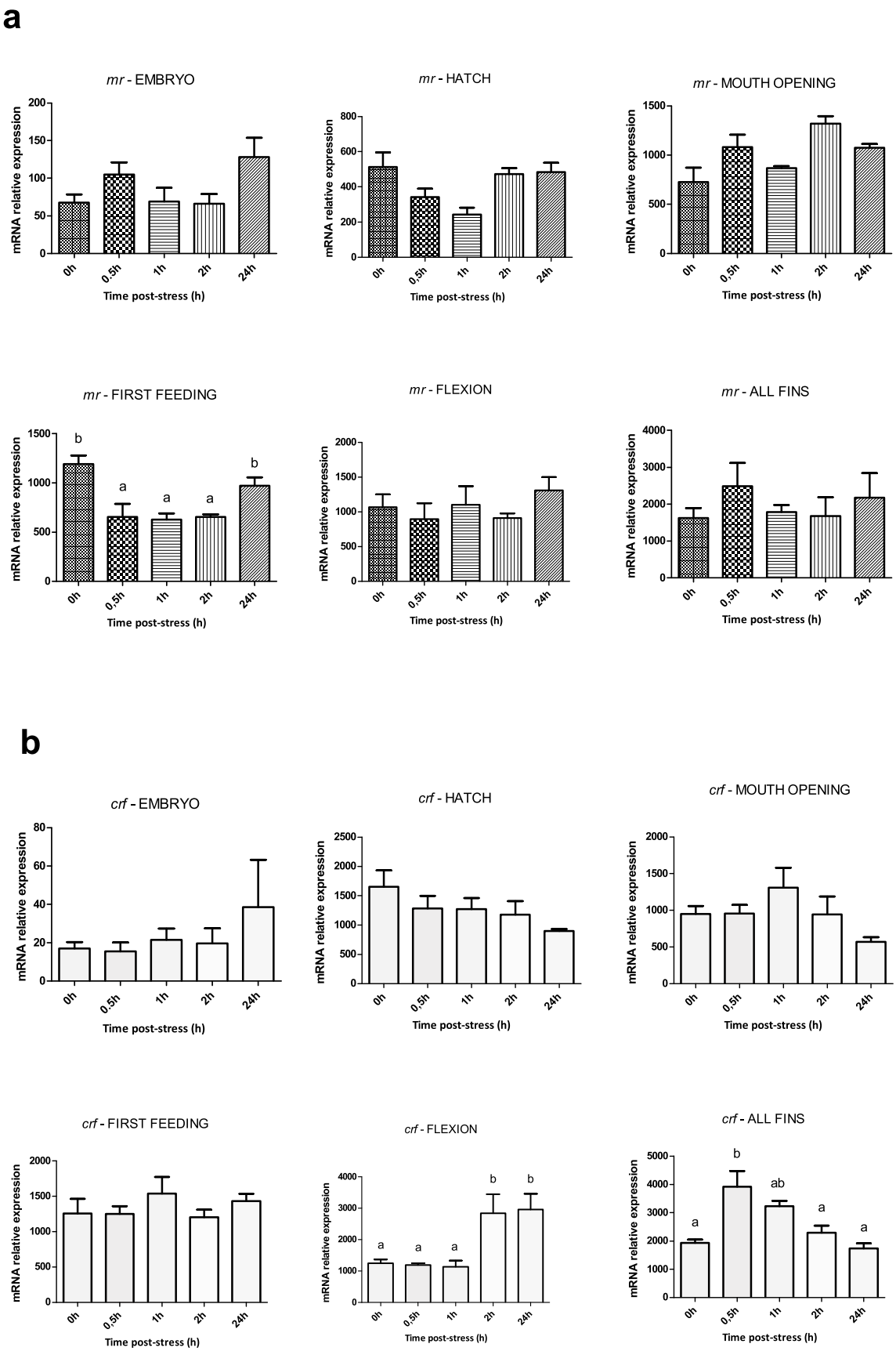

Figure 5 | Expression of *mr* and *crf* after application of a stressor during early ontogeny. (a) Expression profile of *mr* prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. (b) Expression profile of *crf* prior to (0 h) 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 ($P < 0.05$).

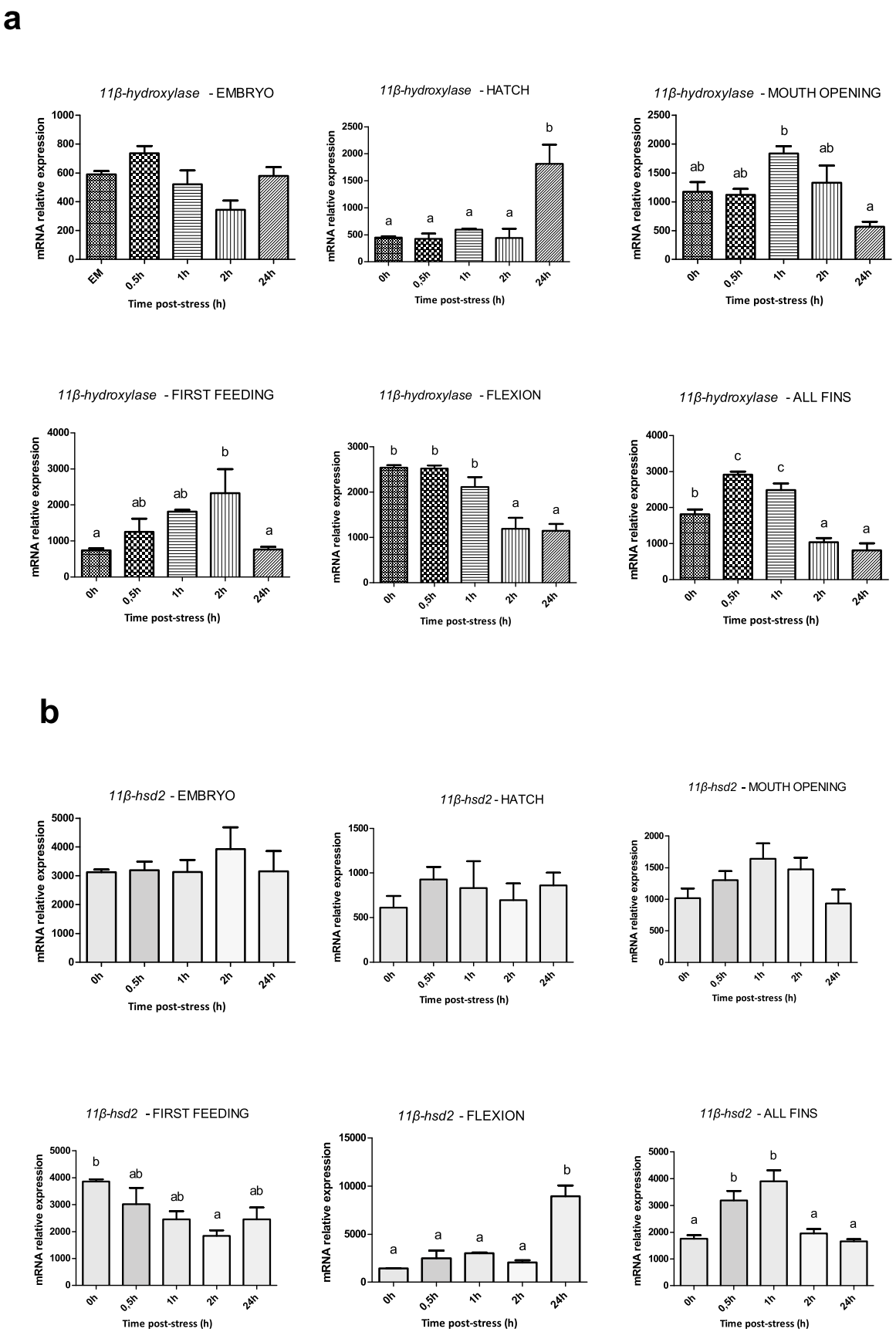

Figure 6 | Expression of *11β-hydroxylase* and *11β-hsd2* after application of a stressor during early ontogeny. (a) Expression profile of *11β-hydroxylase* prior to (0 h) and after (0.5 h, 1 h, 2 h and 24 h) the application of the stressor at the different developmental points/stages. (b) Expression profile of *11β-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 28days 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)[42]. 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 bass[30], rainbow trout[25], the yellow perch[26] and the zebrafish[28]. However, this is the first time that the exact pattern of cortisol response following exposure to acute husbandry 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 *crf*; 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 *crf* produced in the hypothalamic preoptic area (POA), stimulates the pituitary corticotropes to secrete adrenocorticotropic hormone (ACTH)[43,44], which in turn stimulates synthesis and secretion of cortisol into the circulation[45]. *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 carried out in sea bass exposed to very high stocking densities[46], in coho salmon[47], in Atlantic salmon[6], in common carp[48], in the hippocampus of rats exposed to increasing corticosteroid levels[49], in mouse pups with high corticosteroid levels due to 24-h maternal deprivation[50]. 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. *Crf* 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 *crf* 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 larvae[21,24,27] and it is also implicated in neural development and in the induction metamorphosis[16,51]. The inability of the stress response system to respond to the stress-elevated cortisol levels via the *crf* and the *gr*s 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 zebrafish 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* signaling[28]. Studies in mice showed that mice lacking a functional *gr* survive until birth but die shortly thereafter due to impaired lung development[52] and that there is no abnormal embryonic development detected in a mutant zebrafish line that does not develop corticotropic pituitary cells[53]. Other studies demonstrated that knocking down maternal *gr* leads to developmental defects in mesoderm formation in zebrafish[54] and that *gr* signaling is essential for zebrafish muscle development[55]. 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 *gr*s, 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-deoxycortisol[56] 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 fish[57]. 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 stressor[58]. 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 *crf*) 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-

