Cortisol dynamics in European sea bass, *Dicentrarchus labrax* L.: factors inducing variability and its molecular and endocrine regulation

PhD Thesis

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ΕΥΧΑΡΙΣΤΙΕΣ

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Abstract

The aim of the present study was to get a better insight into environmental, husbandry, and genetic factors that affect cortisol regulation and variability, as well as control the cortisol stress response in a Mediterranean marine teleost, the European sea bass, *Dicentrarchus labrax*.

In order to examine how the environment can modify cortisol regulation, the effects of water temperature on cortisol dynamics so before stress as after exposure to acute stress were studied. Three water temperatures were examined, *i.e.* 15, 20, and 25°C, which reflect the range of temperature fluctuation in Mediterranean aquaculture. Fish were acclimated at the respective temperature for 2 weeks before sampling. Blood and water samples were collected prior and at 0.5, 1, 2, 4, and 8 hours post-stress for cortisol analysis. Results showed that water temperature affected the resting and post-stress levels of cortisol. Specifically, higher resting concentrations were observed at 20°C and 25°C than at 15°C. In terms of response, cortisol in all temperatures examined responded with higher post-stress levels. However, the rapidity of the response was greater at the higher temperatures, in both terms of time to peak and to recover, while at the lowest temperature the response was delayed and prolonged. Additionally, the overall outcome of the response, as indicated by the AUC, was greater at 15°C. The release of cortisol in the water was also affected by temperature, being slower and of lower intensity at the low temperature examined. In conclusion, differences in the pattern of response between temperatures for E. sea bass could have resulted from temperature-derived differences in cortisol synthesis and/or clearance rates, but also due to differences in the rate of cortisol release in the water.

A repeated predictable stress protocol of various intensities was developed to investigate how husbandry stress of increasing intensity can exert different allostatic loads on fish and how this could affect cortisol dynamics and peripheral regulation in tissues like liver and head kidney. Fish were left undisturbed (controls) or exposed to three levels of repeated predictable stress for three weeks and then subjected to an additional acute stress test. The stress protocol used a combination of common husbandry practices, such as chasing with a net, confinement and air-exposure, and was categorized as low, medium and high according to its intensity. Specifically, low stress consisted of subjecting fish to confinement at 50 % of the tank for 30 min every 2nd day; medium stress consisted of both confinement (conducted as previously described) and chasing for 5 min with a net every 2nd day; high stress consisted of confining fish at 25% of the tank for 30 min, and chasing for 5 min every 2nd day, coupled with a 1-min

air-exposure stressor once per week. Two days after the end of the application of this protocol, 10 fish per tank were immediately sampled (T0), while the remaining 10 fish were acutely stressed by chasing them for 5 min and exposing them to air for 1 min, and sampled 1 hour later. Results showed that body weight gain was significantly reduced as stress load increased, leading eventually to body weight loss in the high stress group. Feeding was also reduced in all stress groups compared to controls. In terms of cortisol, fish exposed to high stress exhibited high basal cortisol levels and an inability to further respond to acute stress. At the molecular level, upregulation of the expression of adrenocorticotropic hormone (ACTH) receptor, *mc2r*, and of cortisol biosynthesis enzyme *11β-hydroxylase* was observed in the head kidney of all stress load groups compared to controls. Additionally, in the high load group the dysregulation of the balance between the expression of glucocorticoid, *gr*, and mineralocorticoid, *mr*. receptors, as well as the lower hepatic gene expression of *11β-hsd2*, an enzyme that inactivates cortisol, possible indicate the basis behind the high cortisol levels seen in this group.

Genetic background along with the environment is well known to affect the phenotype of individuals. For that reason, it was aimed to assess how the genetic background can influence the variability of cortisol response at both family and individual level and subsequently explore the hepatic transcriptome profile of fish showing consistently low (LR) or high (HR) cortisol responsiveness. The progeny (full sibs) of six families was used, and sampled for plasma cortisol after an acute stress challenge once per month, for four consecutive months. Results suggested that cortisol response was affected by the genetic background, as seen by the family-based differences, and that individual responsiveness was a repeatable trait. Subsequently, LR and HR fish were identified, and showed low or high resting, free and post-stress cortisol concentrations, respectively. These differences could not be explained by differences in the plasma ACTH concentrations. Finally, the liver transcription profiles of LR and HR fish showed some important differences, indicating differential hepatic regulation between these divergent phenotypes. These transcription differences were related to various metabolic and immunological processes, with 169 transcripts being transcribed exclusively in LR and 161 in HR fish.

Mechanisms regulating different cortisol responsiveness between LR and HR individuals have been poorly studied. In this context, it was aimed to study these mechanisms at the level of the head kidney in LR and HR fish of E. sea bass. To do so, initially resting plasma cortisol and ACTH concentrations were estimated in LR and HR approximately 1.5 years after their characterization as such. The head kidneys of these individuals were superfused through an *in vitro* superfusion system, and stimulated with the same dose of ACTH to assess their cortisol

biosynthetic capacity. Moreover, the expression of important for cortisol regulation genes was estimated in the head kidneys. Results showed that LR and HR fish differed in the resting cortisol levels, although no differences existed in the circulating levels of ACTH. Additionally, the biosynthetic capacity of HR was higher than that of LR fish when *in vitro* stimulated with the same concentration of ACTH. At the molecular level, differences in resting cortisol between LR and HR fish could be attributed to a higher expression of the ACTH receptor, *mc2r*, and the 2.3-fold higher expression of *11β-hydroxylase*, an enzyme involved in cortisol biosynthesis in the HR fish. Finally, a significant downregulation of *11β-hsd2*, an enzyme involved in cortisol inactivation was observed in HR when compared to LR fish, indicating for the first time that post-production regulation of cortisol in the head kidney can also explain the differences observed between these divergent phenotypes.

Περίληψη

Σκοπός της παρούσας διδακτορικής διατριβής ήταν η μελέτη περιβαλλοντικών, διαχειριστικών και γενετικών παραγόντων που μπορούν να επηρεάσουν τη ρύθμιση και τη μεταβλητότητα της κορτιζόλης, καθώς επίσης και την απόκριση αυτής στην καταπόνηση σε ένα είδος της Μεσογειακής ιχθυοκαλλιέργειας, το λαβράκι, *Dicentrarchus labrax.*

Για το σκοπό αυτό, αρχικά μελετήθηκε η επίδραση της περιβαλλοντικής θερμοκρασίας νερού στα επίπεδα κορτιζόλης, τόσο πριν όσο και μετά την έκθεση σε οξεία καταπόνηση. Ελέγχθηκαν τρεις περιβαλλοντικές θερμοκρασίες, συγκεκριμένα 15, 20, και 25°C, οι οποίες αντικατοπτρίζουν το εύρος θερμοκρασιών που συνήθως συναντάται στην Μεσογειακή ιχθυοκαλλιέργεια. Τα ψάρια εγκλιματίστηκαν σε αυτές τις θερμοκρασίες για δύο εβδομάδες πριν τη δειγματοληψία. Συλλέχθηκαν δείγματα αίματος και νερού για τον προσδιορισμό της κορτιζόλης τόσο πριν όσο και 0.5, 1, 2, 4 και 8 ώρες μετά την έκθεση σε οξεία καταπόνηση. Τα αποτελέσματα έδειξαν ότι η θερμοκρασία του νερού επιδρά στις συγκεντρώσεις κορτιζόλης, τόσο σε συνθήκες ηρεμίας όσο και μετά την έκθεση σε οξεία καταπόνηση. Συγκεκριμένα, υψηλότερες συγκεντρώσεις κορτιζόλης σε συνθήκες ηρεμίας παρατηρήθηκαν στους 20°C και 25°C συγκριτικά με τους 15°C. Όσον αφορά στην απόκριση στην καταπόνηση, παρατηρήθηκε απόκριση με αύξηση των συγκεντρώσεων κορτιζόλης σε όλες τις θερμοκρασίες που ελέγχθηκαν. Παρ' όλα αυτά, η ταχύτητα της απόκρισης, εκφρασμένη ως ο χρόνος που χρειάζεται για να επιτευχθούν τόσο οι μέγιστες συγκεντρώσεις όσο και η επαναφορά σε φυσιολογικά επίπεδα, ήταν μεγαλύτερη στις υψηλότερες θερμοκρασίες, ενώ αντίθετα η απόκριση στη χαμηλότερη θερμοκρασία ήταν πιο αργή και παρατεταμένη. Επιπλέον, η συνολική απόκριση, όπως αποτυπώθηκε από την Περιοχή Κάτω από την Καμπύλη (AUC), ήταν μεγαλύτερη στους 15°C. Ο ρυθμός απελευθέρωσης κορτιζόλης στο νερό επίσης επηρεάστηκε από τη θερμοκρασία και συγκεκριμένα ήταν πιο αργός και χαμηλότερης έντασης στη χαμηλή θερμοκρασία. Συμπερασματικά, οι διαφορές που παρατηρήθηκαν στο πρότυπο της απόκρισης μεταξύ των διαφορετικών θερμοκρασιών που ελέγχθηκαν θα μπορούσαν να έχουν προκύψει από διαφορές στους ρυθμούς σύνθεσης ή καταβολισμού της κορτιζόλης, αλλά και από διαφορές στους ρυθμούς απελευθέρωσης της κορτιζόλης στο νερό.

Για τη διερεύνηση του κατά πόσο και με ποιο τρόπο οι διαχειριστικές πρακτικές που προξενούν καταπόνηση μπορούν να προκαλέσουν διαφορετικά αλλοστατικά φορτία στα ψάρια και πως αυτό εν συνεχεία μπορεί να επηρεάσει τη ρύθμιση της κορτιζόλης, αναπτύχθηκε και εφαρμόστηκε ένα πρωτόκολλο επαναλαμβανόμενης προβλέψιμης καταπόνησης

διαφορετικής έντασης. Συγκεκριμένα, χωρίστηκαν τέσσερεις ομάδες ψαριών, μία εκ των οποίων δεν υπέστη κανένα χειρισμό (ομάδα ελέγχου), ενώ οι άλλες τρεις εκτέθηκαν σε επαναλαμβανόμενη προβλέψιμη καταπόνηση διαφορετικής έντασης για τρεις εβδομάδες. Το πρωτόκολλο καταπόνησης που αναπτύχθηκε προσομοίαζε συχνές διαχειριστικές πρακτικές όπως κυνηγητό με την απόχη, περιορισμός του διαθέσιμου χώρου και έκθεση στον αέρα και κατηγοριοποιήθηκε ως χαμηλής, μέτριας και υψηλής έντασης. Συγκεκριμένα, η χαμηλής έντασης καταπόνηση περιελάμβανε τη μείωση του διαθέσιμου χώρου της δεξαμενής στο 50 % για 30 λεπτά κάθε δεύτερη μέρα, ενώ η μέτριας έντασης στον περιορισμό των ψαριών με τον ίδιο τρόπο, συνοδευόμενη επιπλέον από 5 λεπτά κυνηγητού με την απόχη. Τέλος, η υψηλής έντασης καταπόνηση αφορούσε στον περιορισμό των ψαριών στο 25 % της δεξαμενής για 30 λεπτά και κυνηγητό με την απόχη για 5 λεπτά κάθε δύο μέρες, ενώ επιπλέον γινόταν και έκθεση των ψαριών στον αέρα για 1 λεπτό, κάθε 7 μέρες. Μετά το πέρας της εφαρμογής του πρωτοκόλλου για το αλλοστατικό φορτίο συλλέχθηκε αίμα από 10 ψάρια από κάθε δεξαμενή χωρίς να τους γίνει οποιοσδήποτε άλλος χειρισμός (T0), ενώ στο υπόλοιπα 10 ψάρια έλαβε χώρα ένα επιπλέον συμβάν οξείας καταπόνησης, κυνηγώντας τα για 5 λεπτά με την απόχη και εκθέτοντάς τα στον αέρα για 1 λεπτό (T1). Τα αποτελέσματα έδειξαν ότι το σωματικό βάρος μετά την έκθεση στο πρωτόκολλο καταπόνησης ήταν αντιστρόφως ανάλογο της ένταση του πρωτοκόλλου, οδηγώντας ακόμα και σε μείωση βάρους, σε σχέση με το αρχικό, στα ψάρια που εκτέθηκαν στην υψηλή καταπόνηση. Επιπλέον, τα ψάρια που εκτέθηκαν στο πρωτόκολλο υψηλής έντασης εμφάνισαν υψηλά επίπεδα κορτιζόλης στο πλάσμα και αδυναμία περαιτέρω απόκρισης μετά την έκθεση σε ένα επιπλέον συμβάν οξείας καταπόνησης. Σε μοριακό επίπεδο, παρατηρήθηκαν αυξημένα επίπεδα γονιδιακής έκφρασης του υποδοχέα της αδρενοκορτικοτρόπου ορμόνης ACTH, *mc2r*, και του ενζύμου 11β-υδροξυλάση, που συμμετέχει στη βιοσύνθεση της κορτιζόλης, στο πρόσθιο τμήμα του νεφρού ψαριών που εκτέθηκαν σε καταπόνηση σε σχέση με την ομάδα ελέγχου. Επιπλέον, στην ομάδα υψηλής καταπόνησης παρατηρήθηκε απορρύθμιση της ισορροπίας μεταξύ της έκφρασης των γλυκοκορτικοειδών (*gr*) και του αλατοκορτικοειδούς υποδοχέων στον υποθάλαμο του εγκεφάλου, καθώς επίσης και μειωμένη ηπατική έκφραση του ενζύμου 11β-υδροξυστεροειδής δεϋδρογονάση τύπου 2 (*11β-hsd2*), που δρα αδρανοποιώντας την κορτιζόλη, υποδεικνύοντας έτσι πιθανούς λόγους που οδήγησαν στα αυξημένα επίπεδα κορτιζόλης στα ψαριών αυτά.

Είναι ευρέως γνωστό ότι το γενετικό υπόβαθρο μαζί με το περιβάλλον διαμορφώνουν το φαινότυπο ενός ατόμου. Για το σκοπό αυτό επιχειρήθηκε να εκτιμηθεί κατά πόσο το γενετικό υπόβαθρο μπορεί να επηρεάσει την παρατηρούμενη μεταβλητότητα στα επίπεδα κορτιζόλης του λαβρακιού, τόσο σε επίπεδο οικογενειών όσο και ατόμων. Επιπλέον, πραγματοποιήθηκε

διερεύνηση του ηπατικού μεταγραφικού προτύπου σε άτομα που δείχνουν σταθερά χαμηλή (LR) ή υψηλή (HR) απόκριση κορτιζόλης. Για το σκοπό αυτό χρησιμοποιήθηκαν οι απόγονοι 6 οικογενειών λαβρακιού, οι οποίοι εκτίθονταν σε οξεία καταπόνηση μία φορά το μήνα και για τέσσερεις συνεχόμενους μήνες με σκοπό να εκτιμηθούν τα επίπεδα κορτιζόλης στο αίμα τους μετά την καταπόνηση. Τα αποτελέσματα έδειξαν ότι η απόκριση της κορτιζόλης επηρεάζεται σημαντικά από το γενετικό υπόβαθρο, όπως αποτυπώθηκε από τις διαφορές μεταξύ των οικογενειών, καθώς επίσης ότι η ατομική απόκριση είναι ένα χαρακτηριστικό με έντονη επαναληψιμότητα και σταθερότητα. Εν συνεχεία, άτομα χαμηλής (LR) και υψηλής (HR) απόκρισης αναγνωρίστηκαν, τα οποία διέφεραν, εκτός από τις συγκεντρώσεις μετά την καταπόνηση, τόσο στα επίπεδα ηρεμίας, δηλαδή απουσίας καταπόνησης, όσο και στα επίπεδα της ελεύθερης, μη δεσμευμένης σε πρωτεΐνες, κορτιζόλης. Οι διαφορές αυτές δεν μπορούσαν να αποδοθούν σε διαφορές στις συγκεντρώσεις της ACTH στο πλάσμα. Τέλος, παρατηρήθηκαν διαφορές στα πρότυπα της ηπατικής γονιδιακής έκφρασης μεταξύ των LR και HR ατόμων οι οποίες υποδεικνύουν διαφορετική ηπατική ρύθμιση μεταξύ των δύο αυτών φαινοτύπων. Οι μεταγραφικές διαφορές σχετίζονταν με διάφορες μεταβολικές και ανοσολογικές διεργασίες, με 169 μετάγραφα να εκφράζονται μόνο στο LR και 161 μόνο στα HR άτομα.