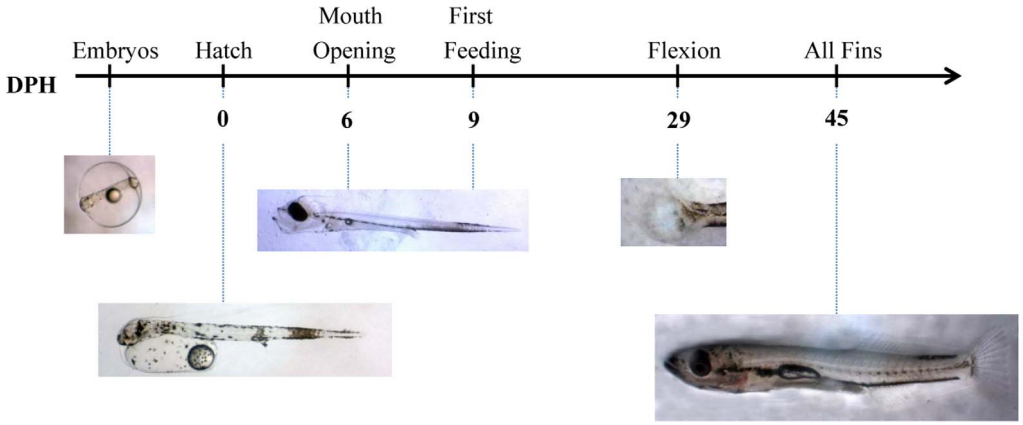

Figure 7 | Sampling design. Ontogeny of the neuroendocrine stress response in European sea bass (DPH: Days Post Hatch). An acute stress test was applied in all developmental points/stages.

neously evident. Whole body cortisol concentrations showed a decline from embryos to hatching, remained at low levels at mouth opening and peaked at first feeding. In addition, around first feeding an effect of stress was evident for the first time on post-stress cortisol concentrations. 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 pseudogreen-water technique[31], in 500 L cylindro-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 initially filled with filtered seawater from a deep well. Aeration was provided by means of a wooden diffuser located in the tank center at a rate of 150–200 ml min^{-1}. Larvae were held during the whole experimental period under mean (±SD) water temperature of 18 (±1.6)°C, dissolved oxygen levels of 7.2 ± 0.8 mg l^{-1}, salinity of 36 and pH of 7.9 ± 0.3. Food was delivered only when inflated swim bladder was observed in more than 80% of the population. Exogenous feeding was based on rotifers (*Brachionus* sp.) at 5 individuals ml^{-1} enriched with proteins and PUFA (INVE Aquaculture S.A., Belgium) until 10 days post hatching (dph) while phytoplankton (*Chlorella* sp.) was supplied until 10 dph. Enriched *Artemia* nauplii (Instar II, EG, Artemia Systems S.A., Belgium) were delivered since 10 dph until 50 dph at 0.5 to 1.0 individual ml^{-1}. From 30 dph, larvae were offered dry feed (PROTON 2–3, INVE Aquaculture S.A., Belgium) using automatic feeders. On day 50 dph, the type of the dry feed changed to PROTON 3–5 (INVE Aquaculture S.A., Belgium). During larval rearing and pre-weaning a sample of 10 larvae was taken daily to determine the morphological characteristics and record of total length while 2 times per week weight measurements were also performed with a sample of 10 individuals. The trial lasted until individuals completed the formation of their fins on 45 days post hatch (dph).

Experimental design. Samples were collected at six different embryonic and larvae phases (embryos, hatching, mouth opening, first feeding, flexion and formation of all fins; Figure 7/Table 1), prior to and after the application of an acute stress test. 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 aeration (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 aeration (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 (3Rs, Replacement, 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 anesthetic (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[32] 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

Development	Description	DPH	Total Length (mm)
Embryos	70% of embryos in 50% epiboly stage	−2	
Hatching	70% of embryos are hatched	0	
Mouth opening	Mouth opens, complete yolk sac absorption	6	4.71 ± 0.09
First feeding	First day of exogenous feeding	9	5.25 ± 0.19
Flexion	65% completed the notochord flexion	29	11.1 ± 0.44
Fins	All fins have been developed	44	15.48 ± 0.21

Whole body cortisol. Samples were homogenized according to Stouthart *et al.*[33]. Cortisol was measured in duplicate using a RIA in a 96-well plate according to Gorissen *et al.*[34]. 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 covered and incubated for one hour at 37°C. After the incubation, 10 µl of standard (4 pg–2048 pg cortisol/10 µl) or 10 µl of undiluted homogenate was added to designated wells and 10 µl assay buffer was added to the non-specifics and B_0. All wells received 90 µl (333 Bq) of 3H-hydrocortisone (PerkinElmer, USA) solution and plates were incubated at room temperature for 4 hours. The 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-emission was quantified by a 3 min count per well using a Microbeta Plus (Wallac/PerkinElmer, USA).

RNA purification and cDNA 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 (Peqlab, Erlangen, Germany), and its integrity was tested by electrophoresis in 1% 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*), Mineralcorticoid Receptor (*mr*), Corticotropin Releasing Factor (*crf*), eukaryotic Elongation Factor 1 (*eEF1a*), 40S Ribosomal protein S30 (*Fau*) and ribosomal 18S RNA (*18S*) were obtained by a previous work of our group[30,35]. Primer design for steroid *11β*-hydroxylase (*11β-hydroxylase*) was based on the available sequence with accession number AF449173.2[36]. Forward and reverse primers for 11-*β*-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′ -GGAGGAGGATTGCTGAGAACG- 3′ and the reverse primer (11β_Rev) an 19-mer primer with the sequence 5′ -AGAGGACGACACGCTGAGA- 3′. For *11β-hsd2*, the sequence of the forward primer (hsd_Fwd) was 5′ -CACCCAGCCACAGCAGGT- 3′ and the reverse primer (hsd_Rev) had the sequnece 5′-ACCAAGCCCCACAGACC- 3′. The products of each primer pair were further checked with sequencing in order to confirm that they amplify 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 MJ 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 (step 2–step 4). Levels of *gr1*, *gr2*, *mr*, *crf*, *11β-hydroxylase* and *11β-hsd2* mRNA were normalized based on the reference genes *18S*, *eEF1a* and *Fau*. 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 *18S*.

Statistical analysis. All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). All data are presented as means ± standard error of the mean (SEM). Data were initially screened for normality and homogeneity. Statistical comparisons of temporal patterns of cortisol and gene expression of unstressed specimens (0 h) between the different developmental stages 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.

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PAPER II

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Early life stress and effects at subsequent stages of development in European sea bass (*D. labrax*)

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ABSTRACT

To investigate the effects of exposure to long term chronic mild stressors during early development on larvae and juvenile performance and cortisol stress response, an unpredictable chronic low intensity stress (UCLIS) protocol was developed for the first time and evaluated in European sea bass, *Dicentrarchus labrax*. UCLIS protocol was based on the unpredictability, variety, frequency and moderate intensity of the applied stressors, providing a relatively realistic model of everyday aquaculture husbandry practices. UCLIS lasted for 14 consecutive days, starting at different phases of early ontogeny (first feeding, flexion and development of all fins). Evaluation was performed through the determination of water-born cortisol concentrations of the larvae rearing tanks at regular intervals, recording of mortality and measurements of growth performance. In addition, its effects on subsequent developmental phases were evaluated by measurement of growth characteristics and by the determination of plasma cortisol in juvenile fish, prior and 30 min after the application of an acute stressor. Our data show that European sea bass larvae are sensitive to mild husbandry stimuli with consequences even at subsequent stages of development, with the stages of first feeding and all fins being the most critical, providing the necessity to reconsider common rearing practices. In particular, UCLIS application resulted in higher water cortisol release rates in all groups compared to the controls proved to be a reliable non-invasive indicator of stress even during early ontogeny. Performance of fish in terms of survival, total length and wet weight was also affected by the stress protocol, as larvae that had been exposed to UCLIS at the beginning of first feeding and the formation of all fins displayed worst performance compared to fish exposed to UCLIS at flexion and compared to the controls. Early life stress did not affect plasma cortisol levels of juveniles exposed to additional acute stressors. However, fish were very sensitive to common handling practice and in addition, significant higher plasma cortisol concentrations were found in juveniles exposed to UCLIS at the stages of first feeding and onwards to flexion and the formation of all fins and onwards to the development of melanophores, compared to the other two groups in accordance with the differences observed in growth rates. Concluding, our data show for the first time that common husbandry practices during early development have an impact both on larvae performance and at later stages of development, as life history affected growth and the stress response in juvenile fish.

1. Introduction

Fish reared under intensive aquaculture conditions are exposed to various high intensity/short duration and/or low intensity/chronic stressors which may affect the physiological stress response and can lead to negative consequences on performance, health status and welfare (Barton and Iwama, 1991; Pickering, 1993; Schreck, 1981; Sumpter, 1993; Wendelaar Bonga, 1997). The fish's primary response to such stressors is regulated through the activation of the hypothalamic–sympathetic–chromaffin cell axis and the hypothalamic–pituitary–interrenal (HPI) axis, resulting in the production of catecholamines and cortisol, respectively (Mommsen et al., 1999; Randall and Perry, 1992; Reid et al., 1998; Wendelaar Bonga, 1997). Cortisol is the most commonly measured indicator of stress in teleosts and usually provides a good reflection of the severity and duration of the stress response (Barton and Iwama, 1991; Fevolden et al., 2002; Wendelaar Bonga, 1997). European sea bass is known to be susceptible to husbandry stressors and characterized by high blood cortisol levels (Fanouraki et al., 2011; Rotllant et al., 2003, 2006) compared to other commercially important aquaculture species such as the gilthead sea bream (Fanouraki et al., 2011; Rotllant et al., 2000; Szisch et al., 2005; Tort et al., 2001), rainbow trout (Barton et al., 1980; Ellis et al, 2004; Ruane et al., 1999), and red sea bream, *Pagrus major* (Biswas et al., 2006). Studies in adult European sea bass have shown that there is a strong positive correlation between cortisol release rate into the water and plasma cortisol concentrations and that cortisol release rate into the water

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($ng\ g^{-1}\ h^{-1}$) can be used as a reliable non-invasive method for the assessment of the stress response (Fanouraki et al., 2008).

Published data in primates, humans and rodents have shown that stress during early development has a profound impact on offspring physiology and behavior with adverse effects on gene expression, physiology, behavior, response to stress, cognitive ability and performance also later in life (Arling and Harlow, 1967; Caldji et al., 1998; Carlson and Earls, 1997; Francis et al., 1999; Glaser, 2000; Harlow et al., 1965; Kalinichev et al., 2002; Lehmann et al., 1999; Liu et al., 1997, 2000; Lovic et al., 2001; Meaney et al., 1985, 1991; Mrdalj et al., 2013; Trickett and McBride-Chang, 1995). However, the effect of early life stress at subsequent stages of development in lower vertebrates with no parental care of offsprings is lacking.

In fish, stress research is focused on the effects of acute or chronic severe noxius stimuli of physical, chemical and husbandry nature applied in juveniles or adult individuals, and there is no information on the effects of early exposure to long term chronic mild stressors on the development and performance of fish at subsequent phases of the life-cycle. In addition, although there are two chronic mild stress protocols developed recently for adult European sea bass (Kollias, personal communication) and zebrafish (Piato et al., 2011) no valid chronic low intensity stress protocol exists for fish at early development.

To this end we developed and evaluated for the first time an unpredictable chronic low intensity stress (UCLIS) protocol, based on everyday aquaculture husbandry practices, applied at three different phases of early ontogeny in European sea bass. The evaluation of the protocol was based on hormonal (water-born cortisol in larvae rearing tanks and plasma cortisol in juveniles) and performance (survival and growth) data during its application period (larvae phase) as well as at subsequent stage of development (juvenile fish).

2. Materials and methods

2.1. Animals and husbandry conditions

Batches of fertilized eggs were obtained from a private fish farm and transferred to the installations of the Institute of Marine Biology, Biotechnology and Aquaculture, HCMR (Heraklion, Crete). Larval rearing was performed applying the pseudogreen-water technique (Papandroulakis et al., 2002), which comprises two phases: (1) the initial phase (lasted 45 days, until formation of all fins), in 500-l cylindro-conical tanks, where both hatching and rearing took place starting with an initial density of 100 eggs l^{-1} and (2) the pre-weaning phase (days 45–60 post-fertilization) in 2000-l cylindro-conical tanks. During the first phase feeding was based on daily supplementation of zooplankton [enriched rotifers (DHASelco, INVE) and Artemia nauplii] and also phytoplankton for a period of 2 weeks. During the second phase feeding was based on Artemia nauplii and the weaning to artificial diets is completed. Tanks were coupled to a biological filter and were initially filled with filtered seawater from a deep well. Water, during embryogenesis, egg hatching and at the autotrophic larval stage, was re-circulated from the bottom of the tank through the biological filter at a rate of 10% h^{-1} and was progressively increased to 70% h^{-1}. Following first feeding the water renewal in the tanks was set to 20% h^{-1} and was gradually increased to 170% at the end of the experimental period. Aeration was provided by means of a wooden diffuser located in the tank center at a rate of 150–200 ml min^{-1}. Larvae were held during the whole experimental period under mean (± SD) water temperature of 18 (± 1.6) °C, dissolved oxygen levels of 7.2 ± 0.8 mg l^{-1}, salinity of 36‰ and pH of 7.9 ± 0.3. During hatching and until mouth opening, tanks were kept in complete darkness while a 12D:12L photoperiod regime (lights on at 08:00) was applied during the rest of the experiment. Following mouth opening and eye development, sea bass larvae were exposed to low light intensity conditions (5–10 lx) in the absence of food for a period of 2 to 4 days to ensure normal swim bladder inflation, while the water surface was also kept free from any oily film by the use of a skimmer. Food was delivered only when an inflated swim bladder was observed in more than 80% of the population. Larvae rearing period lasted for 60 days and then fish were moved into weaning tanks (volume: 10 m^3 each) and kept for about five months under similar husbandry conditions. In particular, the water from a deep well was of constant temperature (19 ± 1 °C) while photoperiod was natural (35°20′N). Feeding was based on artificial diets (INVE SA) that was delivered to satiation with automatic distributors at the beginning and then with demand feeders.

2.2. Experimental design — unpredictable chronic low intensity stress protocol

A chronic low intensity stress protocol was designed and applied for a period of 14 days starting at the beginning of three different early development phases: first feeding (at 9 days post hatch; dph), flexion (at 29 dph) and formation all fins (at 45 dph). Each condition was comprised of two fish groups reared in different tanks. During the same period two more groups of fish were kept undisturbed and served as controls. The stress protocol consisted of optical (increase in light intensity from 60 to 200 lx for 15 min; lights on for 0.5 h during the night; lights off for 0.5 h during the day; exposure to blue or red spectrum for 0.5 h), mechanical (high aeration for 90 s) and social (presence of novel object for 0.5 h) mild stimuli. Two different types of stressors were applied randomly on a daily basis for the total period of 14 days, so that fish were kept under a mild unpredictable chronic stress that minimized the potential for habituation. Full spectrum lights (Phillips, TLD 36 W) were used to approximate natural light and transparent filters to produce the blue (maximum absorption spectrum 450–475 nm) and red (maximum absorption spectrum 620–750 nm) spectrum. The novel objects used were large sized LEGO (5 × 6 cm to 13 × 8 cm) of intense red and green color. Water samples (1 l) were taken at regular intervals (0, 1, 3, 7 and 14 days) from the rearing tanks of the control and experimental groups to evaluate cortisol. During larval rearing and pre-weaning a sample of 10 larvae was taken daily to determine the morphological characteristics and record of total length while 2 times per week weight measurements were also performed with a sample of 10 individuals. At the end of larval rearing (60 dph) the biological performance of each group was evaluated.

At the end of the pre growing phase (about two months after the end of the UCLIS period) and at the end of the experimental period (about five months after the end of the UCLIS period) 20 and 40 fish per group were sampled, respectively, to measure the total length and body weight. In addition, at the end of the experimental period the cortisol stress response was evaluated. Two types of control groups were used; one with minimum handling (addition of a small amount of food in the tank and immediate capture of fish with a net), and another with common handling practice (decrease the water level of the rearing tank, crowding and netting). Then an acute stress challenge was applied, which consisted of crowding (10 min), air exposure (1 min), chasing (5 min), and transfer to 70 l buckets and blood was collected at 1 h post-stress.

All experiments were performed in accordance with relevant guidelines and regulations. The laboratories of the Hellenic Center 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 (3Rs: replacement, 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.

2.3. Plasma cortisol

Free cortisol concentrations were measured using commercial DRG cortisol enzyme immunoassay kits (DRG Diagnostics, Frauenbergstrasse, Germany). All samples were run in duplicate.

2.4. Water cortisol

Water samples (1 l) were peristaltically pumped at circa 10 ml/min through a pre-filter (0.45 μm poresize: AcroCap™, Gelman Sciences, Ann Arbor, MI, USA) and then through an activated solid phase extraction cartridge (Sep-pak® Plus C18, Waters, UK). Cartridges were then stored frozen until assayed. Free corticosteroids were subsequently eluted with 4 ml ethyl acetate. Ethyl acetate was evaporated at 45 °C under nitrogen gas and the residue was re-dissolved in 1 ml of PBS buffer. Free cortisol concentrations were measured using commercial DRG cortisol enzyme immunoassay kits (DRG Diagnostics, Frauenbergstrasse, Germany).

The amount of hormone (H) in ng released over a given time interval (t) in h was calculated according to Ellis et al. (2004), by adapting the equation of Adams and Breck (1990), $H_t = Vkt(C_t - C_0e^{-kt})(1 - e^{-kt})^{-1}$, where V is the water volume (i.e., tank volume minus fish biomass), C_0 and C_t are the hormone concentrations at the beginning and end of the sampling period (over a time interval t) and k is the instantaneous rate of decrease due to dilution from the inflow water. Values for k were derived as R/V, where R is the water inflow rate. The hormone release rate (ng g^{-1} h^{-1}) was then calculated from H_t and fish biomass. The hormone release rate (ng g^{-1} h^{-1}) was subsequently calculated from the differences in the amount of cortisol between sampling points, fish biomass and time, as also described in Fanouraki et al. (2008). Since there is no validated way to measure the exact number of live larvae within the rearing tanks, fish biomass was calculated based on the (a) approximate initial number of incubated eggs, (b) hatching rate, (c) mortality rate, and (d) the mean larvae weight, as estimated by daily measurements of a given sub-sample. The daily number of individuals in each tank was then estimated considering an exponential mortality rate while the estimation of the relevant biomass was made using the above parameters.

2.5. Statistical analysis

All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). Data are presented as means ± standard deviation (SD). For comparison of the growth rates between the different conditions during the larval phase, multiple regression analysis was used. This method was applied for the comparison of both the total length (TL) and the wet weight (WW), following an ln-transform for the second. Statistical comparisons of (i) temporal patterns of water cortisol release rates between the different groups within each respective developmental phase that the stress protocol was applied, (ii) total length and body weight at two and five months after the end of the UCLIS trial, and (iii) plasma cortisol levels between minimum handling, common handling and acute stressed fish were made using one-way ANOVA to assess differences among groups and Tukey's or Dunn's post-hoc tests to assess the level of significance. The significance level used was $P < 0.05$.