Οι μηχανισμοί που ρυθμίζουν τη διαφορετική απόκριση της κορτιζόλης στην καταπόνηση μεταξύ LR και HR ατόμων δεν έχουν μελετηθεί επαρκώς. Στο πλαίσιο αυτό, επιχειρήθηκε να μελετηθεί ο μηχανισμός ρύθμισης των αποκλινόντων αποκρίσεων κορτιζόλης μεταξύ LR και HR ατόμων στο επίπεδο του πρόσθιου τμήματος του νεφρού. Για το λόγο αυτό, αρχικά εκτιμήθηκαν οι συγκεντρώσεις κορτιζόλης και ACTH σε συνθήκες ηρεμίας σε LR και HR άτομα, 1.5 χρόνο μετά τον χαρακτηρισμό τους ώστε να φανεί αν αυτό το χαρακτηριστικό διατηρείται στο χρόνο. Εν συνεχεία, δείγματα από το πρόσθιο τμήμα του νεφρού αυτών των ατόμων διεγέρθηκαν με ACTH σε ένα σύστημα *in vitro* επώασης και έγχυσης (superfusion) ώστε να ποσοτικοποιηθεί η βιοσυνθετική τους ικανότητα για παραγωγή κορτιζόλης. Επιπλέον, μελετήθηκε η έκφραση γονιδίων που συμμετέχουν στη ρύθμιση της σύνθεσης κορτιζόλης από το πρόσθιο τμήμα του νεφρού. Τα αποτελέσματα έδειξαν ότι τα LR και HR άτομα διέφεραν στις συγκεντρώσεις κορτιζόλης ηρεμίας ακόμα και 1.5 μετά τον χαρακτηρισμό τους, ενώ δεν υπήρχαν διαφορές στα επίπεδα της ACTH στο πλάσμα. Επιπλέον, η βιοσυνθετική ικανότητα των HR ήταν υψηλότερη από αυτή των LR ατόμων κατά την *in vitro* διέγερσή τους με την ίδια συγκέντρωση ACTH. Στο μοριακό επίπεδο, οι διαφορές στην κορτιζόλη ηρεμίας μεταξύ LR και HR ατόμων θα μπορούσαν να αποδοθούν στην υψηλότερη έκφραση του υποδοχέα της ACTH, *mc2r*, και την 2,3-φορές υψηλότερη, αν και όχι στατιστικά

σημαντική, έκφραση της 11β-υδροξυλάσης που συμμετέχει στη βιοσύνθεση της κορτιζόλης στα HR άτομα. Τέλος, παρατηρήθηκε μία σημαντική υπό-έκφραση της 11β-υδροξυστεροειδούς δεΰδρογονάσης στα LR άτομα, υποδεικνύοντας έτσι για πρώτη φορά ότι τα επίπεδα της κορτιζόλης μπορεί να ρυθμίζονται στο πρόσθιο τμήμα του νεφρού ακόμα και αμέσως μετά την παραγωγή της ορμόνης εξηγώντας έτσι μέρος της διαφοράς στα επίπεδα της ορμόνης μεταξύ των αποκλινόντων αυτών φαινοτύπων.

Chapter I. General Introduction

1.1. Aquaculture and welfare

The production of fish through aquaculture is an on-growing activity, although at a slowing rate over the last few years (FAO, 2014). The majority of the global aquaculture fish production comes from Asia (88.39% of the total production in tons), followed by the Americas (4.78%) and Europe (4.32%) (data for 2012 as published by FAO, 2014). Although the Mediterranean aquaculture represents a small fraction of the global aquaculture production, it is of major importance for European aquaculture industry. Production forecasts show that by 2030 Mediterranean aquaculture will have shown the highest rate of increase in both tons of production and financial income in Europe (EATiP, 2012). This increase will take place by the expansion and increase of farming sites, increased stocking densities, diversification of the species cultured, better genetic selection of the broodstock, enhanced control over the feed conversion ratio (FCR%) and disease outbreaks. All the above target towards increased production, at the same time, however, it should also be aimed to study how fish will be able to successfully cope to these new husbandry conditions and how their welfare will subsequently be affected in order to achieve an integrated aquaculture plan.

Animal welfare is a term that has relatively recently gained popularity. Although there is no wide acceptable definition for this term, it generally represents the physical and spiritual state of an animal in terms of health, happiness and longevity (Duncan & Fraser, 1997). However, most of the definitions given fall into one of the following three broad categories:

Feelings-based definitions, which are set in terms of subjective mental states. Here, the requirement for good welfare is that the animal should feel well, being free from negative experiences such as pain or fear and having access to positive experiences, such as companionship in the case of social species.

Function-based definitions, which centre on an animal's ability to adapt to its present environment. Good welfare requires that the animal is in good health with its biological systems, and particularly those involved in coping with challenges, functioning appropriately and not being forced to respond beyond their capacity.

Nature-based definitions arise from the view that each animal species has an inherent biological nature that it must express. Good welfare, therefore, requires that the animal is able to lead a natural life and express its natural behaviour.

Many human activities have been suggested to impair fish welfare, including fisheries, aquaculture, sports fishing, scientific research or petting (Huntingford et al., 2006). Although these activities can be perceived as stressors, animal welfare and stress are not the same.

Until recently, welfare was seen as a continuum, ranging from very poor to very good in relation to the amount of environmental challenges (Broom 1988). This notion led to the formulation of the "Five Freedoms" of animal welfare by the UK Farm Animal Welfare Council in 1993. These "Five Freedoms" declare that farmed animals should be free of hunger and thirst; discomfort; pain, injury and disease; fear and distress; as well as free to express their normal behavior.

Recently however, the "Five Freedoms" framework as the basis of animal welfare has become a subject of controversy, as the theory of *allostasis* has provided an integration between the concept of environmental challenges and animal welfare (Korte et al., 2007). According to this model animal welfare must not be seen as a continuum between bad and good welfare, but both too little or too much environmental challenges can lead to bad animal welfare (Fig 1.1.; Korte et al., 2007). Therefore, it is of great importance to first study animal physiology, neuroendocrinology, behavior, and specifically stress physiology and behavior in order to understand and evaluate animal welfare.

1.2. The concepts of stress, homeostasis and allostasis

The concept of homeostasis dates to mid-19th century, and was first conceived by Claude Bernard (1865). However, the terminology *homeostasis* and its definition are coined to Walter Bradford Canon (1932), who defined homeostasis as "the coordinated physiological reactions which maintain most of the steady states of the body". In other words, homeostasis refers to the tendency of an organism or a cell to regulate its internal conditions, usually by a system of feedback controls, so as to stabilize health and functioning, regardless of the external changing conditions.

However, an organism throughout its life faces challenges (or stressors) that threaten its homeostasis. *Stress*, and the response to stress was first described by Seley (1946) as "the non-specific response of the body to any demand placed upon it". According to Seley, when an organism faces a stressor, a response is elicited which can be described by the General Adaptation Syndrome (GAS). The GAS suggests that the stress response is comprised of three distinctive stages, namely (1) the *alarm reaction phase*, constituting of the fight or flight response, (2) the *resistance phase*, where the organism tries to resist to the stressor by

maintaining its homeostasis, and finally (3) the *recovery or exhaustion phase*, where the system's compensation mechanisms have successfully overcome the stressor, yet if the stressor persists beyond coping capacity, the organism exhausts resources and becomes susceptible to disease and death.

Although stress is commonly associated with adverse effect on the organism, a distinction between "good stress" (eustress) and "bad stress" (distress) has been clarified since early on (Seley, 1950). ***Eustress*** refers to the responses of an organism that leads to enhanced survival, performance and adaptation. On the contrary, ***distress*** constitutes of the physiological alteration caused by a stressor that can cause detrimental effects to the organism.

Incorporating this idea, a new theory, called *allostasis*, has been recently proposed. Allostasis, in contrast to homeostasis, suggests that an organism "achieves constancy through change" (Sterling and Eyer, 1988; McEwen and Wingfield, 2003). This is performed by adjusting set points of the regulatory mechanism and involves synthesis of prior knowledge and predicted current needs. The term *allostatic load* is used to describe the capacity of an organism to cope with a certain challenge by adapting its behaviour and physiology, which when exceeded *allostatic overload* occurs and pathologies start to arise (hyperstimulation, **Fig. 1.1**). This is usually seen under chronic stress conditions when individuals are not able to successfully cope with continuous stress challenges (Korte et al., 2007).

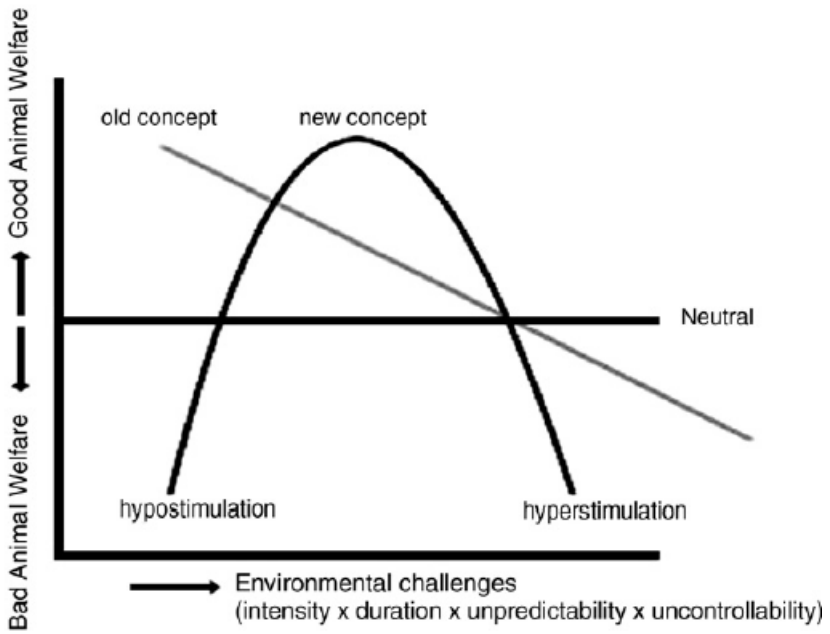


Fig. 1.1. Animal welfare in relation to environmental challenges as shown by the out-dated concept based homeostasis and the new concept based on the inverted U-curve of (di)stress. Figure from Korte et al., (2007).

Comparing the theories of homeostasis and allostasis in terms of welfare, it could be concluded that in homeostasis no or low stress leads to good welfare, while increasing stress leads to an inversely decrease of welfare. The theory of allostasis, however, suggests something different. Specifically, a dinstiction between poor, in cases of both low and high stress, and good welfare in cases of mid-leveled stress is suggested (**Fig. 1.1**; Korte et al., 2007). A very significant aspect of allostasis is that it takes into consideration both the predictability and the controllability of the stressor to define the challenge set to an organism, since after every stress event the organism aquires a prior knowledge to this stressor by the active participation of the brain.

1.3. The stress response in fish

In general, the mechanisms regulating the response to stress are complex, involving the actions of many biological systems of the organism. First, the stressful stimuli are perceived by the central nervous system, which subsequently controls the response. This includes the ***primary responses***, that refer to the hormonal (mainly corticosteroids and catecholamines) response, which in turn activates the ***secondary*** and ***tertiary responses***. Secondary responses refer to the metabolic, haematological, immunological etc. changes, whereas the tertiary responses to alterations concerning the whole organismal level, such as behavior, locomotion, feeding etc. (Wendelaar Bonga 1997. Barton 2002).

1.3.1. Primary responses

Upon perception of a stressful stimulus, fish respond by a cascade of events initiating from the brain. At first, the Hypothalamic – Sympathetic axis is activated, followed by the activation of the Hypothalamus – Pituitary – Interrenal (HPI) axis, which is similar to the Hypothalamus – Pituitary – Adrenal (HPA) axis in mammals. The former results in the release of adrenaline and noradrenaline to induce hyperglycaemia and fuel fight or flight response (Reid et al., 1998; Gorissen and Flik, 2016). The release of catecholamines after stress is rapid due to the existence of reserves stored in the chromaffin cells (Reid et al., 1998; Barton 2002), while the recovery to baseline levels is also fast (Milligan 1996; Wendelaar Bogna 1997).

The activation of the HPI axis begins with the secretion of corticotrophin-releasing factor (CRF) from the preoptic area (POA; Huising et al., 2004; Flik et al., 2006; Chen and Fernald,

2008). The axons of CRF-producing cells project directly to *pars distalis* ACTH cells (Flik et al., 2006), and CRF binds the CRF-receptors (CRF_1R) located on these ACTH cells to stimulate the release of this hormone (Flik et al., 2006). One factor regulating this process is believed to be the CRF-binding-protein (CRF-BP), which binds CRF and subsequently reduces its bioavailability (Flik et al., 2006; Alderman et al., 2008; Manuel et al., 2014). Hypothalamic CRF neurons also project to the pituitary *pars intermedia* and may induce release of α-melanophore-stimulating hormone (α-MSH) (Alsop and Aluru, 2011), and it is suggested that cause an increase in α-MSH release under conditions of chronic stress (Sumpter et al., 1986; Lamers et al., 1992; Metz et al., 2005). α-MSH has been suggested to act as corticotrope signal in some teleost species, such as Mozambique tilapia, *Oreochromis mozambicus*, (Lamers et al., 1994), and barfin flounder, *Vesaper moseri*, (Kobayashi et al., 2011), while this has not been the case in carp, *Cyprinus carpio*, (Metz et al., 2005) indicating that there is species-specificity on the corticotropic actions of α-MSH (Gorissen and Flik, 2016).

After stimulation of pituitary *pars distalis* by CRF, ACTH is secreted into the circulation. This hormone acts via a specific melanocortin receptor type 2 (MC2R), expressed exclusively on interrenal cells in the head kidney of fish (Alsop and Aluru, 2011). This receptor acts as a dimer and is associated with four melanocortin receptor associated proteins (MRAPs; Agulleiro et al., 2010, Faught et al., 2016; Dores et al., 2016). MC2R activates pathways that result in synthesis of cortisol from cholesterol and subsequent secretion to the bloodstream (Butler, 2011).

After synthesis, cortisol is released into the circulation, where it stimulates various target-tissues and regulates their actions. This stimulation by cortisol is regulated by cortisol receptors, which in most teleosts are two glucocorticoid (GRs) and one mineralocorticoid receptors (MR), since teleost fish do not produce aldosterone. Once cortisol is bound, these transcription factors bind specific DNA sequences (GR- and MR-responsive elements) in target-gene promoters and control mineralocorticoid and glucocorticoid activities (Wendelaar Bonga 1997; Mommsen et al., 1999; Schaaf et al., 2008).

The regulation of cortisol synthesis and secretion is achieved by feedback mechanisms throughout the whole HPI axis. Specifically, upon binding to the glucocorticoid receptors in the hypothalamus and pituitary, cortisol exerts a negative feedback regulation in the production of CRH and ACTH respectively (Mommsen et al., 1999). Moreover, cortisol has been suggested to exert an ultra-short-loop negative feedback on the site of cortisol production, the head kidney (Bradford et al., 1992). Finally, ACTH can also regulate its secretion by inhibition of hypothalamic CRH release (Suda et al., 1987).

There are also various other signals that can stimulate cortisol production, such as the hormones arginine vasotocin, AVT (Balment et al., 2006), and α-MSH (Gorissen & Flik, 2016), catecholamines, like adrenaline and noradrenaline (Rotllant et al., 2006), and monoamines like serotonin (Winberg et al., 1997). On the other hand, there are also hormones not belonging to the HPI axis that inhibit the production of ACTH, and therefore cortisol. For instance, somatostatin and leptin is known to exert inhibitory regulation in ACTH production (Eigler & Ben-Shlomo 2014, Gorissen et al., 2012).

Regarding the regulation of cortisol actions, as mentioned above, cortisol exerts its effects upon binding to specific receptors. Most fish species express two glucocorticoid receptors, namely GR1 and GR2, while some species also show a variant splicing in GR1 isoform, resulting in GR1a and GR1b which differ in the presence or absence of a nine-amino acid insert (Greenwood et al., 2003 Stolte et al, 2006; 2008). The most probable explanation for the presence of two GRs in teleost fish is the whole-genome duplication that took place 300-450 million years ago, and only after the divergence of the tetrapods from the fish lineage. Interestingly, there are few teleost species, such as zebrafish (Alsop & Vijayan 2009), that possess only one glucocorticoid receptor. In that species, it is believed that a loss of the gene encoding for GR1 occurred in the late evolutionary history of this species, leading thus to the existence of only one GR (Schaaf et al., 2009).

Apart from the glucocorticoid receptors, cortisol is also a ligand for the mineralocorticoid receptor, MR (Sturm et al., 2005; Prunet et al., 2006; Stolte et al., 2008). This receptor is much more sensitive to the mineralocorticoids aldosterone and 11-deoxyxorticosterone (DOC) than glucocorticoids, such as cortisol. However, due to the lack of aldosterone (Balment & Henderson, 1987; Prunet et al., 2006) and the low DOC circulating concentrations (Campbell et al., 1980; Milla et al., 2008) in fish, cortisol also exerts mineralocorticoid actions upon binding to MR (Prunet et al., 2006). Yet, because of the high sensitivity of MR for DOC, it is has not been ruled out that DOC could also act as a mineralocorticoid in fish (Prunet et al., 2006; Kiilerich et al., 2011; but see McCormick et al., 2008 for controversy).

This complex scheme of three functional receptors for cortisol seems difficult to be resolved. On the one hand, in all species studied so far, no differences in the receptor affinity have been identified for the different receptors (Bury et al., 2003; Stolte et al., 2006). On the other hand, transactivation studies have shown that GR2 is the most potent at low cortisol concentrations, followed by MR and finally GR1 (Bury et al., 2003; Greenwood et al., 2003; Sturm & Burry, 2005; Prunet et al., 2006; Stolte 2008). From all the above, and having in mind that it is difficult to correlate *in vitro* GR transactivation activities with the situation *in vivo*, it

has been suggested that cortisol would preferentially bind to GR2 in basal (i.*e.* unstressed) circulating concentrations of the hormone, and to both GR1 and GR2 in high (*i.e.* stressed) concentration of cortisol (Bury et al., 2003; Prunet et al., 2006). Finally, in mammals, MR is believed to fine-tune the HPA reactivity (de Kloet et al., 1998) and play a role in stress resilience (Heegde et al., 2015).

1.3.2. Secondary and tertiary responses

Secondary responses refer to the primary response-derived alterations that take place in the animal's physiology, including metabolic, biochemical, haematological, immunological, osmoregulatory etc. responses (Barton et al., 2002). These responses mainly act towards the production of energy in order to cope with the stressful event. Initially, catecholamines stimulate the production of glucose via hepatic glycogenolysis and to a lesser degree gluconeogenesis (Wright et al., 1989; Wendelaar Bonga 1997; Fabrri et al., 1998). Cortisol also produces hyperglycemia in a later stage of the stress response, by stimulating hepatic gluconeogenesis (Van der Boon et al*.,* 1991; Vijayan et al*.,* 1993; Wendelaar Bonga 1997; Mommsen et al*.,* 1999). When the energy demands set by the stressor cannot be met by the aerobic metabolism, fish are forced to turn to anaerobic metabolism. This leads to a depletion of muscle glycogen reserves and the accumulation of lactate in the muscles, which subsequently is released into the circulation (Pagnotta & Milligan 1991; Milligan & Girard 1993; Milligan 1996; Van der Salm et al*.,* 2006; Fanouraki et al*.,* 2011; Samaras et al., 2015). This increase in lactate leads to a reduction in the muscle and blood pH, which can negatively affect the transportation of oxygen by the blood (Milligan & Wood, 1987). Catecholamines seem to act against these negative effects by increasing haematocrit and assisting in a faster recovery of the red blood cells pH (Milligan & Wood, 1987; Wendelaar Bonga 1997).

Apart from affecting the carbohydrates metabolism, cortisol exerts proteolytic and lipolytic actions, resulting in that way in increased amino and free fatty acids concentrations in the circulation (Van der Boon et al. 1991; Wendelaar Bonga 1997; Mommsen et al*.,* 1999). In this way, substrates to produce glucose via gluconeogenesis become available.

As mentioned earlier, through binding to the mineralocorticoid receptor, MR, cortisol also plays a crucial role in osmoregulation and ionic equilibrium of fish (Wendelaar Bonga 1997; Mommsen et al*.,* 1999). Moreover, stress and especially cortisol, are believed to increase the susceptibility of an organism to pathogenic diseases (Fast et al., 2008; Tort, 2011). Cortisol,

in particular, is considered to play a role in modulating key components of the innate immune response in fish, such as lysozyme and complement components (Cortes et al., 2013).

Finally, tertiary responses involve changes in the whole animal level, such as swimming behaviour (Juell & Fosseidengen, 2004), reproduction (Schreck, 2010), feeding behaviour and growth (Leal et al., 2011).

1.4. Variation in cortisol response

It has been well documented that basal circulating cortisol levels and the stress response pattern can differ between fish species (Barton, 2000; Fanouraki et al., 2011). It is, however, clear that variance in cortisol concentration can exist between individuals within the same species. In general, this variation can be a result of different environmental conditions, such as temperature (Davis & Parker 1990; Lankford et al. 2003; King et al. 2006; Vargas-Chacoff et al. 2009a; b; Pascoli et al., 2011), photoperiod (Kavadias et al., 2003; Pascoli et al., 2011), and salinity (Tsui et al., 2012), but also husbandry conditions, like stocking density (Pickering, 1992, Ellis et al., 2002; Van der Salm et al., 2004; Lupatsch et al., 2010) and water quality (Santos et al., 2010). Moreover, ontogenetic phase (Pavlidis et al., 2011; Tsalafouta et al., 2014), and genetic origin can affect the stress response within the same species (Pottinger et al., 1992; Tort et al., 2001; Wang et al., 2004; Øverli et al., 2007; Hori et al., 2012a;). Finally, the nature of the stress itself is crucial in the outcome of the response. It is essential to distinguish between acute and chronic stress and among stressors of different intensity, duration (Fatira et al., 2014; Pavlidis et al., 2015), type (Lankford et al., 2013), repeatability, controllability and predictability (Basrur et al., 2010; Madaro et al., 2015) when interpreting results from stress studies.

1.4.1. Environmental and husbandry conditions and allostatic load

Various environmental conditions can affect the stress response of fish, with temperature being of prime importance given the ectothermic nature of most fish species. The effects of temperature on both basal and post-stress cortisol circulating levels have been described in many teleost fish species (Umminger & Gist 1973; Sumpter et al, 1985; Barton & Schreck 1987; Davis & Parker 1990; Lankford et al. 2003; King et al. 2006; Vargas-Chacoff et al. 2009a; b). Specifically, there is a tendency for higher concentrations at high temperatures in several fish species (Sumpter et al, 1985; Barton & Schreck 1987; Davis & Parker 1990;

Lankford et al. 2003; King et al. 2006; Vargas-Chacoff et al. 2009a; b), while the pattern of the stress response is usually faster, in terms of both time to peak and to recovery, and more intense in higher temperatures. However, there are fish species that deviate from this general pattern, such as goldfish, *Carassius auratus* (Umminger & Gist 1973) and chinook salmon, *Oncorhynchus tshawytscha* (Barton & Schreck 1987) which show a lessened response in some of the examined physiological traits, like glucose and liver glycogen.

Husbandry conditions can also affect the stress status of fish and their capacity to respond to stressors. It has been suggested that higher than the optimum stocking densities can become a source of stress for fish (Pickering, 1992, Ellis et al., 2002; Van der Salm et al., 2004; Lupatsch et al., 2010) or can lead to an alteration in the stress response when faced with an additional stressor (Di Marco et al., 2008). Water deterioration also consists a factor that affects stress status and response in fish, but is often studied in collaboration with increased stocking densities (Santos et al., 2010). Pickering & Pottinger (1987), however, were the first to distinguish between these two factors by altering water quality without changes in the stocking conditions. That study revealed that the nature of water deterioration defines the subsequent alterations in cortisol response, and specifically that some factors, such as the combination of elevated ammonia with reduced pH increased, while others like the reduced oxygen, elevated CO_2 and ammonia suppressed the cortisol response in brown and rainbow trout (Pickering & Pottinger, 1987).

Moreover, husbandry conditions also include common aquaculture practices performed by the farming personnel, such as netting, short-timed confinement, sorting, exposing to air, anaesthetizing etc., which can be stressful for the fish. It becomes, thus, important to quantify how such common practices can exert allostatic load on fish, and can therefore affect the stress status and response of fish.

Such practices can be either acute or chronic, with the distinction being mainly based on the severity, duration and repetition of the stressor. Specifically, an acute stressor is usually severe, unpredictable and short in time, while a chronic stressor can be severe or mild, predictable or unpredictable, long lasting or short but repeated, controllable or uncontrollable etc. The nature of the stressor is therefore essential in defining the allostatic load exerted on fish.

In terms of fish response to acute or chronic stress, cortisol, the so-called "stress hormone", is considered to be a very accurate indicator of the former (Wendelaar Bonga, 1997; Mommsen et al., 1999; Fanouraki et al., 2011), while when chronic stress is considered cortisol is not reckoned as a potent indicator (Mommsen et al., 1999; Martínez-Porchas et al., 2009;

Gorrisen & Flik, 2016). Indeed, a recent study in carp has clearly indicated that chronically stressed fish showed no differences in the plasma levels of commonly used stress indicators, such as cortisol, glucose and lactate (Aerts et al., 2015). This study suggested that instead of plasma cortisol, the concentration of the hormone accumulated to the scales can reflect the stress level experienced by fish over time (Aerts et al., 2015).

Therefore, it seems important to understand how different allostatic loads induced by common aquaculture processes, such as confinement, netting and air exposure, affect the stress status of the fish in order to provide husbandry for optimum performance and welfare.

1.4.2. Genetic background, coping styles and cortisol responsiveness

In general, the effects of a stressor depend heavily on how an animal perceives and processes the stressful stimuli and how it can cope with it (Koolhaas, 1999). In this context, much attention has been given during the last decades to the fact that individuals of the same species may show consistently divergent physiological and behavioral responses to a stimuli or stressor, and how this is connected to the genetic background of individuals. These sets of responses have been described as coping styles (Koolhaas, 1999), behavioral syndromes, personalities or temperaments, with a more or less synonymous use (Castanheira et al., 2015). Two main categories of coping styles have been identified in vertebrates, namely the proactive and reactive (Koolhaas, 1999), that differ in many aspects of their behavior and physiology. Specifically, proactive animals seem more aggressive, display the fight-flight response and show low behavioral flexibility, in contrast to reactive animals that tend to be non-aggressive and cautious, adopt the freezing response strategy and are behaviorally flexible. In terms of physiology, proactive animals have been linked to low stress axis output, regulated by low mRNA expression of CRH in the hypothalamus, and low glucocorticoid (cortisol or corticosterone depending on the taxa) production. Moreover, these animals show low parasympathetic and high sympathetic activity compared to reactive individuals which show the reverse pattern (Korte et al., 2005). Therefore, identification of such intra-specific differences in cortisol responsiveness and better understanding of their impact in the animal performance and fitness would be beneficial towards better husbandry and selection of breeding stocks in selective breeding programmes.

Divergence in the cortisol response has been described in many fish species (Pottinger et al., 1992; Tort et al., 2001; Weil et al., 2001; Fevolden et al., 2002; Wang et al., 2004; Øverli et al., 2007; Hori et al., 2012a; Castanheira et al., 2015) and is also observed in tetrapods,

including amphibians (Narayan et al., 2013), reptiles (Øverli et al., 2007), birds (Cockrem et al., 2013), and mammals (Benus et al., 1991; Sgoifo et al., 1996). It has thus been suggested that intra-species diversity neuroendocrine stress-responsiveness and behaviour has been maintained by natural selection throughout the vertebrate sub-phylum (Øverli et al., 2007). Fish are among the most widely studied taxa in this aspect, and consistent differences in the cortisol response between individuals of the same species have been described (Pottinger et al., 1992; Tort et al., 2001; Weil et al., 2001; Fevolden et al., 2002; Wang et al., 2004; Hori et al., 2012a), leading to the identification of low (LR) and high (HR) responding fish. In these fish, cortisol responsiveness has also been associated with behavioral differences and coping styles (Øverli et al., 2005; Ruiz-Gomez et al., 2011), as well as animal performance and fitness, such as differential growth (Fevolden et al., 2002; Pottinger, 2006; Weber & Silverstein, 2007), and metabolic functions (Tort et al., 2001; Trenzado et al., 2006; Pemmasani et al, 2011).

1.5. European sea bass (*Dicentrarchus labrax*)

European sea bass (*Dicentrarchus labrax*, L.) is a demersal teleost fish species inhabiting coastal waters to a depth of 100 m, as well as brackish waters in estuaries and coastal lagoons for spawning (Pérez-Ruzafa & Marcos, 2014). It is an euryhaline species, inhabiting waters ranging from hyperhaline to brackish, occasionally even entering rivers, and shows oceanodromous behaviour. E. sea bass is also eurythermal, inhabiting waters with temperatures between 8-24°C, while it has been observed in areas with water temperatures as low as 5°C and as high as 32°C (Pérez-Ruzafa & Marcos, 2014). This euryhaline and eyrythermic capability allows this species to have a wide geographic range of distribution along the North Eastern Atlantic coasts, from Norway to Senegal, to the Mediterranean Sea and the Black Sea (Pérez-Ruzafa & Marcos, 2014).

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Perciformes
Family	Moronidae
Genus	*Dicentrarchus*
Species	*Dicentrarchus labrax*

Maturity in nature comes at the age of 2- to 3- years old for the males, while females mature one year later. Adult E. sea bass can reach an age of 30 years and a maximum weight and length of 15 kg and 1 m, respectively (Kottelat and Freyhof, 2007).

Adults usually move from coastal feeding zones to deep areas to spawn, a phenomenon that occurs during winter (December to March) in the Mediterranean Sea (Pèrez-Ruzafa & Marcos, 2014). After hatching, larvae return to coastal areas, and subsequently enter nurseries such as lagoons, harbours and marinas (Dufour et al., 2009). At the juvenile and "adolescent" stage of the life cycle, E. sea bass' spatial movements are relatively low, while adult fish perform extensive seasonal migrations between feeding and spawning areas (Pérez-Ruzafa & Marcos, 2014).

As regards to the feeding ecology of the species, E. sea bass is a highly adaptable and opportunistic predator (Pérez-Ruzafa & Marcos, 2014). Feeding behavior and preferences are related to the size and the life cycle of the individuals. In general, juveniles and adults hunt as shoals, while solitary hunters can also be observed (Millot et al., 2014). Juveniles feed mainly on crustaceans, such as peracarida, copepod, amphipoda and isopoda, while to a lesser extent on decapoda, molluscs, and small fishes (Pérez-Ruzafa & Marcos, 2014). Adult fish mainly consume crustaceans, such as crabs and shrimps, molluscs and fish, but also polychaetes, seagrasses, seaweeds, aquatic insects and snails though in lesser amounts (Pérez-Ruzafa & Marcos, 2014).

1.6. European sea bass in aquaculture

E. sea bass has been historically cultured in coastal lagoons, by trapping schools of fish that lived in these areas (FAO, 2016). However, mass production of this species started around the late 1960s to early 1970s by the development of reliable mass-production techniques (FAO, 2016). E. sea bass was the first non-salmonid fish species to be cultured in Europe, and is still one of the most widely cultured species in the Mediterranean area, where along with gilthead sea bream, *Sparus aurata*, represent the 90% of the total finfish production (EATiP, 2012).

E. sea bass can be farmed in seawater ponds and coastal lagoons, but mass-production is usually achieved through sea cage farming. In cage farming, hatchery-produced juveniles move to net-pen sea cages at a size of approximately 5 g for on-growing up to the commercial size (300-500 g) for 18-24 months. Sea cages in E. sea bass farming usually range between 1,000 to 15,000 m^3 in volume, at a depth of 6-8 m (Scott & Muir, 2000; FAO, 2016).

The total production of E. sea bass in the Mediterranean Sea was 111,000 tons in 2010, with Turkey and Greece being the main producers with 50,796 and 45,000 tons, respectively (EATiP, 2012; FEAP, 2015). It has been provisioned that the total production in the area will have more than doubled by 2030 (EATiP, 2012).

However, E. sea bass culture can still be improved if better growth rates and more appropriate breeding is achieved. Additionally, there are still problems that need to be resolved, with disease outbreaks being one of the most important (Hillen et al, 2014; FAO, 2016). Moreover, this species shows an intense stress response (Fanouraki et al., 2011) and is considered susceptible to stress by inducing reproductive dysfunctions and disease outbreaks (FAO, 2016), which, in turn, hamper production.

1.7. European sea bass stress response

As indicated above, E. sea bass is characterized by an intense stress response, as depicted by the high cortisol concentrations observed upon exposure to acute stress (Fanouraki et al., 2011; Ellis et al., 2012). Apart from the intense response, this species shows high resting (*i.e.*, without stress) circulating levels of cortisol compared to other teleost fish species studied so far (Fanouraki et al., 2011; Ellis et al., 2012). This intense response has been suggested to derive from an increased capacity of the interrenal cells to produce cortisol, as well as from an extended sensitivity of these cells to ACTH (Rotllant et al., 2003b).

Moreover, this species shows high variability in the basal circulating cortisol levels, as well as the post-stress levels, both between different studies (reviewed by Ellis et al., 2012), as within the same study (Fanouraki, 2010). It is, of course, true that a direct comparison of data from different studies is not feasible, since different research groups might use different analytical methods to assess cortisol, such as ELISA and RIA assays, while different assays within the same method are also being used; still however the trend derived from such a comparison can reveal the variation of cortisol. Such a high variability in both basal and response levels of cortisol can prevent accurate prediction of the stress status of fish, as well as their ability to cope with subsequent stressors (Ellis et al., 2012).

Apart from the analytical component of variation in cortisol levels, there are many factors that can induce variation in stress-related parameters. In short, these factors could roughly be categorized into environmental, husbandry, and genetic, few of which have been attempted to be examined in E. sea bass.