3. Results

3.1. Effect of treatment on water-born cortisol concentrations

Water cortisol release rates (Fig. 1) showed statistically significant higher values throughout the experimental period in larvae exposed to UCLIS protocol at the phase of first feeding (STR-FF) compared to the corresponding sampling days of the control group (CON-FF). Although the cortisol release rates for both groups were similar for days 0 to 1 (CON-FF D0–D1 = 5.72 ± 0.24 ng g^{-1} h^{-1}; STR-FF D0–D1 = 8.75 ± 1.4 ng g^{-1} h^{-1}), during days 1 to 3 the stressed group showed peak values ($P < 0.001$; STR-FF D1–D3 = 12.78 ± 0.02 ng g^{-1} h^{-1}), whereas for the control group the values decreased ($P < 0.05$; CON D1–D3 = 3.89 ± 0.5 ng g^{-1} h^{-1}). Then a gradual decrease was observed, however, during days 3 to 7 water cortisol release rates decreased but remained statistically significant higher in the stressed

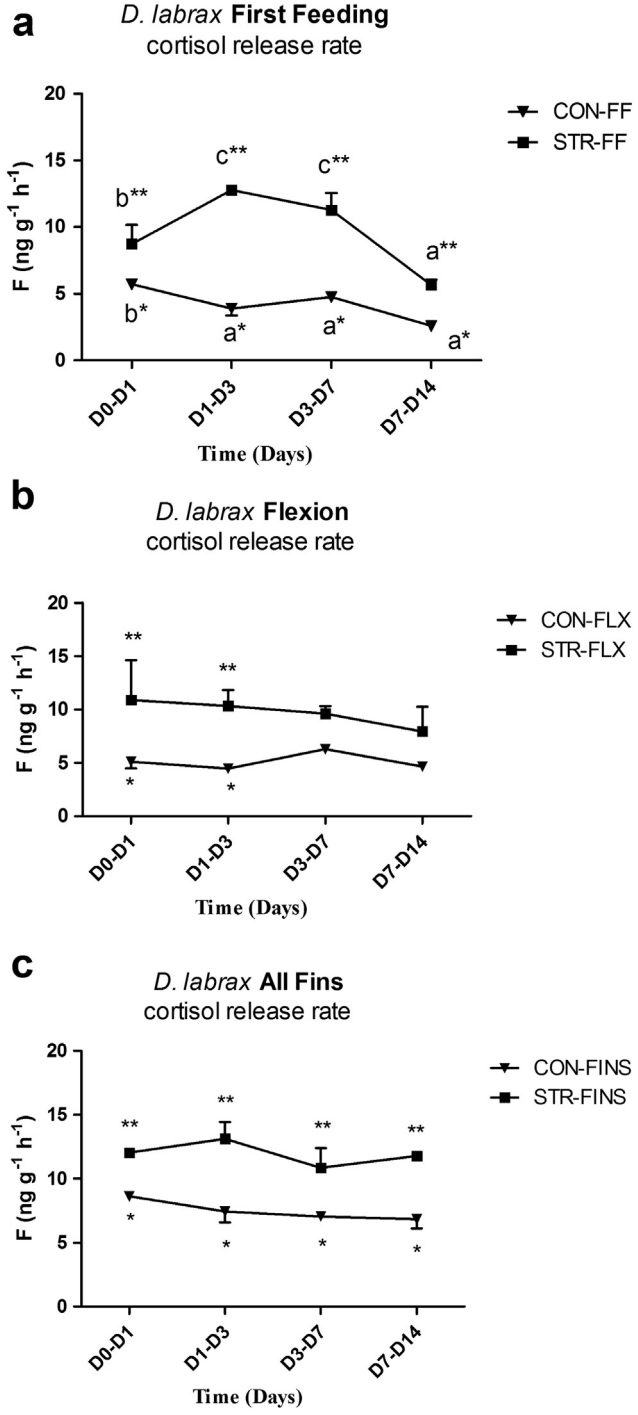

Fig. 1. Water-born cortisol release rates in the holding tanks during early ontogeny of European sea bass. The UCLIS protocol was applied at different periods, i.e. from first feeding to flexion (group STR-FF), from flexion to the formation of all fins (group STR-FLX), and from the formation of all fins to the full cover of body with melanophores (group STR-FINS). One group remained undisturbed and served as the control (CON-FF, CON-FLX, CON-FINS). All conditions were performed in duplicate tanks. Letters indicate differences between days within the same group whereas asterisks indicate differences between the stressed and the control group.

group ($P < 0.001$) than in the controls (CON-FF D3–D7 = 4.77 ± 0.3 ng g^{-1} h^{-1}; STR-FF D3–D7 = 11.3 ± 1.27 ng g^{-1} h^{-1}). The same pattern occurred in the period from days 7 to 14. Higher water cortisol release rates were also observed during the first three days following exposure to UCLIS in larvae at the phase of flexion (STR-FLX D0–D1 = 10.88 ± 3.73 ng g^{-1} h^{-1}; STR-FLX D1–D3 = 10.31 ± 1.50 ng g^{-1} h^{-1}), compared to the controls (CON-FLX D0–D1 =

5.11 ± 0.63 ng g^{-1} h^{-1}; CON-FLX D1–D3 = 4.46 ± 0.36 ng g^{-1} h^{-1}), as well as in larvae that have developed the formation of all fins (STR-FINS D1–D3 = 13.12 ± 1.34 ng g^{-1} h^{-1}; CON-FINS D1–D3 = 7.44 ± 0.85 ng g^{-1} h^{-1}).

3.2. Fish performance

During larval rearing there was no significant difference in terms of survival, with a mean value at the end of the experimental period of 11% in all groups. The multiple linear regression analysis for both total length and wet weight showed that treatment, i.e. the application of the unpredicted chronic low intensity stress protocol (UCLIS), affected significantly the growth rate of larvae at the end of the period that it was applied (until 60 dph) (Fig. 2). In particular, higher growth rate in terms of total length and body weight was observed in the control group (CON) and those exposed to UCLIS for 14 days after the formation of all fins (STR-FINS) compared to those exposed to UCLIS after the onset of first feeding (STR-FF) and flexion (STR-FLX).

In addition, during the pre-growing period and at approximately two months after the end of larval rearing (UCLIS trial period), significant differences ($P < 0.05$) in growth performance of juvenile fish were observed among the experimental groups. In particular, higher mean total length was observed in fish of the STR-FLX (6.7 ± 0.63 cm) and the control (6.5 ± 0.73 cm) groups compared to those of the STR-FF (5.8 ± 0.7 cm) and STR-FINS (5.9 ± 0.58 cm) groups. Higher mean body weight was found in fish of the STR-FLX (3.5 ± 1.05 g) and the controls (2.9 ± 0.95 g) compared to the STR-FF (2.0 ± 0.67 g) and STR-FINS (2.2 ± 1.02 g) groups (Fig. 3a).

Similarly, significant differences ($P < 0.05$) in growth performance among the experimental groups were also observed five months after the end of the UCLIS trial period (juveniles up to the size of approximately 16 cm). In particular, higher mean total length was observed in juveniles of the STR-FLX (13.5 ± 1.26 cm) and the control (12.9 ± 1.15 cm) groups compared to those of the STR-FF (12.1 ± 0.7 cm) and STR-FINS (11.8 ± 0.86 cm) groups, and higher mean body weight was found in fish of the STR-FLX (34.1 ± 10.57 g) and the control (30.0 ± 8.03 g) groups compared to the STR-FF (26.0 ± 4.66 g) and STR-FINS (18.6 ± 4.83 g) groups (Fig. 3b).

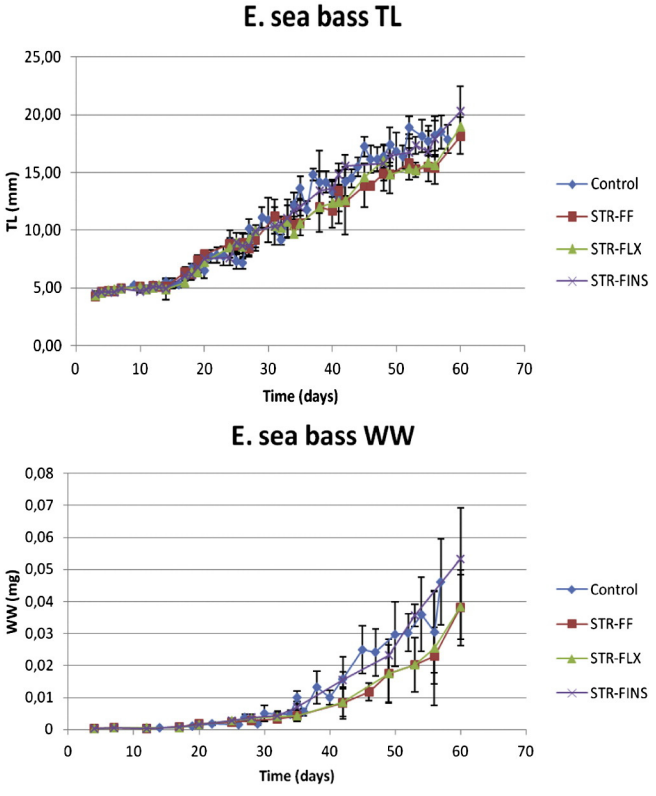

Fig. 2. Growth performance (TL: total length; WW: wet weight) during the period that the unpredicted chronic low intensity stress (UCLIS) protocol was applied (days 5 to 60 post-hatching). Control: control larvae; STR-FF: larvae exposed to UCLIS from first feeding onwards flexion; STR-FLX: from flexion onwards formation of all fins; STR-FINS: from the formation of all fins onwards full cover of body by melanophores. Values are given as mean ± S.E.M. ($n = 10$ per group and sampling point).