1.8. Aims of the study

The aim of the present study was to get a thorough insight of genetic, environmental and husbandry factors that affect cortisol variability and regulate the cortisol stress response in E. sea bass. Specifically, the objectives of this thesis were to:

- examine how water temperature affects circulating cortisol at both pre- and post- stress conditions (Chapter II),
- define the allostatic load through the development of a repeated predictable stress protocol of various intensities and study how this affects cortisol dynamics and peripheral regulation in organs such as liver and head kidney (Chapter III),
- study how the genetic background affects cortisol response to stress at both family and individual level, and whether cortisol responsiveness is a repeatable trait (Chapter IV).
- examine possible differences in the hepatic transcription profile of individuals with consistently different basal and post stress cortisol concentrations (Chapter IV),
- investigate the mechanisms regulating divergence in cortisol responsiveness between individuals of E. sea bass (Chapter V).

Chapter II. Water temperature modifies cortisol acute stress response of European sea bass, *Dicentrarchus labrax* L.

2.1. Introduction

Temperature widely affects the physiology and performance of ectotherm organisms. Fluctuations of physiological parameters, such as hormones and metabolites, in relation to the annual cycle due to temperature, photoperiod and the physiological state of the fish have been described in many teleost species (Vargas-Chacoff et al., 2009b; Gómez-Milán et al., 2011; Fregeneda-Grandes et al., 2013), including E. sea bass (Gutiérrez et al. 1987; Pascoli et al., 2001). Since fish are ectotherm organisms, such environmental challenges can significantly affect the circulating levels of metabolites in the blood and result in high variability in these traits. However, fish, and particularly eurythermal species, are capable of functioning adequately well in a wide range of temperatures, exhibiting rates of metabolic activity more similar than would be predicted from Q10 relationships (Somero, 2004). This phenomenon, known as temperature compensation of metabolism, is mostly achieved by the alteration of enzyme requirements for activation energy and catalytic rates according to temperature, as well as by using a variety of different enzyme orthologs (Somero, 2004). Although fish can function in a spectrum of water temperatures, there is a thermal preferendum for each species, a range over which the performance of the animal is maximized (Beitinger & Fitzpatrick, 1979). Since different aspects of physiology and function of an organism can have different thermal preferendums, the whole-animal optimum is determined by (a) the integration of various functions and systems preferendum of the organism, (b) the optimum of the most sensitive function, or (c) may be governed by the masking influence of other environmental factors (Beitinger & Fitzpatrick, 1979). In E. sea bass, the thermal optimum has been suggested to be around 25°C for growth, 25-29°C for feed intake and 19-25°C for feed efficiency ratio (Person-Le Ruyet et al., 2004), between 20-25°C for metabolism (Claireaux & Lagardère, 1999), from 19.3 to 29.6°C for swimming performance (Koumoundouros et al., 2002) and 22-28°C for the whole animal performance (Dügler et al., 2012). Spawning, on the other hand, is performed in much lower temperatures; specifically, between 9 and 18°C, with the optimal temperatures ranging between 13-15°C (Mañanós et al., 2009).

Moreover, rearing temperatures have been referred to alter the rate of many biological processes of an organism, including mechanisms that regulate the stress response. Many

physiological parameters commonly used as stress indicators, such as cortisol, glucose, lactate and osmolality, show a tendency for higher concentrations at high temperatures in several fish species (Sumpter et al., 1985; Barton & Schreck, 1987; Davis & Parker, 1990; Lankford et al., 2003; King et al., 2006; Vargas-Chacoff et al., 2009a; b). In addition, the pattern of the stress response is usually faster, in terms of both time to peak and to recovery, and more intense in higher temperatures. However, there are fish species that deviate from this general pattern, such as goldfish, *Carassius auratus* (Umminger & Gist, 1973) and chinook salmon, *Oncorhynchus tshawytscha* (Barton & Schreck, 1987) which show a lessened response in some of the examined physiological traits, like glucose and liver glycogen.

During the last years, non-invasive indicators of stress, such as water cortisol released from the fish, have been developed (Ellis et al., 2004; Ellis et al., 2005; Scott & Ellis 2007) and evaluated in many fish species (Ellis et al., 2004; Fanouraki et al., 2011; Pavlidis et al., 2013). However, the effects of temperature upon the release of cortisol have been poorly examined. To our best knowledge, there is only one study in this respect, which showed reduced release after an acute temperature drop in common carp, *Cyprinus carpio* (Jaxion-Harm & Ladich 2014). However, plasma cortisol was not quantified in that study, and therefore it cannot be concluded whether this reduced cortisol release was due to an impaired release or to lower plasma values.

The aim of the present study was to examine the effects of acclimation temperature on cortisol dynamics and release rate in the water, by applying a previously validated acute stress protocol and by keeping other environmental parameters such as photoperiod constant.

2.2. Materials and Methods

2.2.1. Fish and husbandry conditions

Juvenile E. sea bass individuals were hatched and raised in the Hellenic Centre for Marine Research (HCMR, Greece, Crete), and were transferred and maintained in 3 x 250 L aquaria at the installations of the Fish Physiology Laboratory, University of Crete. The aquaria were continuously aerated and the water was recirculated through a biological and mechanical filter (Eheim external canister filter, EHEIM GmbH & Co. KG, Germany). Temperature was controlled by thermostat heaters (Resun®, China) and chillers (Sfiligoi, S.R.L., Italy) while the photoperiod was set at 12L:12D.

Initially, 252 fish (mean weight ± S.E.M.: 29.6 ± 0.6 g) were transferred to the experimental aquaria, and divided into 84 fish per aquarium, resulting in a density of 9.80 ± 0.06 kg m^{-3}, and were held at a water temperature of 20°C, similar to the holding and transportation temperature. After one week of acclimation to the novel environment, and after fish showed resumption to feeding, a protocol of mild temperature change was performed. In particular, in two of the aquaria the desired temperatures of 15°C and 25°C were reached by appropriately changing the temperature by 1°C every second day, with the whole procedure lasting for 10 days. In the third aquarium the temperature was held constant at 20°C. Subsequently, fish were left for an extra period of two weeks to acclimate to the new conditions prior to sampling, with the mean temperatures in each aquarium during this time being 15.05 ± 0.03°C; 19.80 ± 0.08°C; 25.03 ± 0.36°C; termed hereafter as 15, 20 and 25 °C groups respectively.

Fish were fed daily a constant quantity (2% of their body weight) of a commercial dry pellet diet (Irida S.A., Greece) consisting of approximately 44% protein and 19% lipids. During the experiment water parameters were monitored daily (temperature, pH) or weekly (ammonia, nitrate, nitrite).

2.2.2. Experimental design

In order to study the resting and post-stress levels of cortisol in fish acclimated to different temperatures 70 fish from each temperature treatment were used. Specifically, 10 fish from each aquarium were immediately captured (0h, unstressed fish), euthanatized in 2-phenoxyethanol (500 ppm; Merck, 807291) and bled from the caudal vein using heparinized syringes. Additionally, two water samples were collected from each respective tank (0.5 L). The remaining fish were acutely stressed by lowering the water to approximately 1/3 of its initial volume and then by chasing with a net for 5 min. After the completion of the stress protocol, fish were divided in 6 x 30 L tanks (10 individuals per tank), filled up with the water from the home aquarium, covered with a lid in order to keep fish in the shade and provided with aeration to avoid reductions in oxygen concentration. At each sampling point of 0.5, 1, 2, 4 and 8 h post-stress all the fish from one tank were sampled as described for the unstressed fish. Fish sampled at 8 h post-stress were held in two tanks (10 individuals per tank) for the purposes of water sampling (described below), and so 5 fish per duplicate tank were randomly chosen for blood sampling. Following blood collection, blood was centrifuged (2,000 g; 10 min) and the resulting plasma was stored at -20°C until the analysis.

Water samples (V = 0.5 L) were collected at the same time points as blood samples from the duplicate tanks holding fish that were sampled at 8 h post-stress. By collecting successive water samples from the same tank it was possible to calculate the release rate of cortisol from the fish in that tank, which would not be possible if the water samples were collected from different tanks at each sampling point. Care was taken when removing water at each sampling point in order to avoid disturbing and further stressing the fish. For that reason, the water was collected using a catheter tube without removing the cover of the tank or exposing fish to any disturbance. Immediately afterwards, the water that was removed during the sampling was replaced, using the same way, in order to maintain the same stocking density throughout the trial. This water removal and renewal was considered when estimating the release rate of cortisol in the water. So, the amount of hormone release, expressed as ng g^{-1} of fish biomass, was calculated from the difference in the amount of cortisol between successive sampling points per unit of fish biomass. Specifically, the equation used was:

$$H_t = [V * C_t - (V * C_{t-1} - V_s * C_{t-1})]/B,$$

Where, H_t is the hormone released per fish biomass (ng g^{-1})

V the volume of the tank (l)

C_t the concentration of cortisol at time *t*, which is the time of sampling (ng l^{-1})

C_{t-1} the concentration of cortisol at time *t-1*, *i.e.* the previous sampling (ng l^{-1})

V_s the volume of the sampled (removed) water (l)

B the biomass of the fish (g)

Subsequently, the release rate of cortisol (ng g^{-1} h^{-1}) was calculated in respect to the time intervals.

During the experimental period (8 h), fish were held in the same temperature as the one they had been acclimated to, by setting the environmental room temperature at the respective level. No significant reduction in water quality, in terms of oxygen concentration and ammonia accumulation, was observed.

2.2.3. Analytical measurements

Plasma and water-born cortisol were estimated using a commercial enzyme-linked immunoassay kit (DRG® Cortisol ELISA, DRG® International Inc., Germany). The

performance of the kit has been evaluated with linearity and recovery tests in E. sea bass plasma samples. Specifically, sequential dilutions (1:2; 1:4; 1:8 and 1:16) of low (≈100 ng ml^{-1}) and high (≈600 ng ml^{-1}) plasma samples have shown high parallelism (r^2=0.994 and r^2=0.986, respectively), while the recoveries of added cortisol in these samples were 99.6 ± 2.3% and 108.3 ±14.4% (mean ± SEM, n=2) when 200 ng ml^{-1} and 400 ng ml^{-1} of cortisol were added, respectively.

Cortisol from water samples was extracted as described by Ellis et al. (2004). Briefly, water samples were pumped with a peristaltic pump at ca. 10 ml min^{-1} through a pre-filter (0.45 μm pore-size, AcroCap™, Gelman Sciences, MI, USA) and then through an activated solid phase extraction cartridge (Sep-pak® Plus C18, Waters Ltd., UK). Subsequently, cortisol was eluted from the extraction cartridges with ethyl acetate, which was evaporated at 45°C under nitrogen gas and the residue was re-dissolved in 1 ml of phosphate buffer saline (which has shown no interference with the specific ELISA analysis). All samples were run in duplicate.

2.2.4. Statistical analysis

Statistical analysis was performed using the SigmaStat 3.1 statistical package and the graphs were created using GraphPad Prism 6.0 software (GraphPad Software, USA). Results are presented as means ± standard deviation (SD). One-way Analysis of Variance (ANOVA) tests were used to check for significant effects of *temperature* on the resting concentration of the examined parameters, and two-way ANOVA tests to check for significant effects of the factors *time post-stress*, *temperature*, and their interaction. When significant differences existed, at a level of $P < 0.05$, Tukey's post-hoc multiple comparison analysis were subsequently performed. Prior to analysis, data from the samples collected from the duplicate tanks at 8 h post-stress were compared using t-test, and since no significant differences were found in cortisol, they were pooled together. Moreover, before performing the one way or two-way ANOVA data were checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test and, when necessary, data were log-transformed before analysis. In the case of the resting values of osmolality the one-way ANOVA assumptions were not met even after data transformation and therefore the non-parametric Kruskal-Wallis test was used followed by Dunn's post-hoc analysis.

In order to estimate the magnitude of the response of each physiological trait as a whole, the Area Under the Curve (AUC) was calculated based on the trapezoidal method, using the GraphPad Prism 6.0 software (GraphPad Software, USA). Specifically, the total AUC (AUC_t)

was calculated by counting the total area between the ground (y = 0) and the curve. Additionally, since fish at different temperature conditions showed different baseline values, the AUC with respect to increase from the baseline levels (y = value of the physiological trait at 0 h) (AUC_i), was also calculated. In details, AUC_i calculates the area with respect to the baseline measurement subtracting the area that goes below that baseline, when such area exists, emphasizing in that way on the changes over time (Fekedulgen et al. 2007). In addition, the slope from baseline to peak (SBP), defined as the difference between the baseline and peak value divided by the time interval between these two points (in min) was calculated as a descriptive indicator of the speed of the response.

2.3. Results

2.3.1. Resting concentrations

One-way ANOVA analysis showed that the mean resting values for cortisol differed between fish reared at different temperatures ($F_{2,27} = 8.278$; $P = 0.002$; **Table 2.1**). Specifically, lower values were observed at 15°C compared to those at 20°C ($P = 0.048$) and 25°C ($P < 0.001$; **Table 2.1**).

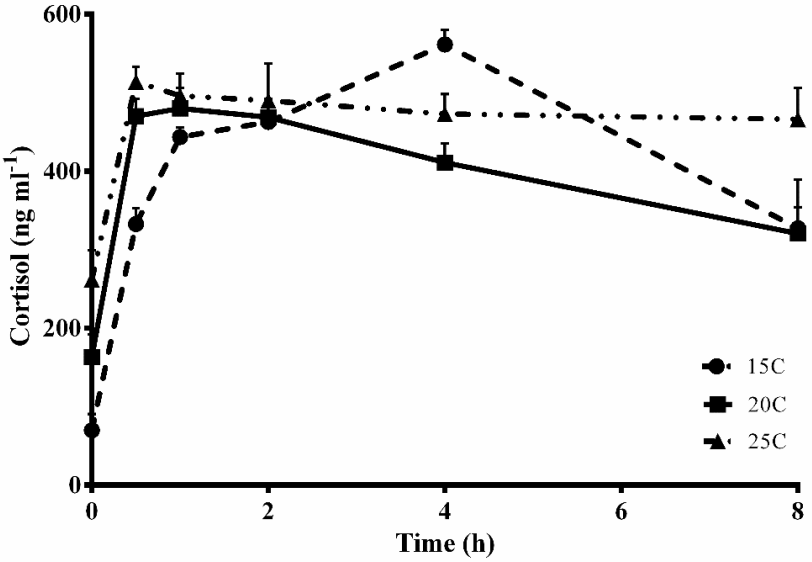
Table 2.1. Descriptive statistics of the resting levels of plasma cortisol (n = 10). The range expresses the max-min value, while 5-95% represents the max-min value of the data belonging to 5-95% of the distribution to avoid extreme outliers. Different letters in the mean column indicate statistically significant differences between the groups ($P < 0.05$).

Temperature	Mean (ng ml^{-1})	SD	CV%	Min	Max	Range	5-95% Range
15°C	69.9^{a}	62.1	88.8	5.6	186.7	181.1	140.8
20°C	189.1^{b}	116.3	61.5	16.2	423.3	407.1	211.6
25°C	262.4^{b}	118.5	45.2	73.0	417.6	344.7	297.7

2.3.2. Stress response

In all temperature conditions, a cortisol response was evoked after acute stress, yet however significant interactions between the *temperature* and *time* were presented ($F_{2,158} = 4.774$; $P < 0.001$). More specifically, fish acclimated at 20°C and 25°C reached the highest mean concentrations of plasma cortisol at 0.5 h post-stress, showing approximately 2.5 and 4 times higher slope to peak than 15°C, respectively (**Table 2.2** & **Fig. 2.1**), and remained

unaltered until 4 h post-stress in the former and 8 h post-stress in the latter conditions. In contrast, at 15°C a continuous increase was observed until 4 h post-stress, with signs of recovery afterwards. Apart from the variations in the resting values, differences in cortisol concentration existed both at 0.5 h and 4 h post-stress, with lower and higher concentrations, respectively, in the 15°C group ($P < 0.01$ in all cases) (**Fig. 2.1**).



	0 h	0.5 h	1 h	2 h	4 h	8 h
15°C	a*	b*	bc*	c*	c*	b*
20°C	a#	b#	b*	b*	bc#	c*
25°C	a#	b#	b*	b*	b#	b*

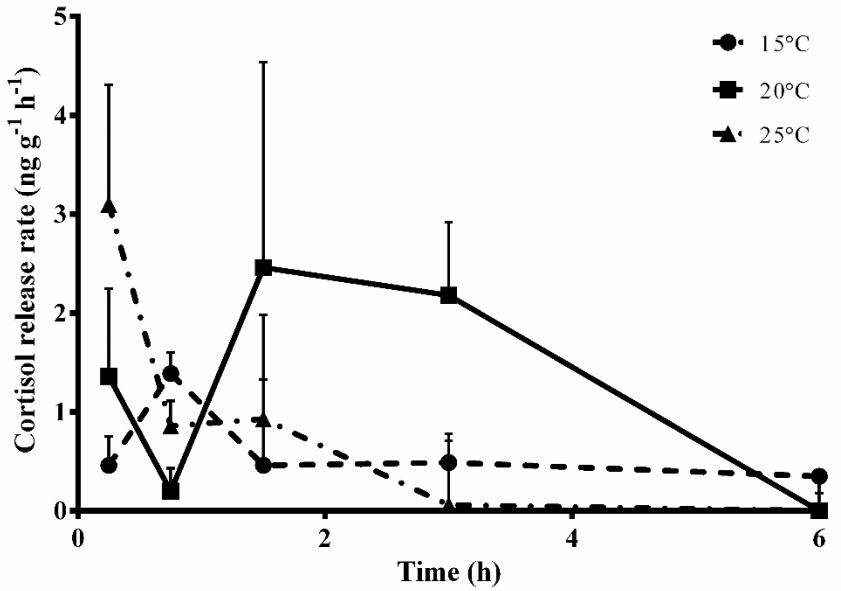
Fig. 2.1. Cortisol concentrations after acute stress in fish acclimated at the three different temperatures. Values are given as mean + SD (n = 10). Within each temperature treatment different letters indicate statistically significant differences, while different symbols (*, #, +) indicate differences between temperatures at each sampling point.

The magnitude of cortisol response, as depicted by the Area Under the Curve with respect to increase from the baseline value (AUC_i), was highest at 15°C group, followed by fish reared at 20°C and then 25°C (**Table 2.2**).

Table 2.2. Descriptive statistics of the stress response curve of cortisol, in fish acclimated at different temperatures. AUC_t: total Area Under the Curve; AUC_i: Area Under the Curve with respect to increase; SBP: slope from baseline to peak.

Temperature	AUC_t	AUC_i	SBP
15°C	3531	2972	122.9
20°C	3203	1751	315.9
25°C	3488	1389	503.9

The rate of cortisol release into the water was affected by the interaction of *temperature* and *time* ($F_{8,15} = 3.215$; $P = 0.025$; **Fig. 2.2**). Specifically, the 25°C group showed the highest maximum values of release rate (3.1 ± 1.2 ng g^{-1} h^{-1}) compared to the other two groups (2.5 ± 2.1 ng g^{-1} h^{-1} and 1.4 ± 0.2 ng g^{-1} h^{-1} at 20°C and 15°C, respectively). The pattern of cortisol release into the water also differed, with fish reared at 25°C showing an intense and fast release, being highest at the interval 0 to 0.5 h post-stress ($P < 0.01$), and declining to almost non-traceable release after 2 h. On the other hand, fish acclimated at 20°C showed the highest release of cortisol into the water at the intervals between 1 and 4 h, showing no release during 4 to 8 h. Finally, at 15°C no differences were observed in the release rate of cortisol ($P = 0.28$), there was, however, a constant release of cortisol even after 8 h post-stress.



	0 to 0.5 h	0.5 to 1 h	1 to 2 h	2 to 4 h	4 to 8 h
15°C	a*	a*	a*,#	a*	a*
20°C	ab*,#	ab*	a*	ab*	b*
25°C	a#	ab*	ab#	b*	b*

Fig. 2.2. Cortisol release rate into the water after acute stress in fish acclimated at the three different temperatures. Within each temperature treatment different letters indicate statistically significant differences, while different symbols (*, #, +) indicate differences between temperatures in each sampling point.

2.4. Discussion

The results of the present study demonstrate that the environmental water temperature can affect the resting circulating levels of cortisol as well as the timing, magnitude, and duration of the stress response in E. sea bass. It is therefore an environmental factor that could add part

of the variability observed in cortisol resting and post-stress levels, especially when the results of different studies are under discussion. Previous studies have shown that temperature can affect the stress response in a species-specific way (Barton & Schreck, 1987; Davis & Parker, 1990; King et al., 2006; Costas et al., 2012), depending mostly on the biology and thermal tolerance and preference of each species. Apart from temperature, however, other environmental factors such as photoperiod (Pavlidis et al. 1999; Biswas et al. 2006) and season (Pascoli et al. 2011; Maricchiolo et al. 2011; Danzmann et al. 2016) are also significant in regulating the physiological status of fish. Specifically, the concentrations of cortisol and glucose have been closely correlated with photoperiod in E. sea bass (Pascoli et al. 2011; Kavadias et al. 2003). In addition, diel fluctuations in physiological traits due to the circadian rhythm also exist in fish (Pickering & Pottinger, 1983; Fatira et al., 2014). In E. sea bass, a peak in cortisol concentration has been observed at dusk (*i.e.* when the lights turn off) (Fatira et al., 2014). For these reasons, in the present study apart from the differences in water temperature, the other environmental conditions in the tanks, such as photoperiod and water quality were held constant, while the tanks were covered with a lid to avoid diel fluctuations.

In the present study, resting cortisol levels were affected by temperature, being lower in 15°C than the two other temperature regimes. A positive relationship between cortisol and water temperature and photoperiod has been suggested in this species (Planas et al., 1990; Pascoli et al., 2011; Samaras et al., 2015), which is in accordance with the present results. This pattern has been observed in other fish species as well, like the Atlantic cod, *Gadus morhua*, and haddock, *Melanogrammus aeglefinus* (King et al. 2006), the striped bass, *Morone saxatilis* (Davis & Parker 1990), and the Adriatic sturgeon *Acipenser naccarri* (Cataldi et al., 1998). On the other hand, the absence of temperature effects on cortisol levels has also been reported in some species, like chinook salmon, (Barton & Schreck, 1987) and green sturgeon, *Acipenser medirostris* (Lankford et al., 2003). Finally, a more complex pattern was observed in Senegalese sole, *Solea senegalensis*, where higher cortisol values were achieved in either higher or lower than the control temperatures (Costas et al., 2012).

Moreover, a response in cortisol was observed in all acclimation temperatures, however with differences in both the rapidity and magnitude of the response. Specifically, at 25°C and 20°C the time to peak was shorter, accompanied by a greater slope to peak than at 15°C, where a prolonged response until 4 h post-stress after was observed. This could be the result of a higher metabolic rate in the high temperatures (Claireaux & Lagardère 1999; Claireaux et al. 2006), or be caused by a possible temperature-derived effect on cortisol dynamics through altering the rate of cortisol synthesis and clearance (Barton & Schreck 1987). In general, a

faster cortisol response at higher temperatures has been observed in many fish species (King et al. 2006; Lankford et al. 2003; Barton & Schreck 1987; Sumpter et al. 1985; Davis & Parker 1990), being in line with the present study in E. sea bass.

The response at 15°C, although delayed, was of a higher magnitude as described by the high AUC_i. Davis and Parker (1990) have suggested that in striped bass both the magnitude of the response and the rapidity of return to pre-stress conditions reflect the imbalance that a stressor causes to the organism. Based on that, they have suggested that stressing striped bass at temperatures lower or higher than the optimum augments the response, which might also be the case when stressing E. sea bass at low temperatures, such as 15°C.

Apart from plasma cortisol, temperature also affected the release rate of cortisol into the water. The differences in the response of plasma cortisol were in accordance with the pattern of release rate of cortisol into the water. Specifically, fish reared at 25°C showed the fastest and most intense cortisol release rate, followed by those at 20°C and finally those at 15°C, which showed a constant and stable low rate of cortisol release. Reduced release rate in lower temperature has also been observed in a freshwater fish species, the common carp (Jaxion-Harm & Ladich, 2014), however a direct comparison cannot be made since in that study fish were not acclimated to the lower temperature but faced an acute reduction temperature of 6°C through a time interval of 3.5 hours.

One possible explanation for the decreased release of cortisol in low temperatures could be the reduced gill permeability (Ellis et al., 2004). In rainbow trout, *Oncorhynchus mykiss*, gill arches cultured *in vitro* showed a negative effect of temperature on the permeability of the gills for non-electrolyte substances (Isaia, 1979). Another important factor is the branchial blood flow and the ventilatory water flow, since in both cases higher flow could result in increased release of cortisol (Ellis et al., 2004; Scott & Ellis, 2007). In this respect, cardiac output seemed to increase with temperature in rainbow trout (Farrell et al. 1996), as did the ventilatory flow in common carp (Klyszejko et al. 2003). In E. sea bass, an increase in temperature within the thermal preferendum of the species (from 18°C to 22°C), has led to increased heart beat rate, yet this increase was compensated by a lower maximum stroke volume, leading to the same maximum cardiac output (Farrell et al. 2007).

In conclusion, this study clearly showed that water temperature significantly affects the basal levels, timing, magnitude and duration of peak values of stress indicators in E. sea bass, and is therefore a factor that can induce variability in cortisol in this species. The release rate of cortisol into the water was also significantly affected by temperature, being faster and of a higher intensity in the highest temperature examined. These results may provide a basis to

understand the important differences observed in stress intolerance, disease susceptibility, and performance of E. sea bass individuals, especially when reared in temperatures close to the lower and upper limits, in which they may be exposed in open sea cage rearing conditions or in the wild. Finally, these differences should be taken into consideration when evaluating the stress status of fish either in laboratory or field conditions.

Chapter III. Cortisol dynamics and peripheral regulation in European sea bass, *Dicentrarchus labrax* L., upon exposure to increasing allostatic loads.

3.1. Introduction

The concept of allostasis, which states that animals 'achieve constancy through change' (adjusting set points of regulatory loops to prevailing needs; Sterling and Eyer, 1988; McEwen and Wingfield, 2003) is gaining popularity in stress physiology. Allostasis involves synthesis of prior knowledge with predicted current needs and resetting of one or more physiological set points accordingly. A successful stress response involves the reorganisation of the organism´s energy budget, their immune system, and neural and endocrine mechanisms to successfully cope with a given stressor. If the response fails or is inadequate, an allostatic overload occurs. This is usually seen under chronic stress conditions when individuals are not able to successfully cope with continuous stress challenges (Korte et al., 2007). The term *allostatic load* is used to describe the capacity of an organism to cope with a certain challenge by adapting its behaviour and physiology. Stress responses meant to be compensatory and adaptive, and that allow the animal to overcome the threat are referred to as eustress. However, when an animal is facing an intense or chronic stress, the stress response might lose its adaptive significance, become dysfunctional and ultimately result in adverse effects such as inhibition of growth, failure to reproduce, and impeded resistance to pathogens (distress; Pickering and Pottinger 1989; Iwama et al., 2004). Such effects of the allostatic load on the stress response can add substantial variability in the outcome of the response, so within populations due to individual differences, as among populations or contexts due to different allostatic loads.

In this context, the aim of the present study was to define allostatic load by developing a protocol of repeated predictable stress of various intensities and to examine cortisol dynamics and peripheral regulation under such conditions in E. sea bass. To do so, fish were exposed to increasing levels of repeated stress episodes combining common aquaculture stressors, such as confinement, chasing and air-exposure and then subjected to an additional acute stress test. Fish were sampled prior to this acute stressor for resting values and one-hour post-stress to assess interrenal steroid production capacity.

3.2. Materials and Methods

3.2.1. Fish and husbandry conditions

Hatchery-produced E. sea bass (14-month-old) were provided by the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Centre for Marine Research (HCMR). In total, 160 seabass of 28.7 ± 4.0 cm (mean ± SD) fork length and 380.0 ± 83.1 g body mass were used. Fish were kept at HCMR in Gournes, Crete, Greece. Duplicate groups of fish were divided according to body weight over 8 cylindrical 500 L tanks with flow-through filtered seawater at a final stocking density of 16.2 ± 0.2 kg m^{-3}. The fish were then left to acclimatize for three weeks before the start of the experiment. The water temperature was kept at 19°C and the photoperiod was set at 12L:12D. Fish were fed *ad libitum* using a commercial diet (Irida S.A., Greece) of approximately 44% protein and 19% lipids. The quantity of consumed food was measured daily per tank, by collecting uneaten pellets within 1 hr after feeding.

3.2.2. Experimental design

The experimental treatment consisted of exposing fish groups to 3 different stress protocols, varying in intensity, over a period of 21 days. The stressors used were chosen in a way that would reflect common aquaculture practices and have been previously shown to elicit stress responses in E. sea bass. Specifically, these stressors were confinement (Di Macro et al., 2008; Karakatsouli et al., 2012), confinement and chasing (Castillo et al., 2008; Samaras et al., 2015) and a combination of confinement, chasing and air-exposure (Fanouraki et al., 2011) (**Table 3.1**).

Table 3.1. Description of the stressors applied to E. sea bass in the 3 different stress loads.

			Stress Load		
Stressor	**Time (min)**	**Frequency**	**Low**	**Medium**	**High**
Confinement*	30	Every 2 days	√	√	√
Chasing	5	Every 2 days		√	√
Air exposure	1	Every 7 days			√

Confinement and chasing were performed once per two days; air-exposure was performed once per week.
*Confinement in the Low and Medium stress groups was performed by restraining fish to the 50 % of the initial water volume, while in the High stress group to the 25 % of the volume.

In details, the low stress regime consisted of subjecting fish to a confinement stressor for 30 min every 2nd day; this was accomplished by placing a net-trap (**Fig 3.1**) into the tank to decrease the available space to 50 %. The medium load consisted of subjecting fish to both confinement (conducted as previously described) and chasing of the fish for 5 min with a net every 2nd day. The high load consisted of confinement (to only 25 % of the tank volume) for 30 min, chasing for 5 min every 2nd day, and air exposure for 1 min once per week.

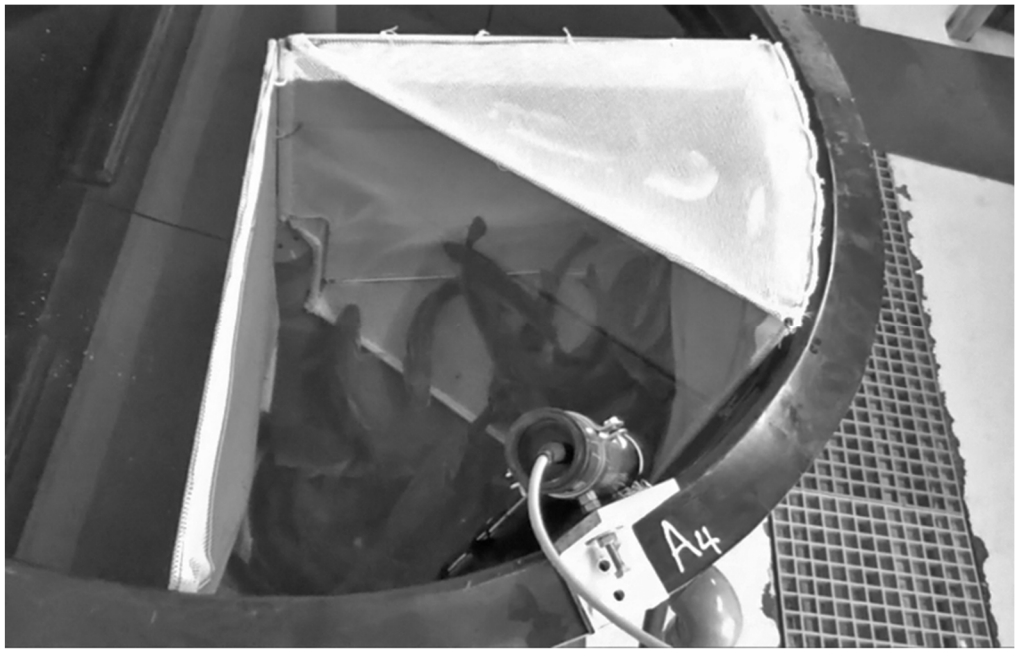


Fig. 3.1. The prototype net-trap designed by Dr. N. Papandroulakis at HCMR. This photo depicts the confinement of fish to 25 % of the volume, as performed at the high load group.

Two days after the end of the application of the stress protocol 10 out of 20 fish per tank were immediately sampled using the net-trap and deeply anesthetized in 500 ppm of 2-phenoxyethanol. These fish will hereinafter be referred to as T0 fish. Blood was drawn via heparinized syringes, centrifuged (2,000 g for 10 min) and the plasma was stored at -80°C until further analysis. The spinal cord was cut to kill the fish and liver and head kidney samples were collected, snap-frozen in liquid N_2, and stored at -80°C. The 10-remaining fish were acutely stressed by subjecting them to chasing for 5 min and air-exposure for 1 min. Subsequently, the fish were then left undisturbed for 1 h (*i.e.* at peak cortisol values following a stress event; Fanouraki et al., 2011) and then deeply anesthetized before sampling (T1 fish), as described above.

3.2.3. Analytical procedures

Plasma cortisol analysis was performed by radioimmunoassay in a 96-wells plate as described by Gorissen et al., (2012), using ^{3}H-hydroxotisone (PerkinElmer, USA).

3.2.4. RNA purification and cDNA synthesis

Liver and head kidney samples (20-30 mg) were 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 using the RNeasy Plus Mini Kit (Qiagen, Valencia, USA), according to manufacturers' instructions. RNA yield and purity were determined by measuring the absorbance at 260 and 280 nm, using Nanodrop® ND-1000 UV-Vis spectrophotometer (Peqlab, Erlangen, Germany), while its integrity was tested by gel electrophoresis in 1% agarose. Reverse transcription (RT) was performed using 1 µg RNA with the QuantiTect Reverse transcription kit (Qiagen, Valencia, USA), according to manufacturers' instructions.