3.3. Acute stress response

Handling affected significantly plasma cortisol levels of juveniles (Fig. 4). In particular, minimum mean cortisol concentrations were found, regardless the early life history, in fish that were caught by minimum handling (18.3 to 36.8 ng ml^{-1}), compared to individuals caught by common handling (72.3 to 140.0 ng ml^{-1}) or acute stressed fish (195.1 to 207.9 ng ml^{-1}). In addition, early life stress affected the cortisol response of fish caught by common handling, with higher ($P < 0.001$) plasma cortisol levels in the STR-FF (139.6 ± 27.5 ng ml^{-1}) and the STR-FINS (140 ± 24.9 ng ml^{-1}), compared to the control (72.9 ± 35.6 ng ml^{-1}) and the STR-FLX (81.9 ± 30.7 ng ml^{-1}) groups.

4. Discussion

Fish reared in intensive aquaculture are exposed to various husbandry-related acute stressors of short duration and high intensity like transportation, sorting, handling, confinement, chasing, and air exposure. In addition, common aquaculture practices may include long term chronic stressors and non optimum rearing conditions like unpreferable stocking densities, water quality deterioration, unfavorable social hierarchies, exposure to pathogens which affect the physiological stress response and may lead to severe negative consequences on performance, disease resistance and welfare (Ashley, 2007; Barton and Iwama, 1991; Conte, 2004; Di Marco et al., 2008; Ellis et al., 2002; Mauri et al., 2011; Pickering, 1993; Sammouth et al., 2009; Schreck, 1981; Sumpter, 1993; Wendelaar Bonga, 1997).

Stress has been associated mostly with negative events and consequences, however the recently introduced stress concept of allostasis (McEwen, 2000; McEwen and Wingfield, 2003), used to explain the adaptive progress for actively maintaining stability through change (Sterling and Eyer, 1988), has placed the stress concept into a new context. Organisms in response to a stressor change their allostatic state by altering the activity levels of the primary mediators (e.g. glucocorticosteroids) in order to adjust to both predictable and unpredictable events (McEwen and Wingfield, 2003). 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 fitness (Barton and Iwama, 1991; Schreck, 1981; Wendelaar Bonga, 1997). Cortisol exerts a central role in several physiological actions as adaptation to stressful stimuli, by virtually acting on all tissues and affecting a wide variety of biological responses including control of intermediary metabolism, ionic and osmotic regulation, growth and immune functions (de Jesus and Hirano, 1992; de Jesus et al., 1991; Mommsen et al., 1999; Vazzana et al., 2002). Therefore, cortisol is the most commonly measured indicator of stress in teleosts and usually provides a good reflection of the severity and duration of the stress response (Barton and Iwama, 1991; Fevolden et al., 2002; Wendelaar Bonga, 1997), and the development of non-invasive methods for cortisol measurement are of prime importance. In European sea bass, a species known to be sensitive to husbandry stressors and characterized by high blood cortisol levels (Fanouraki et al., 2011; Rotllant et al., 2003, 2006) there is a strong correlation between plasma cortisol and water-born cortisol in adults (Fanouraki et al., 2008). However, data for the use of water-born cortisol as a reliable non-invasive indicator of stress in larvae are still lacking.

Studies in primates, humans and rodents have shown that stress during early development can alter offspring physiology and behavior

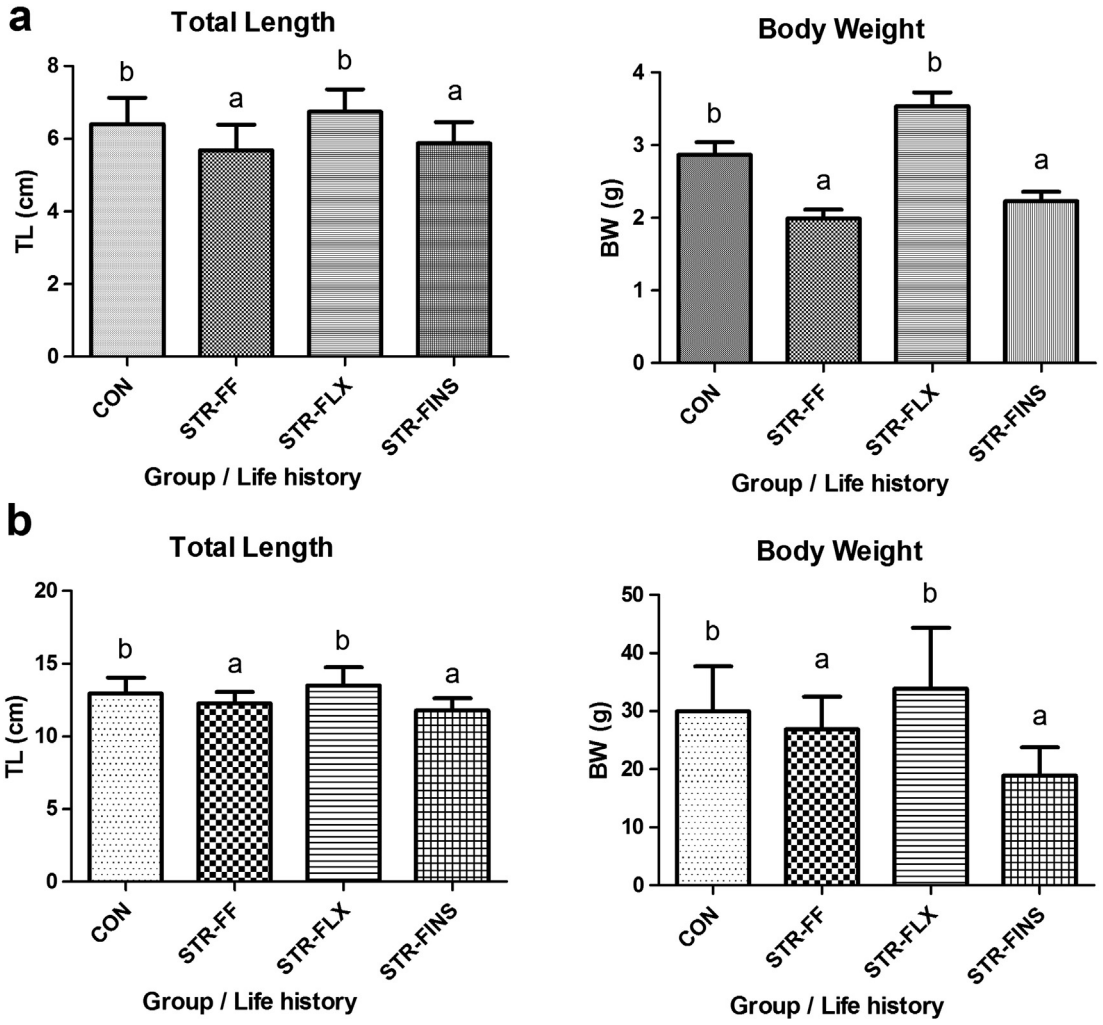

Fig. 3. Growth performance (Total Length, Body Weight) of juvenile European sea bass (a) two months and (b) five months after the end of the UCLIS period. All fish were held under the same weaning and pre-growing rearing conditions. Groups are indicated according to their early larvae history. Box and whiskers (min to max) is the outcome of 20 and 40 measurements per group, for the two and five month fish, respectively.

and affect cognition and social interactions (Arling and Harlow, 1967; Carlson and Earls, 1997; Glaser, 2000; Harlow et al., 1965; Kalinichev, et al., 2002; Lehmann, et al., 1999; Lovic et al., 2001; Trickett and McBride-Chang, 1995). On the contrary, studies in rodents have shown that when the stress applied is kept at low levels this can have a positive effect and lead to a decreased the stress response and improved performance of offspring and enhanced learning and memory (Meaney et al., 1985, 1991).

In order to investigate the effect of early exposure to long term chronic mild stressors on development and performance of fish, an unpredictable chronic low intensity stress (UCLIS) protocol for the early development of European sea bass was developed for the first time. The UCLIS consisted of optical, mechanical and social mild stressors applied randomly on a daily basis for a period of 14 days starting at the beginning of three selected developmental stages (first feeding, flexion, all fins). The UCLIS application resulted in higher water cortisol release

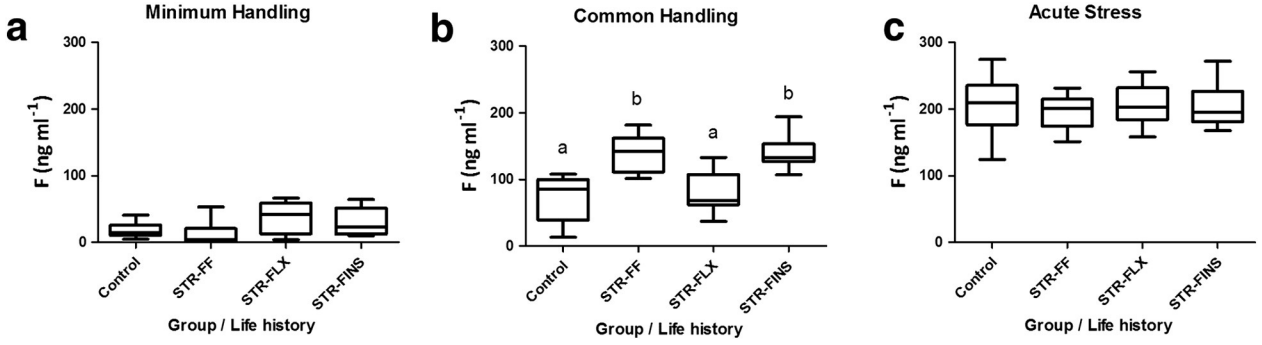

Fig. 4. Plasma cortisol levels ($x \pm$ S.E.M., $n = 20$) of juvenile E. sea bass according to early life stress (CON: control; STR-FF, STR-FLX, STR-FINS: chronic mild stress for 14 days after the beginning of first feeding, flexion and the formation of all fins, respectively) and sampling method (minimum handling, common handling and 1 h post acute stress).

rates (1.5 to 4.3 fold) throughout the experimental period in the rearing tanks of stressed larvae compared to the controls, in all developmental phases applied. Our data demonstrate for the first time that water born cortisol shows good relation between parameters indicative of stressed status of the larvae in the holding tank, therefore, it may be used as a reliable indicator of stress not only in juvenile and adult fish but also during early ontogeny.