3.2.5. Quantitative real-time PCR (qPCR)

The mRNA expression of genes encoding for *gr1*, *gr2*, *mr*, *11β-hx*, *11β-hsd2* and *mc2r* was determined with quantitative polymerase chain reaction (qPCR) assays using the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Willmington, USA). Oligonucleotides used in the qPCR analysis have been published by Tsalafouta et al., (2014) and are shown in **Table 3.2**. Reactions were cycled and the resulting fluorescence was detected with MJ Mini Thermal Cycler (Bio-Rad) under the following parameters: (1) 95˚C for 3 min (HotStarTaq DNA Polymerase activation step), (2) 94˚C for 15 s (denaturation step), (3) 60˚C for 30 s (annealing step), (4) 72˚C for 20 s (extension step), cycling steps (2) to (4) for 40 cycles. Levels of *gr1*, *gr2*, *mr*, *11β-hx* and *11β-hsd2* mRNA were normalized based on the reference genes *β-actin*, *eEF1α*, and *18s*. A relative standard curve was constructed for each gene, using 4 sequential dilutions (1:5) of a pool of all the cDNA samples. geNORM analysis was also performed to validate which were the most suitable reference genes to serve as internal control and showed that *β-actin*, and *eEF1α* were the most appropriate.

Table 3.2. Primer sequences used in qPCR.

Gene	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
β-actin	CGCGACCTCACAGACTACCT	AACCTCTCATTGCCGATG
eEF1α	GCCAGATCAACGCAGGTTACG	GAAGCGACCGAGGGGAGG
18s	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCACA
gr1	GAGATTTGGCAAGACCTTGACC	ACCACACCAGGCGTACTGA
gr2	GACGCAGACCTCCACTACATTC	GCCGTTCATACTCTAACCAC
mr	CCTGTCTCCTCATGAATGG	AATCTGGTAATGGAATGAATGTC
11β-hsd2	CACCCAGCCACAGCAGGT	ACCAAGCCCCACAGACC
11β-hx	GGAGGAGGATTGCTGAGAACG	AGAGGACGACACGCTGAGA
mc2r	CATCTACGCCTTCCGCATTG	ATGAGCACCGCCTCCATT

3.2.6. Statistical analysis

Statistical analysis was performed using the SPSS v.22 (IBM Corp., Armonk, NY, USA) statistical package and the graphs were created using Graphpad Prism 6.0 software (GraphPad Software, USA). Results are presented as means ± standard deviation (SD). Data from plasma analyses were assessed by nested two-way Analysis of Variance (ANOVA), analyzing factors *allostatic load* and *acute stress*, with factor *tank* being nested in *allostatic load*. Gene expression data were tested using nested one-way ANOVA with the factor *tank* being nested in the factor *allostatic load*. When significant differences existed, at a level of $P < 0.05$, Tukey's post-hoc multiple comparison analysis was subsequently performed. Prior to analysis, data were checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test and, when necessary, data were log-transformed before analysis.

3.3. Results

3.3.1. Chronic stress and food intake

In E. sea bass mass gain seemed to decrease with increasing stress intensity, leading even to weight loss at the highest intensity, though no statistical analysis was performed due to the small sample size (N=2). In feed consumption, there was a significant interaction between stress and time ($F_{6,95} = 2.36$; $P = 0.037$), with higher feed consumption in the control group compared to stress groups in the 2nd and 3rd week of the experiment (**Fig. 3.1**).

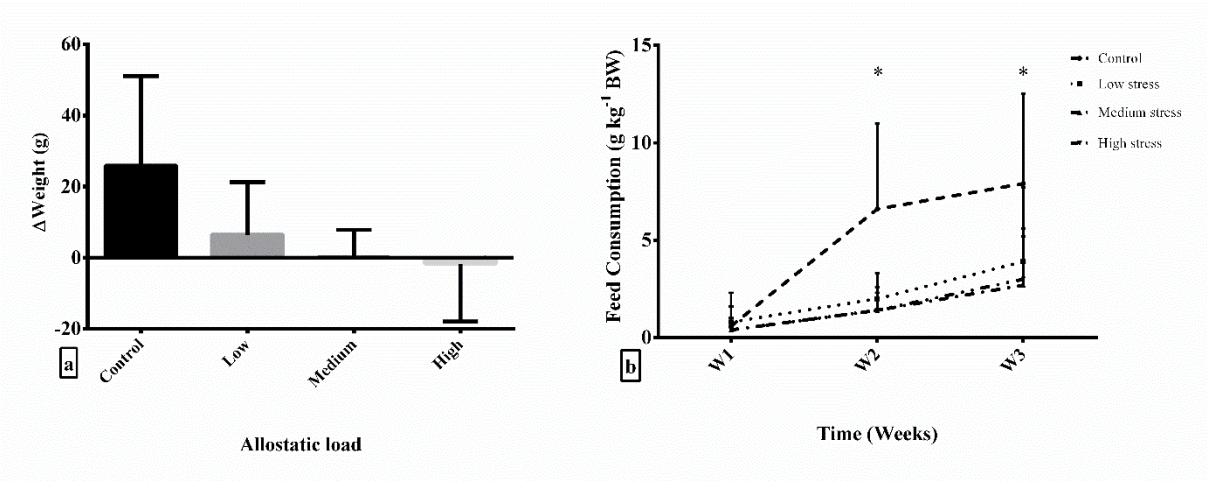


Figure 3.1. Body weight gain in E. sea bass (a) in controls and after exposure to low, medium and high allostatic load (mean + SD; n = 40). Daily food consumption of seabass (b) expressed as grams of dry food per kg of fish (n = 2). Two-way ANOVA showed significant differences between control and the rest of the groups in food consumption (* $P < 0.05$).

3.3.2. Plasma cortisol

Allostatic load resulted in increased variability of cortisol among individuals, indicating that some individuals were more susceptible to stress than others within the same allostatic load group (**Table 3.3**). Specifically, the T0 minimum values in the low and medium load groups were at the same level with those of the control group, whereas their maximum levels were more that 2-fold higher, and this resulted in a much higher range (**Table 3.3**). In the high load group, however, both minimum and maximum levels were much higher than the other groups. In T1, the opposite was true, with much higher minimum values in the control than all the stress groups (**Table 3.3**).

In terms of mean concentrations, there was a statistically significant interaction between *allostatic load* and *acute stress* ($F_{3,147} = 29.27$; $P < 0.001$) (**Fig. 3.2**). Specifically, when comparing T0 cortisol levels between groups, the group with the highest stress load showed significantly higher concentrations than the other groups. However, when comparing T1 levels between the different groups, a gradual decrease with increasing load was observed (**Fig. 3.2**). Finally, within each respective allostatic load group, a cortisol increase was observed at T1 (after an additional acute stress episode) when compared to T0, in all groups except the highly-stressed fish, which did not respond to acute stress with a cortisol response, rather cortisol levels decreased (**Fig. 3.2**).

Table 3.3. Descriptive statistics of cortisol levels in E. sea bass in controls and after exposure to chronic- low, medium, and high allostatic load, before (T0) and after an acute stress test (T1). The range expresses the max-min value, while 5-95% represents the max-min value of data belonging to 5-95% of the distribution to avoid extreme outliers.

	Mean (ng ml^{-1})	SD	CV%	Min	Max	Range	5-95% range
			T0				
Control	79.4	45.0	56.7	18.7	168.8	150.1	133.7
Low	140.5	82.0	58.4	30.3	386.8	356.5	340.0
Medium	118.7	94.3	79.4	12.1	375.1	363.0	254.8
High	252.1	116.3	46.1	115.8	596.4	480.8	296.4
			T1				
Control	457.4	116.5	25.5	220.5	680.5	460.0	309.9
Low	287.8	192.7	67.0	51.3	750.2	698.9	520.3
Medium	293.0	123.3	42.1	52.3	497.5	445.2	370.5
High	143.0	88.6	62.0	38.4	379.0	340.6	238.9

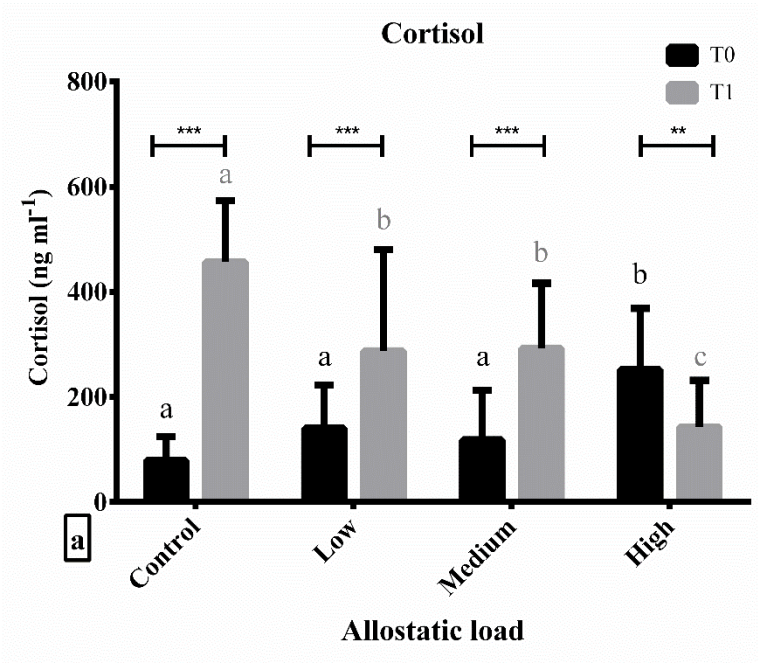


Fig. 3.2. Cortisol concentrations in controls and after exposure to low, medium, and high allostatic load, before (T0) and after an acute stress test (T1). Values are presented as means + SD (n = 20). Different letters indicate statistically significant differences in plasma cortisol concentrations between treatment groups at T0 (black columns/lettering) and T1 (grey columns/lettering) ($P < 0.05$), while asterisks indicate significant differences between T0 and T1 within each treatment group ($^{**}P < 0.01$; $^{***}P < 0.001$).

3.3.3. Gene expression in liver and head kidney

Allostatic load did not affect the hepatic expression of the glucocorticoid receptors ($F_{3,16}$ = 0.176; P = 0.911 for *gr1*, and $F_{3.14}$ = 2.101; P = 0.146 for *gr2*) (**Fig. 3.3 a** & **b**) and the mineralocorticoid receptor ($F_{3,15}$ = 2.036; P = 0.155) (**Fig. 3.3 c**). However, there were significant differences in the ratio of *gr1* to *mr* ($F_{3,15}$ = 5.619; P = 0.009), specifically being higher in the high when compared to the low load group (**Fig 3.3 d**). The ratio between *gr2* and *mr*, on the other hand, did not differ ($F_{3,13}$ = 1.318; P =0.311) (**Fig. 3.3 e**). Finally, *11β-hsd2*, the enzyme that catabolizes cortisol, was significantly affected by allostatic load ($F_{3,15}$ = 3.777; P = 0.033), being lower in the high than the low and medium groups (P = 0.022 and 0.048, respectively) (**Fig. 3.3 f**).

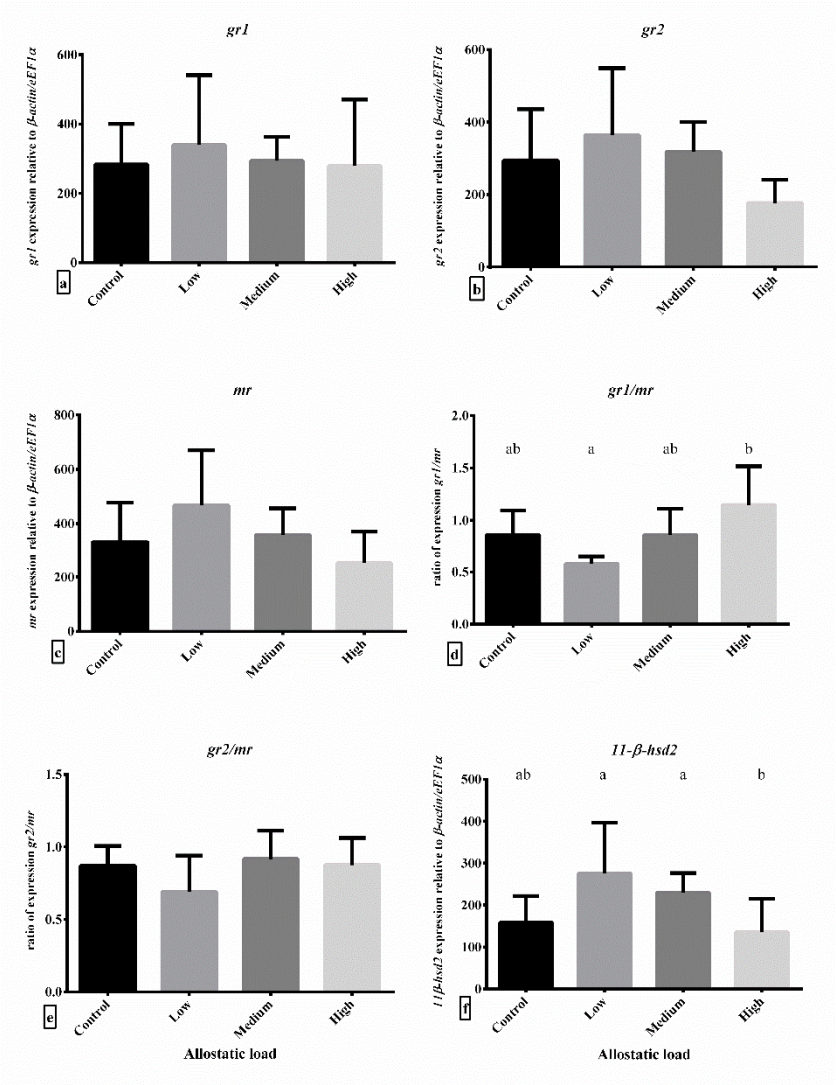


Fig. 3.3. Hepatic mRNA transcript levels of *gr1*, *gr2*, *mr*, *gr1/mr*, *gr2/mr*, and *11β-hsd2* for control fish and groups previously subjected to low, medium and high allostatic load. Values are presented as means + SD (n = 6). Means with different letters differ significantly from one another (P < 0.05).

In the head kidney, allostatic load did not have any effect on the expression of *gr1* and *gr2* (*gr1*: $F_{3,12} = 1.251$; $P = 0.335$; and *gr2*: $F_{3,13} = 0.944$; $P = 0.448$) (**Fig. 3.4 a** & **b**) and *mr* ($F_{3,12} = 1.145$; $P = 0.371$) (**Fig. 3.4 c**). The same results were observed for the ratios between *gr1* to *mr* ($F_{3,11} = 1.068$; $P = 0.402$), and *gr2* to *mr* ($F_{3,12} = 1.572$; $P = 0.247$) (**Fig. 3.4 d** & **e**).