During larvae rearing and based on the growth rate, in terms of total length and wet weight, first feeding and flexion stages appeared to be more sensitive to the stimuli applied. However, at about two months after the end of the stress period, the first sampling at the juvenile stage, showed that the UCLIS did also have an impact when it was applied on larvae that were at the stage of all fins. Similarly, during the second sampling, i.e. approximately five months after the end of the stress period, comparison of growth between the different groups clearly showed that fish exposed to UCLIS at the beginning of first feeding and the formation of all fins displayed worst performance than fish exposed to UCLIS at the flexion and the controls. Thus, and based on survival and growth data of both larvae and juvenile fish, the most critical period for exposure to noxius stimuli appeared to be that of first feeding and all fins. In a recent study on European sea bass, based on histological, hormonal (cortisol) and molecular data, it was shown that the first appearance of a distinct and functional hypothalamo–hypophysial–interrenal axis is observed at first feeding, but the stress response becomes fully functional and mature around the stage of all fins (Tsalafouta et al., 2014). This may explain the differential effects of the UCLIS depending on the developmental stage applied. In addition, our data show for the first time that not only juveniles and adults but also European sea bass larvae are sensitive to mild husbandry stimuli with consequences even at subsequent stages of development.

Based on personal observations showing a high sensitivity of European sea bass to ordinary handling practices, we tested two different sampling methods; a common handling practice which includes decrease of the water level of the rearing tank, crowding and netting, and a minimum handling practice by which the fish were immediately caught with a net. Our data, based on plasma cortisol, clearly verified that sea bass juveniles are very sensitive to common handling practices and that this may be one explanation for the huge variability in basal cortisol concentrations reported for this species up to date (Cerdá-Reverter et al., 1998; d'Orbcastel et al., 2010; Fanouraki et al., 2011; Fatira et al., 2014; Filiciotto et al., 2012; Maricchiolo et al., 2008; Marino et al., 2001; Peruzzi et al., 2005; Planas et al., 1990; Rotllant et al., 2003, 2006; Vazzana et al., 2002) and emphasizes the need for the development of new handling procedure for sea bass in order to minimize stress during sampling. Furthermore, whether early life stress has an impact also on the sensitivity to stress at subsequent stages of development, as indicated by the differences observed in cortisol concentrations based on early life history in fish caught by common handling practice, remains to be thoroughly investigated.

In conclusion, European sea bass, which is known to be very susceptible to husbandry stressors and characterized by high blood cortisol levels (Fanouraki et al., 2011; Roche and Bogé, 1996) compared to other species, proved to be sensitive to mild husbandry stressors applied during the larval period, as well as to common sampling practices at the juvenile phase. Common husbandry practices during early development appeared to have an impact not only when applied, but also at subsequent developmental phases, providing the necessity to reconsider common rearing practices. Water cortisol proved to be a promising non-invasive stress indicator during larvae rearing, but a more thorough validation needs to be carried out. This is the first time that an unpredictable chronic low intensity (UCLIS) protocol was developed and applied in the early development of fish. The UCLIS protocol should be evaluated in other fish species, to provide a useful tool to investigate the neuro-endocrine mechanisms implicated in the stress response. It is worth to notice that the offspring of pelagic spawners in contrast to mammals, rodents and many other fish species receive no parental care, providing in this way a very good model to study genomic–environmental stress interactions in early development.

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PAPER III (submitted)

Development and regulation of the α-MSH stress response during early ontogeny in European sea bass, *Dicentrarhus labrax*.

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Abstract

The α-MSH ontogenetic pattern, its response to stress during early ontogeny and the molecular mechanisms involved 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 α-MSH whole body concentrations and the expression of genes involved in the hypothalamic–pituitary–interrenal (HPI) axis activation and regulation were determined. An α-MSH stress response characterized by elevated levels is evident for the first time around the stage of mouth opening showing a specific pattern that becomes established around the formation of all fins. Moreover, mRNA transcript levels of *pomc* and *mc2r* were altered after the acute stress application in a consistent elevated pattern especially as development proceeds, at the stages of flexion and after the formation of all fins, showing at the same time a strong correlation with the whole body αMSH concentrations. The acute stress application had no effect on the expression of *mc1r* but in the case of *mc4r* resulted in an increased transcription even at the stage of mouth opening. Concluding, our data give us for the first time a more thorough view on the importance of the α-MSH stress response, an additional to cortisol pathway regulating stress in teleosts, in the early development of European sea bass.

Keywords: stress, ontogeny, α-MSH, *pomc*, *mc2r, mc1r, mc4r*

1. Introduction

In fish, stress leads to the activation of the hypothalamic-pituitary-interrenal (HPI) axis which stimulates the pituitary's corticotrope cells in the *pars distalis* and the melanotrope cells in the *pars intermedia* to synthesize and secrete pro-opiomelanocortin (POMC)-derived peptides involved in the mediation and regulation of the stress response (Wendelaar Bonga 1997, Slominski *et al.* 2000). The *pomc* gene is mostly expressed in the pituitary gland and is translated into a single protein product, which is the precursor molecule of other neuropeptides, among which, adrenocorticotropic hormone (ACTH) and alpha-melanocyte stimulating hormone (α-MSH) (Smith & Funder 1988). In European sea bass (*Dicentrarhus labrax*) a single form of a functional *pomc* gene has been cloned and characterized (Varsamos, 2003). The melanocortins exert their physiological role by binding to a family of specific G protein-coupled receptors (GPCR) that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R-MC5R), although in teleost fish the number of receptors diverges (Cerdá-Reverter et al., 2011). The melanocortin 2 receptor (MC2R) is specifically activated by ACTH, while the other MCRs can be activated by the MSHs as well as ACTH (Schiöth et al., 2005).

During HPI axis activation, ACTH secreted by the stimulated corticotrope cells of the *pars distalis* is a key regulator of the stress response as it stimulates the interrenal cells via MC2R in order to synthesize and secrete corticosteroids, which then enter into the circulation and are distributed to target tissues (Barton, 2002; Wendelaar Bonga, 1997; Schiöth et al, 2005). Cortisol is the main glucocorticoid and mineralocorticoid and the most commonly used hormonal indicator of stress in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Few studies have focused on the

function and characterization of MC2R in fish (Agulleiro et al., 2010; Klovins et al., 2004; Metz et al., 2005; Aluru and Vijayan, 2008) and a recent study conducted in European sea bass suggested the existence of a negative feedback on MC2R expression that could be involved in long-term stress adaptation (Agulleiro et al., 2013). The interaction of α-MSH and melanocortin 1 receptor (MC1R) plays a key point in the control of the pigmentation and mutations of MC1R are responsible for reduced melanization, whereas the expression of melanocortin 4 receptor (MC4R) is thought to play a role in the regulation of the energy balance in fish through the modulation of feeding behavior (Cerdá-Reverter et al., 2003a, 2003b, 2011; Song and Cone, 2007).

Apart from ACTH, α-MSH is also involved in the stress response in fish (Wendelaar Bonga 1997) and studies in gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) have shown that air exposure induced an increase in α-MSH levels (Arends *et al.* 1999; Sumpter *et al.* 1986). Moreover, studies, carried out in salmonids have shown that ACTH and α-MSH cells are differentially activated. In these studies, HPI axis activation by handling and confinement led to elevated plasma concentrations of ACTH only, but when these stressors were combined with a thermal shock also α-MSH was increased (Sumpter et al., 1985, 1986). Similarly, the latter is supported by other studies in tilapia (*Oreochromis mossambicus*), where although long-term netting had no affect on ACTH concentrations, plasma cortisol levels were elevated, but when netting was combined with confinement both cortisol and ACTH were increased (Balm et al., 1994). Taken together, these results suggest a functional role for α-MSH during stress, but also suggest that α-MSH is not corticotropic.

Recently, the cortisol stress response and its molecular regulation during early ontogeny has been studied in Euorpean sea bass (Pavlidis et al., 2011; Tsalafouta et

al., 2014). However, there are no data in fish species about α-MSH ontogenetic pattern, its response to stress during early ontogeny and the molecular mechanisms involved. To this end, we examined α-MSH temporal patterns at various points, phases and stages of early development in European sea bass and the expression profiles of *pomc*, *mc1r*, *mc2r* amd *mc4r* genes prior and after exposure to acute stressors.

2. Materials and methods

2.1. Ethics statement

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). 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 (3Rs, Replacement, 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.

2.2. 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 pseudogreen-water technique (Papandroulakis et al., 2002), in 500 L cylindro-conical tanks, with an initial density of 100 eggs L^{-1} in which both hatching and rearing took place. A biological filter was coupled to the tanks which

were initially filled with filtered seawater from a deep well. Water, during embryogenesis, egg hatching and at the autotrophic larval stage, was re-circulated from the bottom of the tank through the biological filter at a rate of 10% of the tank volume per h and was progressively increased to 70% of the tank volume per h until the end of the trial. Aeration was provided by means of a wooden diffuser located in the tank center at a rate of 150 - 200 ml min^{-1}. Larvae were held during the whole experimental period under mean (± SD) water temperature of 18 (± 1.6) °C, dissolved oxygen levels of 7.2 ± 0.8 mg l^{-1}, salinity of 36‰ and pH of 7.9 ± 0.3. During hatching and until mouth opening, tanks were kept in complete darkness while a 12D:12L photoperiod regime (lights on at 08:00 h) was applied during the rest of the experiment. Following mouth opening and eye development, sea bass larvae were exposed to low light intensity conditions (5–10 lx) in the absence of food for a period of 2 to 4 days to ensure normal swim bladder inflation, while the water surface was also kept free from any oily film by the use of an air-blower skimmer. Food was delivered only when inflated swim bladder was observed in more than 80% of the population. Exogenous feeding was based on rotifers (*Brachionus* sp.) at 5 individuals ml^{-1} enriched with proteins and PUFA (INVE Aquaculture S.A., Belgium) until 10 days post hatching (dph) while phytoplankton (*Chlorella* sp.) was supplied until 10 dph. Enriched *Artemia* nauplii (Instar II, EG, Artemia Systems S.A., Belgium) were administered from 10 dph until 50 dph at 0.5 to 1.0 individual ml^{-1}. From 30 dph, larvae were offered dry feed (PROTON 2–3, INVE Aquaculture S.A., Belgium) using automatic feeders. The trial lasted until individuals completed the formation of their fins on 45 days post hatch (dph).