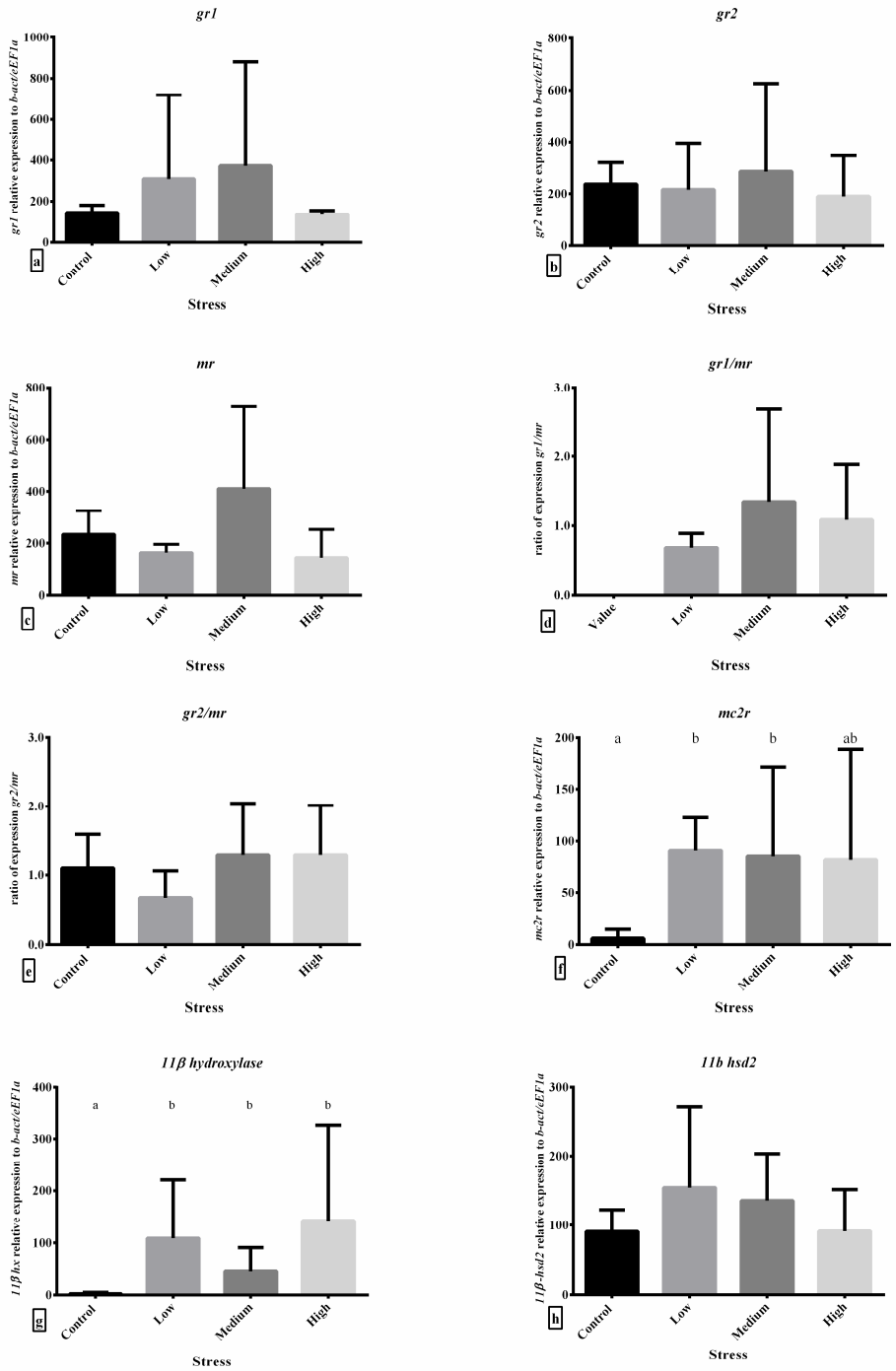


Fig. 3.4. Head kidney mRNA transcript levels of *gr1*, *gr2*, *mr*, *gr1/mr*, *gr2/mr*, *mc2r*, *11β-hydroxylase* and *11β-hsd2* for control fish and groups previously subjected to low, medium and high allostatic load. Values are presented as means + standard deviation (n = 6). Means with different letters differ significantly from one another ($P < 0.05$).

The expression of *mc2r* gene, on the other hand, was affected by allostatic load in the head kidney ($F_{3,12}$ = 4.078; P = 0.033) (**Fig. 3.4 f**), showing an up-regulation in the low and medium load groups when compared to the control (P < 0.05). The same was true for the expression of *11β-hydroxylase* ($F_{3,12}$ = 5.706; P = 0.012) (**Fig. 3.4 g**), with an over-expression in all stress regimes when compared to controls (P < 0.05). Finally, *11β-hsd2* expression was not affected by allostatic load ($F_{3,13}$ = 0.780; P = 0.526) (**Fig. 3.4 h**).

3.4. Discussion

This is the first time that the concept of allostasis was attempted to be applied in this species of high cortisol response and variability. Insight in fish stress handling is crucial to guarantee welfare and product quality (Van de Vis et al., 2012). Moreover, understanding the mechanisms of response to increasing load of chronic repeated handling stress is of importance in order to assess the welfare and variation of cortisol under such treatments.

It is well known that stress leads to decreased food consumption and growth in fish (Barton et al., 1987; McCormick et al., 1998; Leal et al., 2011). Indeed, fish in this study decreased their feed consumption due to exposure to stress, while their body mass also decreased with increasing allostatic load. It is known, that E. sea bass is sensitive to common aquaculture practices such as tank cleaning, which can lead to reduced feed intake (Rubio et al., 2010), and reduction in growth (Leal et al., 2011). It is, in general, believed that reduced feeding intake induced by stress is regulated by various biological functions, combining behavioral and physiological adaptations to stress (Bernier, 2006), accompanied with increased energy expenditure reallocation (Barton et al., 1987; Leal et al., 2011), which may in turn lead to growth reduction. Cortisol, specifically, seems to be an important factor leading to reduced feeding and growth in fish (Gregory & Wood, 1999; Leal et al., 2011).

Cortisol in fish combines glucocorticoid and mineralocorticoid actions, by redistributing energy away from growth and reproduction towards survival mechanisms including regulation of hydromineral balance (Colombe et al., 2000; Takei and Hwang, 2016). Therefore, high concentrations of circulating cortisol can affect a wide range of metabolic, immune and reproductive functions (Mommsen et al., 1999; Wendelaar Bonga 1997). In the present study when E. sea bass fish were subjected to a high level of allostatic load, resting plasma cortisol levels were significantly elevated compared to both control and lower stress load groups. Moreover, there were no differences in cortisol levels between non-stressed groups, and the low and medium stress groups. Taken together, this might indicate that in the strongly stressed

fish, which showed increased cortisol, the stress intensity exceeded the coping ability of the fish and led to a dysregulation of the HPI axis (Schreck, 2010; Sørensen et al., 2013). Notably, the high-stressed E. sea bass group was also not able to surmount an increase in cortisol levels to an acute stressor, suggesting stress overload (Gorissen and Flik, 2016). In fact, cortisol levels in these fish were lower after acute stress indicating possible exhaustion of the interrenals (Ram and Singh, 1988; Hontela et al., 1992; Madaro et al., 2016).

One major site of regulation of the cortisol stress response is the brain. For that reason, the expression of HPI-related genes has also been estimated in the pre-optic area (POA) and telencephalon of these fish, though these data were not analyzed in the context of the present thesis (Samaras & Espirito-Santo et al., submitted). These data showed an overexpression of *crf* in POA of the highly-stressed fish and a dramatic increase in the *gr1* and *gr2* to *mr* ratio in this brain area. This ratio shift was directly the result of increased *gr1* expression and decreased *mr* expression, which may be explained by differential feedforward and feedback mechanisms of cortisol on these receptors, respectively. Shifts in *gr*/*mr* ratio are indicators of impaired appraisal, poor learning and fear avoidance in vertebrates (de Kloet et al., 1999; Lupien & Lepage, 2001; Joëls et al., 2008). In zebrafish (Manuel, 2015) and trout (Johansen et al., 2011) chronic stress increased the brain *gr* to *mr* ratio and this was associated with diminished cognitive quality and inhibitory avoidance learning. In mammals, *gr* to *mr* ratio shifts make the brain prone to steroid-induced pathologies (de Kloet et al., 2005), and it is tempting to speculate that the same holds for fish. In this context, the *gr* to *mr* ratio can be considered a good indicator of allostatic overload. Notably, this seems to be confirmed by the impaired cortisol response to an additional acute stressor in the highly-stressed fish.

Two other sites of regulation of cortisol are the liver, due to its catabolic actions, and the interrenal tissue in the head kidney, since it is the site of cortisol production. In both tissues, however, allostatic load seemed to have no effect on the expression of cortisol receptors, *gr1*, *gr2*, and *mr*, except a lower hepatic *gr1* to *mr* ratio in the low stress compared to the high stress group. Results from the head kidney are in line with data from Atlantic salmon faced with unpredictable chronic stress (Madaro et al., 2015).

In general, cortisol is believed to autoregulate its receptor, *gr*, by exerting a negative feedback in the liver (Mommsen et al., 1999; Sathiyaa & Vijayan, 2003; Vijayan et al., 2003), as well as exert an ultra-short-loop negative feedback in cortisol production from the interrenal tissue (Bradford et al., 1992). Specifically, chronically increased stocking density stress in E. sea bass has led to reduced expression of the *gr* gene in the liver (Terova et al., 2005), while both *in vivo* cortisol administration (Vijayan et al., 2003) and *in vitro* culture of rainbow trout

hepatocyte in medium containing cortisol (Sathiyaa & Vijayan, 2003) showed increased *gr* expression, but reduced actual abundance of the receptor protein. This autoregulation may be crucial in the physiological stress response process, especially regulating glucocorticoid-dependent metabolic processes, such as gluconeogenesis and protein and fatty acids metabolism (Sathiyaa & Vijayan, 2003). However, no regulation in the expression level of the hepatic and head kidney *gr* was observed in the present study in fish under different allostatic loads, although fish in the high stress group showed higher cortisol levels than the other groups.

It is important to note, however, that studies in rainbow trout have shown that chronically high cortisol levels via cortisol implants can lead to a mismatch in *gr* mRNA expression and GR protein abundance (Vijayan et al., 2003). In details, these studies suggest that although *gr* mRNA expression can be upregulated by cortisol, an inhibition in the translational machinery and/or post-translational modifications can result in enhanced GR protein breakdown, and thus lower abundance of GR protein (Sathiyaa & Vijayan, 2003; Vijayan et al., 2003). It has been suggested that the proteasome is involved in this cortisol-mediated GR breakdown in trout hepatocytes (Boone & Vijayan, 2002). It seems therefore, that autoregulation in the expression of the *gr* gene and the actual protein abundance can show a mismatch under cortisol treatment, making the interpretation of the gene expression results complex.

The liver apart from regulating many metabolic and energy-dependent processes, is also a key target organ for cortisol disposal (Mommsen et al., 1999). One of the enzymes catabolizing cortisol is *11β-hydroxysteroid dehydrogenase type 2* (*11β-hsd2*) which inactivates cortisol to cortisone (Mommsen et al., 1999). It has been proposed that *11β-hsd2* can inactivate 30-40% of the daily cortisol production (Chapman et al., 2013) and inhibition of its actions has led to elevated cortisol levels in zebrafish (3.8-fold increase; Alderman & Vijayan, 2012). In mammals, this enzyme is essential in MR-rich tissues, such as the kidney, where it inactivates cortisol in order to prevent inappropriate activation of the MR, which binds both glucocorticoids and aldosterone with similar or greater affinity (Edwards et al., 1996; Alderman & Vijayan, 2012). Teleost fish, however, do not have the capacity to synthesize aldosterone and up till now the only potent ligand of MR is cortisol, with 11-deoxycorticosterone being a candidate for consideration (Sturm et al., 2005; Prunet et al., 2006; Stolte et al., 2008). Therefore, the few studies on the distribution of this enzyme in fish tissues show a multi-tissue expression of this enzyme (Kusakabe 2003; Alderman & Vijayan, 2012).

In the present study, the gene encoding this enzyme was significantly less expressed in fish exposed to high when compared to low and medium stress loads, but not the controls. The mechanisms regulating the expression of this gene are limited in both fish and mammals,

especially when it concerns hepatic regulation. In other tissues, such as the mammalian kidney factors like IGF-1 (Huang et al., 2010), and estrogen (Chapman et al., 2013) can regulate the expression of this enzyme, as does the administration of human Chorionic Gonadotropin (hCG) in trout testis (Rasheeda et al., 2010). Therefore, given the lack of knowledge in this respect, it cannot be elucidated whether stress, increased cortisol levels, reduced feeding or any other factor led to this downregulation in the high stress when compared to the low and medium stress groups.

Although the regulatory mechanism leading to this reduced expression of *11β-hsd2* is unknown, this downregulation could be one possible factor explaining the high cortisol levels observed in the highly-stressed fish, induced by a lower inactivation rate in the liver when compared to the other stress groups. This could be further supported by the fact that although the head kidneys of fish of stressed groups seemed to over-express genes encoding for the receptor of ACTH (*mc2r*) and the hydroxylase involved in the synthesis of cortisol (*11β-hydroxylase*), higher plasma cortisol levels were only observed in the high stress group. Additionally, *11β-hsd2* gene expression was not differentially regulated between control and stress groups, leading to hypothesize that there was no effect of allostatic load on cortisol catabolism in the head kidneys. These data suggest that allostatic load can affect the sensitivity of the head kidneys to ACTH, as seen by the over-expression of *mc2r*, and the biosynthetic capacity, as seen by the over-expression of *11β-hydroxylase*, but no difference existed between the stress load groups which could explain the increased plasma cortisol concentrations observed only in the high stress group. Taking all the above into consideration, combined with results from the hepatic gene expression of *11β-hsd2*, it could be suggested that the high cortisol levels seen in this group could partly be a result of reduced cortisol inactivation in the fish liver.

Regarding the regulation of expression in the head kidney, literature concerning the control of *mc2r* by stress is not comprehensive. Up until now, there is only one study in line with the present study, showing an up-regulation of *mc2r* in isolated interrenal tissue after 5 days of exposure to chronic stress (Fierro-Castro et al., 2015). Previous studies in E. sea bass have shown that *mc2r* was unaffected by chronic stress (defined as tank cleaning once of three-times per week for 33 days) (Agulleiro et al., 2013), which was also the case in Atlantic salmon faced with unpredictable chronic stress (Madaro et al., 2015). Down-regulation of *mc2r*, on the other hand, was observed in carp confined for 7 days, which, however, was not accompanied by an alteration of the interrenal synthesis and release capacity (Metz et al., 2005). In general, it has been suggested that ACTH positively regulates the expression of *mc2r* mRNA *in vitro* (Aluru & Vijayan, 2008), while cortisol administration has the opposite result (Agulleiro et al.,

2013). Given, however the divergent results presented between the different studies and the fact that there is no antibody that allows quantification of *mc2r* protein, it seems difficult to exclude solid conclusions on the regulatory role of this receptor in chronic stress.

Enzymes involved in the biosynthesis of cortisol have been reported to be regulated upon chronic stress. In line with the present study that showed an upregulation of *11β-hydroxylase* upon exposure to chronic stress, cortisol biosynthetic enzymes (*StAR; P450; 3β-hsd; 11β-hydroxylase*) were significantly upregulated in chronically stressed rainbow trout (Fierro-Castro et al., 2015). In Atlantic salmon, however, unpredictable chronic stress had no effect in the expression of *StAR* (Madaro et al., 2015). ACTH administration *in vitro* has also been shown to cause an upregulation in cortisol biosynthetic enzymes, such as *StAR* and *P450* (Aluru & Vijayan, 2008).

In summary, fish exposed to high allostatic load showed signs of allostatic overload, reflected by a reduction in the bodyweight, an inability to regulate resting and post-stress cortisol levels, and an upregulation in *gr* and downregulation in *mr* expression in hypothalamic POA. Moreover, the hepatic expression of *11β-hsd2* in the high stress group was lower than the other stress groups, denoting the possibility of reduced cortisol inactivation in this group. Finally, although the expression of *mc2r* and *11β-hydroxylase* were upregulated after exposure to the stress protocol, except *11β-hydroxylase* in the high-stress group, there were no differences between the stress regimes that could explain the high cortisol levels observed in the high stress group. Therefore, no solid conclusions regarding the biosynthetic capacity of the head kidney under different allostatic loads can be extracted. Further study in the level of the head kidney using both *in vivo* (e.g. ACTH or ACTH + Dexamethasone administration) and *in vitro* (i.e. superfusion experiments) approaches, would be required to get a better insight in the effects of chronic stress in this tissue. Moreover, apart from mRNA studies, it is essential to quantify the abundance of proteins that are in fact present in the interrenal tissue, as well as their activities, as far as enzymes are concerned, and affinities, when receptors are under study. In this respect, it is also important to notice that the head kidney is a multi-tissue organ, with mixed cellular diversity and multiple functions. A recent study has shown that the use of isolated interrenal cells instead of whole head kidney extracts substantially increases the detection of regulatory processes that take part in these cells (Fierro-Castro et al., 2015). Such separation in future studies would be essential in leading to more accurate results.

Chapter IV. Genetic component and individual repeatability of the cortisol stress response in European sea bass, *Dicentrarchus labrax* L.; transcriptional differences between individuals with divergent responses

4.1. Introduction

One of the most classic concepts in biology is that the phenotype of an individual can be defined as the sum of the effects of its genotype and the environment

phenotype (P) = genotype (G) + environment (E),

while more recent models also incorporate the interaction between the genotype and the environment, leading to a more elaborate model of

phenotype (P) = genotype (G) + environment (E) + genotype (G)*environment (E).

Therefore, it had become clear since the early days of farming that optimum environmental conditions would lead to improved yields. Moreover, selection of the more suitable genotypes, by selecting animals of the desired phenotype to use as breeders, was also practiced since early in farming. Especially after the description of the laws of inheritance by Gregor Mendel and the more recent advances in quantitative genetics and genetic engineering, selective breeding has become a very important aspect in agricultural industries and sciences. Although the first record of selective breeding in carp goes back to around 500 B.C. (Hershberger, 1990), the majority of genetics-based selective breeding in fish farming did not start before 1950s, considerably later than plants and terrestrial farm animals (Gjedrem, 1983; Gjedrem, 2012).

Since the early days of selective breeding in aquaculture, the traits of interest have mainly been directly economically-defined, such as growth rate, food conversion efficiency, resistance to disease, meat quality and age at maturation (Gjedrem, 1983). However, attention to possible side effects of selection based on these criteria, such as heart failures, has been raised in salmonid fish (Vandeputte et al., 2016). Moreover, there are other traits of interest that can indirectly affect those of economic value, such as stress, which can affect growth and food intake (Barton et al., 1987; Leal et al., 2011), disease resistance (Pickering & Pottinger, 1989)

and flesh quality (Pottinger, 2000; Poli et al., 2005). Specifically, strong genetic correlations between cortisol and lysozyme (Fevolden et al., 1999) and phenotypic correlations between cortisol, weight, and specific growth rate (Fevolden et al., 2002) have been observed in rainbow trout. It seems therefore, that new selection criteria should also be evaluated and incorporated in selective breeding programs, especially in traits that show a considerable amount of variability among individuals, such as cortisol responsiveness in E. sea bass.