2.3. Experimental design

Samples were collected at six different points during early life development (embryos, hatching, mouth opening, first feeding, flexion and formation of all fins), prior to and after the application of an acute stress test. Based on the tolerance of larvae at the particular developmental stages different stressors were applied. In particular, embryos were exposed to transportation for 8 hours at a density of 50 g L^{-1} and then to netting followed by air exposure for 1 min until distribution into the incubation tanks. Stress for pre-larvae (hatching, mouth opening, first feeding) consisted of chasing with a net for 20 sec and high aeration (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 aeration (as above), chasing with a net for 20 sec, confinement (collection in beakers), and air exposure for 5 sec. Samples for molecular (embryos, hatched eggs and larvae samples: n=3 pools of *ca.* 30 mg; juveniles: pools of 1–2 fish) and α-MSH (n=3 pools of *ca.* 250 mg) analysis were collected with a net at 0, 0.5h, 1h, 2h and 24h post-stress, flash frozen in liquid N_2 and stored at -80 ^{o}C.

2.4. α-MSH radioimmunoassay

Whole-body α-MSH concentrations were measured *via* radioimmunoassay. α-MSH was labeled with ^{125}I using the iodogen method (Salacinski *et al.* 1981). Labeled α-MSH was purified by solid phase extraction (C8 Bakerbond column, J.T. Baker, Center Valley, PA, USA). The antiserum shows 100% cross reactivity with des-, mono- and di-acetyl- α-MSH (van Zoest *et al.* 1989), and was used in a final concentration of 1:22,500. The second antibody to precipitate immunocomplexes was a sheep-anti-rabbit anti-body (Fitzgerald, Acton, MA, USA) and was used at a final dilution of 1:15.

2.5. RNA purification and cDNA 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 a Nanodrop® ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany), and its integrity was tested by electrophoresis in 1% agarose gels. Reverse transcription (RT) was carried out using QuantiTect Reverse transcription kit (Qiagen) using 1 μg of total RNA, according to the manufacturer's instructions.

2.6. Primer design for mc2r and pomc genes

Primers for *mc4r* were as described by Sanchez et al. (2009), whereas the reference genes *eukaryotic Elongation Factor 1* (*elf1a*) and *ribosomal 18S RNA* (*18S*) were obtained by a previous work of our group (Kollias et al., 2010). Primer design for *melanocortin 1 receptor* (*mc1r*), *melanocortin 2 receptor* (*mc2r*) and *pro-opiomelanocortin* (*pomc*) was based on the available sequences with accession numbers FN377856.1 (Sanchez et al., 2010), FR870225.1 (Agulleiro et al., 2013) and AY691808.1 (Varsamos et al., 2003), respectively.

Mc1r forward and reverse primer sequences were a 18-mer with the sequence MC1R_Fwd 5′CTCCACCTCATCCTCATC 3′ and a 18-mer with the sequence MC1R_Rev 5′ GAAGCACCAAGAACACAG 3′, respectively. In the case of *mc2r*, the 5′ primer (MC2R_Fwd) was a 20-mer with the sequence 5′ CATCTACGCCTTCCGCATTG 3′ and the 3′ primer (MC2R_Rev) an 18-mer primer with the sequence 5′ ATGAGCACCGCCTCCATT 3′. For *pomc*, 5′ primer

(POMC_Fwd) was a 19-mer with the sequence 5′ TCTCTTCCTCCTCCTCTCC 3′ and the 3′ primer (POMC_Rev) an 18-mer primer with the sequence 5′ TTCGTCCAACAGGCTTCC 3′. The products of each primer pair were further checked with sequencing in order to confirm that they amplify the desired genes.

2.7. Real-time quantitative PCR (qPCR)

Relative gene expression of *mc1r,mc2r, mc4r* and *pomc* was determined with quantitative polymerase chain reaction (qPCR) assays using the *KAPA SYBR® FAST* qPCR Kit (Kapa Biosystems), according to the manufacturer's instructions. The resulting fluorescence was detected with CFX Connect Thermal Cycler (Bio-Rad) under the following cycling parameters: 95 °C for 3 min, 94 °C for 15 sec, 60 °C for 30 sec (for *mc2r and mc4r*)/ 55°C for 30 sec (for *pomc and mc1r*), 72 °C for 20 sec, 40 cycles. Levels of *mc1r, mc2r, mc4r* and *pomc* mRNA were normalized based on the reference genes *18S* and *elf1a*. A standard curve was constructed for each gene, using 4 serial dilutions (1:5) of a pool of all cDNA samples by graphing the negative log of the dilution factor against the relative cycle threshold value. To be considered suitable for analysis, each primer pair was required to have a linear standard curve with an r^2 value above 0.98 and primer amplification efficiency between 90% and 100%.We performed geNORM analysis (Vandesompele et al., 2002) in order to validate the reference genes that served as internal control (M values < 0.5).

2.8 Statistical analysis

All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). Data are presented as means ± standard deviation (SD). Statistical comparisons of α-MSH concentration and gene expression of unstressed specimens (0h) between the different

developmental points/stages and statistical comparisons of temporal patterns of α-MSH concentrations and gene expression between the different time points following exposure to a stressor at each developmental point/stage were made using one-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 $P < 0.05$.

3. Results

3.1 Temporal patterns of α-MSH content and gene expression at early ontogeny

European sea bass embryos had low basal α-MSH content (44.5 ± 13.5 pg g^{-1}) that subsequently increased at first feeding (172 ± 93.5 pg g^{-1}), but the differences were not statistically significant. A significant increase ($P < 0.05$) was observed at flexion (307 ± 101 pg g^{-1}) and remained at higher values onwards to the formation of all fins (408.8 ± 41.1 pg g^{-1}). All genes were expressed in all developmental points examined (Fig. 1). Transcripts of *pomc* showed a decreased mRNA abundance from embryos till first feeding and a statistically significant increase at the stage of flexion ($P < 0.001$) that remained high during all fins. Expression levels of *mc1r* showed no statistically significant differences between the developmental stages examined, whereas expression of *mc2r* showed minimum levels from embryos till mouth opening and peak values were observed at the stage of first feeding ($P < 0.001$), after which expression levels gradually dropped at flexion ($P < 0.05$) and all fins. Expression levels of *mc4r* remained low from the embryo stage until mouth opening showing a statistically significant increase at the stage of first feeding and remained at the same levels till the formation of all the fins ($P < 0.05$).

3.2 Ontogeny of the α-MSH stress response and molecular onset of pomc, mc1r, mc2r and *mc4r*

Figures 2-6 show the α-MSH response and the expression profile of the *pomc*, *mc1r*, *mc2r* and *mc4r* genes prior to (0h) and after (0.5h, 1h, 2h and 24h) the application of a stressor during early ontogeny. There was no statistically significant effect of stress on α-MSH levels at embryos and hatch (Fig. 2). A statistically significant effect of the stressor on α-MSH concentrations was first observed at mouth opening, where α-MSH from basal values at 0h (100 ± 18.4 pg g^{-1}) peaked at 2 h (501.6 ± 94.5 pg g^{-1}) and started to decline at 24h (331.4 ± 113.8 pg g^{-1}) post stress ($P < 0.05$). At first feeding, basal α-MSH values at 0h (172 ± 93.5 pg g^{-1}), showed a statistically significant increase at 0.5h and 1h ($P < 0.05$), reached a peak at 2h (508.7 ± 60.2 pg g^{-1}), to decline at 24h post stress. During flexion, a different pattern of change was observed, with low levels from 0h to 0.5h, minimum at 1h (268.2 ± 46.7 pg g^{-1}), a statistically significant increase at 2h and a peak at 24h (400.5 ± 16.3 pg g^{-1}) ($P < 0.05$). Finally, at the formation of all fins, there was a gradual increase to a maximum at 2h (855.7 ± 84.7 pg g^{-1}) followed by a minimum (190.6 ± 31.9 pg g^{-1}) at 24h post stress ($P < 0.05$).

Transcripts of *pomc* (Fig. 3) showed no statistically significant changes following exposure to the stressors in embryos and hatching. However, at mouth opening there was a 2-fold up-regulation from basal values at 2h ($P < 0.05$). In first feeding, a statistically significant 2.4-fold up-regulation ($P < 0.05$) compared to controls was observed at 0.5h post-stress, which dropped afterwards to the basal values. Similarly, in flexion *pomc* transcripts peaked at 0.5h (1.4-fold up-regulation, $P < 0.001$) and gradually dropped to reach control values at 24h post stress. Finally, at the stage of all

fins a statistically significant maximum (1.8 up-regulation) compared to controls was observed at 1h post stress ($P < 0.05$) that dropped to resting values at 24h post stress. The acute stress application had no effect on *mc1r* at any of the stages applied (Fig. 4). No statistically significant changes following exposure to the stressors were detected for the transcript levels of *mc2r* in embryos, hatched eggs and larvae at the stage of mouth opening (Fig. 5). The first effect of the applied stressors on the mRNA levels of *mc2r* was observed at first feeding where a 2.7-fold down-regulation ($P < 0.001$) compared to controls was observed at 1h and 2h post-stress to reach basal levels at 24h post stress. In flexion, a different pattern to that of first feeding was observed with minimum mRNA abundance at 0h and maximum levels at 0.5h post stress (2-fold up-regulation, $P < 0.001$) to return again to the basal values immediately after. Finally, in all fins, the pattern observed for *mc2r* expression consisted of low basal values at 0h that increased gradually at 0.5h to reach a maximum at 1h post stress (3.8-fold up-regulation, $P < 0.001$). No statistically significant changes where observed after the acute stress application for the transcript levels of *mc4r* in embryos and hatched eggs (Fig. 6). The first effect of the applied stressors on the mRNA levels of *mc4r* was observed in larvae at the stage of mouth opening where a 2.3-fold up-regulation ($P < 0.05$) compared to controls was observed at 1h post-stress to reach basal levels at 24h post stress. In first feeding, the acute stress had no effect on the levels of *mc4r*. However, the elevated pattern of *mc4r* expression appeared again at the stage of flexion with peak values (1.5-fold up-regulation, $P < 0.05$) at 0.5h post stress and the same pattern remained until the stage of the full formation of all the fins where a peak in the transcript levels appeared at 1h post stress (1.7-fold up-regulation, $P < 0.05$) to gradually reach basal values at 24h post stress.

4. Discussion

During ontogenesis, the temporal changes of whole-body α-MSH levels of sea bass showed a gradual increase from low levels during the first stages to maximum values at the stages of flexion and development of all fins. There are very few data available on α-MSH during early ontogeny apart from a study carried out during the early developmental stages in scyliorhinid dogfish (*Scyliorhinus torazame*) that showed a gradual increase of the MSH-producing cells in the adenohypophysis (Chiba and Oka, 2002). The observed increase in whole-body α-MSH concentrations at the advanced stages of early development may reflect the involvement of α-MSH in the formation of melanophores and the coloring of the body which consists with the period around the formation of the fins, as α-MSH is also involved in the control of skin pigmentation and melanophore formation (Eberle, 1988; Fujii and Oshima 1986, 1994; Lu et al, 1998). Expression of *pomc* increases at the stage of flexion and its peak is in line with the first statistically significant elevation of α-MSH levels. *Mc2r* abundance remains at low levels until the stage of first feeding where it reaches a maximum and then decreases gradually at the later stages of development. Previous studies of our group have shown that sea bass larvae begin to synthesize cortisol around the stage of first feeding (Pavlidis et al., 2011; Tsalafouta et al, 2014), which coincides with the expression profile observed for *mc2r*. Similar results have been obtained in zebrafish, where the expression of *mc2r* is upregulated immediately before the rise in whole-body larvae cortisol concentrations (Alsop and Vijayan, 2008). *Mc1r* expression was not altered depending on the developmental stage, whereas expression levels of *mc4r* appeared low from the embryo stage until mouth opening showing an increase at the stage of first feeding and remained at similar levels thereafter till the stage of the full formation of all fins.

The acute stress challenge tests did not have any effect on α-MSH levels in embryos and hatch stages. The first α-MSH response is observed at the stage of mouth opening with peak values at 2h post stress. Similarly, at the stage of first feeding α-MSH concentrations start to rise at 0.5h post stress to reach maximum values at 2h. At the stage of flexion the response is characterized by elevated α-MSH levels at 2h to reach a peak at 24h after application of the stressor, indicating a prolonged response compared to the cortisol stress response where peak values were reported to occur at 2h post stress (Tsalafouta et al, 2014). As development proceeds, the pattern of the α-MSH response to stress seems to become established until the stage of all fins where the magnitude of the response is higher, showing a gradual increase of α-MSH levels to peak values at 2h which fall to resting values at 24h post stress. These results are supported by other studies in adult gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*), which have shown that the application of acute stress led to an increase of α-MSH levels (Arends *et al.* 1999; Sumpter *et al.* 1986).

α-MSH is a POMC-derived peptide, so in order to reveal the molecular mechanisms related to the onset of the α-MSH stress response, qPCR experiments were carried out in order to analyze transcript levels of *pomc*. Expression levels of *pomc* after the acute stress application appear to be altered for the first time at the stage of mouth opening, where its abundance increases and shows maximum values at 1h post stress. This pattern continues in the later stages of development, where mRNA expression of *pomc* is upregulated along with α-MSH levels, indicating a strong relation between *pomc* mRNA expression and α-MSH production. At the stages of first feeding and flexion, peak values for *pomc* are observed at 0.5h to return afterwards to resting levels, whereas at the stage of all fins the pattern of *pomc* stress response is characterized by maximum values at 2h post stress that gradually drop to resting

levels at 24h. These results are in accordance with the results obtained from a study in adult channel catfish, where an up-regulation of *pomc* mRNA was observed after water was drained at the fish eye-level, i.e. crowding stress (Karsi et al., 2005).

The melanocortins exert their physiological role by binding to melanocortin receptors (MC1R-MC5R). MC2R is specifically activated by ACTH, while the other MCRs can be activated by the MSHs as well as ACTH (Schiöth et al., 2005). In the present study, the acute stress application had no statistically significant effect on *mc2r* mRNA levels in embryos and pre-larvae at hatching and larvae at mouth opening. However, at first feeding a down-regulation of the receptor was observed with minimum values at 1h and 2h post stress, which at 24h return to control (0h) levels. This down-regulation of *mc2r* after stress at first feeding had no effect on the up-regulation of cortisol which showed peak values at 0.5h post stress (Tsalafouta et al., 2014). During stages of flexion and all fins a different pattern was observed, where transcript levels of *mc2r* were up-regulated after application of the stressor, with peak values at 0.5h and 1h post stress, respectively, showing similar patterns with the observed cortisol patterns obtained under the same conditions (Tsalafouta et al., 2014). The different pattern obtained for *mc2r* at the stage of first feeding might reflect the differences in the magnitude and duration of the cortisol response which is evident as development proceeds. The observed up-regulation of *mc2r* after stress at the stages of flexion and development of all fins is in accordance with the data obtained in a study conducted in rainbow trout where application of an acute stressor led to increased levels of *mc2r* transcripts (Aluru and Vijayan, 2008). Up-regulation of *mc2r* after acute stress is further supported by a study by Tokarz and colleagues (2013) in zebrafish, where the expression level of *mc2r* increased significantly until about 30 min after the stressor and subsequently decreased to the mRNA levels of

unstressed fish (Tokarz et al., 2013). Both *mc1r* and *mc4r* recognize α-MSH and are involved in the control of the pigmentation and the modulation of food intake, respectively (Cerdá-Reverter et al., 2003a, 2003b, 2011; Song and Cone, 2007). *Mc1r* expression was not altered following application of the stressor at any of the stages examined, whereas *mc4r* expression appeared to be affected by stress even as early as at the stage of mouth opening showing peak values at 1h post stress, which coincides with the first α-MSH response to stress observed. In first feeding, the acute stress had no effect on the levels of *mc4r*. However, the elevated pattern of *mc4r* expression appeared again at the stage of flexion with peak values at 0.5h post stress and the same pattern remained until the stage of the full formation of all the fins where transcript levels peaked at 1h post stress. The inability to respond to the stress applied at the stage of first feeding via *mc4r*, might reflect the role of *mc4r* in the modulation of the feeding behavior in E. sea bass even at early development as the survival of the larvae at this crucial step of the transition from the autotrophic state to exogenous feeding is of critical importance.

In summary, we characterized for the first time in a Mediterranean marine teleost, the European sea bass (*Dicentrarchus labrax*), the temporal pattern of whole body α-MSH and the expression profile of *pomc*, *mc1r*, *mc2r* and *mc4r* genes during ealy ontogeny. Additionally, sea bass embryos, pre-larvae and larvae were exposed to acute stressors and the temporal patterns of whole body α-MSH and the expression of *pomc*, *mc1r*, *mc2r* and *mc4r* genes prior to and 0.5h, 1h, 2h, and 24h after application of the stressor, were determined. Overall, these data, combined with data on the cortisol response during early ontogeny (Pavlidis et al., 2011; Tsalafouta et al., 2014) give us for the first time a more thorough view on the two mechanisms involved in the stress response in E. sea bass with similar patterns observed for α-MSH and cortisol.

α-MSH is a truly pleiotropic hormone, with, among others, effects on skin coloration, feed intake and metabolism. To what extent α-MSH contributes to each of these processes separately in early development remains to be determined, but our results indicate that the α-MSH stress response is paramount in early development of early vertebrates on earth, the teleostean fishes.

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Figure Legends

Fig. 1. Temporal patterns of α-MSH content and gene expression at early ontogeny of European sea bass. Changes in resting whole body α-MSH and mRNA transcript levels of *pomc* and *mc2r* at 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 ($P < 0.05$).

Fig. 2. The α-MSH stress response during early ontogeny. European sea bass embryos, pre-larvae and larvae at specific points/stages of development were exposed to acute stressors and the whole body α-MSH content was analyzed prior to (0h) and after (0.5h, 1h, 2h and 24h) the application of the stressor. Values are means ± standard error (n=3). Means with different letters differ significantly from one another ($P < 0.05$).

Fig. 3. Molecular onset of *pomc* in response to stress. Expression profile of *pomc* prior to (0h) and after (0.5h, 1h, 2h and 24h) the application of the stressor at the different early 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 ($P < 0.05$).

Fig. 4. Molecular onset of *mc2r* in response to stress. Expression profile of *mc2r* prior to (0h) and after (0.5h, 1h, 2h and 24h) the application of the stressor at the different early 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 ($P < 0.05$).