Divergence in the cortisol response has been described in many vertebrates, including fish (Øverli et al., 2007; Castanheira et al., 2015), amphibians (Narayan et al., 2013), reptiles (Øverli et al., 2007), birds (Cockrem et al., 2013), and mammals (Benus et al., 1991; Sgoifo et al., 1996). Fish, in particular, are among the most widely studied taxa in this aspect, and consistent differences in the cortisol response between individuals of the same species have been described (Pottinger et al., 1992; Tort et al., 2001; Weil et al., 2001; Fevolden et al., 2002; Wang et al., 2004; Hori et al., 2012a). Consequently, low (LR) and high (HR) responding fish have been identified in respect to the intensity and consistency of their cortisol response after exposure to acute stressors. Cortisol responsiveness has also shown moderate to high heritability in rainbow trout, ranging between 0.22 to 0.56 (Pottinger & Carrick, 1999; Fevolden et al., 1999; Weber et al., 2008; Vallejo et al., 2009), with strains of low and high responding individuals having been established (Pottinger & Carrick, 1999) though still merely used for research purposes. This divergence in cortisol responsiveness has also been associated with behavioral differences and coping styles (Øverli et al., 2005; Ruiz-Gomez et al., 2011), as well as animal performance and fitness. Specifically, differences in growth (Fevolden et al., 2002; Pottinger, 2006; Weber & Silverstein, 2007), plasma metabolites (Tort et al., 2001; Trenzado et al., 2006), hepatic carbohydrates reserves and enzymatic activity (Trenzado et al., 2006), and differential expression in many genes involved in physiological functions including metabolic and immune functions (Pemmasani et al, 2011) have been observed between LR and HR individuals.

Still however, individual divergence in cortisol responsiveness has not been identified in E. sea bass, a species of major economic importance in the Mediterranean aquaculture. This species shows an intense stress response (Fanouraki et al., 2011) and is considered susceptible to stress by inducing reproductive dysfunctions and disease outbreaks (FAO, 2016), which, in turn, hamper production. Moreover, cortisol concentrations are characterized by high variability in both basal and response levels, so between studies (Ellis et al., 2012) as between individuals of the same population (Fanouraki 2010). Consequently, it is of great importance to gain better knowledge on the stress physiology of this species by investigating the

divergence of the response among individuals, since stress responsiveness can affect their performance (Fevolden et al., 2002; Pottinger, 2006). Previous studies have shown low (h^2 = 0.08; Volckaert et al., 2012) to moderate (h^2 = 0.34 Vandeputte et al., 2016) heritability of post-stress cortisol concentrations in this species, while LR and HR fish have not been identified. Yet, three suggestive Quantitative Trait Loci (QTL) for cortisol stress response have been identified (Massault et al., 2009), indicating the existence of a genetic background regulating this response. It could be therefore suggested that an important confounding aspect in the studies showing low heritability (Volckaert et al., 2012) or no consistency in the cortisol response (Fanouraki, 2010) in E. sea bass might have been that the progeny used was produced from a breeding population with a small effective size and hence low genetic variability.

In this context, the main objectives of this study were to investigate the variability of the cortisol stress response in E. sea bass families and individuals, to characterize possible differences in free cortisol concentrations among LR and HR individuals, and to get a better insight into the liver transcription profile of LR and HR fish. Such results could provide better knowledge of the stress physiology of the species, which could also constitute a potentially new selection trait to consider in selection programs for the species in aquaculture. Finally, analysis of the hepatic transcriptome between LR and HR fish could provide better insight to the metabolic and immune mechanisms that accompany the LR and HR phenotypes.

4.2. Material and Methods

4.2.1. Fish and husbandry condition

Six full sib families of E. sea bass were randomly chosen out of 96 families from a Greek fish farm (Nireus S.A.) family-based breeding program. All families were created on the same day from different parents (6 females and 6 males). Each of the 6 families consisted of 12 sexually immature fish (17 months-old at the beginning of the experiment), randomly chosen from the whole progeny of each respective family (mean weigh ± SD of 93.2 ± 20.5 g). Each family was reared separately in open circulation tanks, at Nireus S.A. research facilities at Pyrgoulaki, Euboea. Fish were fed twice per day, for 6 days a week, using a commercial diet (Blue Line 45:20 3.5 mm, Feedus S.A., Greece). Throughout the experimental period, the photoperiod was set at 12L:12D, the water temperature was 18.20 ± 0.03°C, and the salinity 27. Oxygen and pH ranged between 6 – 10 mg L^{-1} and 7.20 – 7.40, respectively.

4.2.2. Sampling

In the first sampling, all fish per tank were confined by lowering the water of the tank and then chased with a net for 5 min. Fish were left confined for 30 min, and then carefully netted, immediately anaesthetized in 2-phenoxyethanol (300 ppm; Merck; 807291) and then weighed and tagged using unique glass PIT tags. Blood was immediately collected from the caudal vessel via heparinized syringes and centrifuged (2,000 g; 10 min), and the resulting plasma was stored at -20°C until analyzed. The stress protocol was repeated once a month for four consecutive months, termed hereafter as S1 to S4.

Fifteen days following the completion of the last stress experiment (S4), a final sampling was performed to assess resting cortisol values and to collect liver samples for mRNA analysis (S5). Fish were immediately captured, euthanized with high doses of anesthetic, and their blood was sampled as described previously. Liver samples collected for molecular genetic analysis were immediately frozen in liquid nitrogen and subsequently stored at -80°C until analyzed.

4.2.3. *z*-score and repeatability statistic calculation

In order to correct for variations in the cortisol data between the samplings the *z*-score was used instead of the raw data. By calculating *z*-scores of the individual post-stress cortisol levels for each sampling, the cortisol concentrations of each individual fish were standardized in respect to the overall mean and standard deviation of each respective sampling, as described below (Weil et al., 2001).

$$z = (x - \mu)/\sigma$$

where x is the cortisol concentration of each individual in each sampling, while μ and σ represent the mean and standard deviation of the population in each sampling, respectively.

The repeatability of the post-stress standardized cortisol levels was assessed using the repeatability statistic, *r* (Lessells & Boag, 1987). Repeatability, *r*, was calculated as

$$r = s_A^2/(s^2 + s_A^2)$$

where s_A^2 is the among-groups variance component and s^2 is the within-group variance components. These variance components were calculated from the mean squares in the analysis of variance as

$$s^2 = MS_w$$

and

$$s_A^2 = (MS_A - MS_w)/n_0$$

where MS_w refers to the within-groups mean squares, MS_A represents the among-groups mean squares and n_0 is a coefficient related to the sample size per group. Specifically, n_0 equals to the sample size when the group have the same sample size, as is the case in the present study. If the group sizes are not equal, n_0 is smaller than the mean group size, and the equation for this calculation can be found at the manuscript of Lessells & Boag (1987).

Therefore, in order to estimate the repeatability, a nested analysis of variance (ANOVA) was performed. The ANOVA model that was analyzed consisted of the factors *individual*, which was nested in the factor *family*. The null hypothesis was that individual *z*-scores were inconsistent within individuals and hence the variable was not repeatable. In other words, the consistency of the response within an individual across the four samplings was tested.

4.2.4. LR and HR fish identification

For the identification of LR and HR individuals, the sum of the *z*-scores (z_{sum}) of all samplings was calculated for each individual fish. If the cortisol response is a consistent trait, high (or low) responders should have high (or low) post-stress cortisol levels in all sampling. In this way, high responders should always have a positive and high z_{sum}, while low responders should always show lower cortisol concentrations than the overall mean, and have negative values of z_{sum}. Finally, fish with either intermediate or no consistent response should have intermediate value of z_{sum}. In this manner, the $z_{sum}$of all individuals were ranked, and fish belonging to the upper quartile of the distribution of z_{sum}were identified as HR, and those belonging to the lower quartile as LR fish. This method ensures that both the intensity and the consistent of the cortisol response were taken into consideration when characterizing these types of responses.

4.2.5. Analytical procedures

Plasma total and free (*i.e.* unbound) cortisol were measured using a commercial enzyme-linked immunoassay kit (DRG® Cortisol ELISA, DRG® International Inc, Germany). The separation of free and bound to proteins cortisol was performed based on the ultrafiltration method. Ultrafiltration is a membrane filtration system where the separation of solutes results from forces like pressure or concentration gradients. Specifically, proteins, and thus protein-bound hormones, become selectively partitioned into the fraction of the sample, not being able to pass through the low-adsorptive hydrophilic membranes and O-rings. Free ligands, on the other hand, pass unhindered through the membrane along with solvent (Robin et al., 1978). This method ensures the maintenance of the equilibrium conditions of the initial plasma, and does not require dilution compared to other methods, like equilibrium dialysis and gel filtration (Robin et al., 1978; Lentjes et al., 1993).

The protocol used for the separation of free and bound to proteins cortisol in the plasma, was based on that of Hosoya et al., (2007). Briefly, 200 μl of plasma were loaded in centrifugal filter units (Centrifree®-MPS, Micropartition devices, Millipore Corp, USA) and centrifuged at 2,000 g for 30 min at 18°C. Ultrafiltration is, in general, temperature sensitive (Spencer, 2010), and high centrifugal temperatures can result to increased levels of free cortisol (Robin et al., 1978; Lentjes et al., 1993). For that reason, and after trials of ultrafiltration in different temperatures, it was chosen to perform the ultrafiltration in the temperature that fish where reared, *i.e.* 18°C. After ultrafiltration, the resulting filtrate with the free cortisol was stored at -20°C until analyzed. Determination of free cortisol was performed in a subsample (n =10) consisting of the LR and HR individuals with the lowest and highest z_{sum}.

Plasma ACTH concentrations were estimated by a commercial, specialized for fish, enzyme-linked immunoassay kit (CUSABIO® Fish Adrenocorticotropic hormone [ACTH] ELISA kit, CUSABIO BIOTECH Co. Ltd, China). This assay is based on competitive inhibition reaction between the ACTH of the samples and externally added biotin-conjugated ACTH, with the pre-coated antibody specific for ACTH. According to manufacturer's instructions, this assay has high sensitivity and excellent specificity for the detection of fish ACTH, with no significant cross-reactivity or interference between fish ACTH and its analogues. Both intra- and inter-assay variation were less the 15% (CV% < 15). The standard curve and the extrapolation of the results were analyzed using the Curve Expert 1.3 software (CurveExpert, USA), as recommended by the manufacturer. The standard curve was analyzed by a four-parameter logistic curve-fit.

4.2.6. RNA extraction

Liver tissue from the 3 LR and 3 HR fish with the lowest and highest sum of z_{sum}, respectively, was subjected to RNA extraction. Disruption of the samples was performed in liquid nitrogen using a mortar and pestle. After adding lysate buffer, the lysate was homogenized by passing it through a 20G needle attached to a sterile plastic syringe 5 times. RNA was subsequently extracted using the RNA extraction Kit II of Macheray Nagel (Dueren, Germany), according to manufacturers' instructions. RNA concentrations were determined using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington USA) and the quality was assessed by gel electrophoresis as well as by RNA Nano Bioanalysis chip (Agilent 2100 Bioanalyzer).

4.2.7. NGS sequencing

Total RNA was submitted for Illumina 100 bp reads paired-end High Throughput sequencing (RNA-Seq) in the Norwegian Sequencing Centre. Tags reads were separated using a mulitplex identifier (MID). Evaluation of the reads was assessed using the freely available FastQC software program (version 0.10.0; http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Low quality reads as well as adaptors were removed by Trimmomatic software (Lohse et al., 2012). Read assembly was obtained by applying Trinity, version 2012-06-08 (Grabherr et al., 2011). Raw sequence data as well as metadata were submitted to the Short Read Archive (SRA) database of NCBI available under the accession number SRP064240.

4.2.8. Expression analysis

Paired end reads were mapped against the constructed reference transcriptome in order to assess expression abundances quantified by RSEM version 1.2.3 (Li & Dewey, 2011). Contigs with low read support were excluded from downstream analysis. Transcript expression profiles were assessed by the estimation of pairwise abundance of transcript between the two types of response using the R Bioconductor package DESeq (Anders & Huber, 2010). The most stringent dataset of transcripts was used, by analyzing transcripts that were exclusively

expressed in one condition (no transcripts identified in the other) and in all three replicates, with $P < 0.05$ and FDR-value < 0.05.

4.2.9. Functional annotations and gene ontology

All transcripts obtained were submitted against the non-redundant protein database (nr) as well as the non-redundant nucleotide database (nr/nt) using the standalone BLAST tools (version 2.2.25); threshold cut-off e-values of 10^{-6} and 10^{-10}, respectively, were chosen. Annotations and GO terms assignment were retrieved by Blast2Go software.

4.2.10. Statistical analysis

Statistical analysis was performed using the SigmaStat 3.1 statistical package (Systat Software, San Jose, CA, USA) and SPSS v.22 (IBM Corp., Armonk, NY, USA). Results are presented as means ± standard deviation. In all statistical tests data were examined for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test prior to analysis.

Repeatability was estimated based on a nested analysis of variance (ANOVA). The ANOVA model that was analyzed consisted of the factors *family* and *individual*, with *individual* being nested in the factor *family*. The null hypothesis was that individual *z*-scores were inconsistent within individuals and hence the variable was not repeatable. In other words, the consistency of the response within an individual across the four samplings was tested.

Variance components analysis was performed based on the Restricted Maximum Likelihood (REML) method, using the VARCOMP command in SPSS. The model consisted of the factors *individual* and *family*, with *individual* being nested in the factor *family*, as random factors, and the factor *sampling* as fixed factor, thus taking into consideration the interaction between *sampling*family*.

For the comparison of cortisol levels between the selected LR and HR fish, repeated measures analysis of Variance (ANOVA) tests were performed. The factors *type of response* (LR vs HR) and *sampling time* (S1 to S4) were used as fixed factors, with their interaction being also checked, and the *individuals* as subjects. The assumption of sphericity was checked using Mauchly's test of sphericity, and if violated the Greenhouse-Geisser correction (when $\varepsilon < 0.75$) or the Hyunh-Feldt correction (when $\varepsilon > 0.75$) were used. For the comparison of free

cortisol and ACTH (in S4) and resting cortisol levels (in S5) between LR and HR individuals t-tests were used.

4.3. Results

4.3.1. Variability of cortisol response

The cortisol stress response of all the individuals from the 6 families used in this study showed high variability in all samplings (**Fig. 4.1**). Statistically significant differences in the mean cortisol concentrations of the experimental population between the successive samplings were observed, as shown by the Huynh-Feldt F-test ($F_{2.59,162.94} = 3.894$; $P = 0.014$).

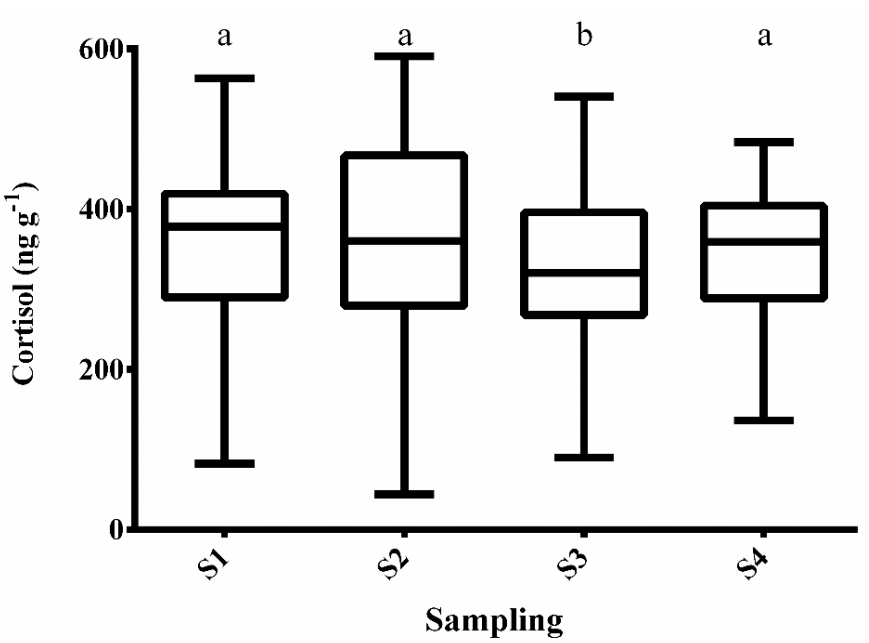


Fig. 4.1. Post-stress plasma cortisol concentrations in the four monthly samplings. The box-plot represents the interquartile range between the 1st and 3rd quartiles, while the horizontal line inside the box-plot represents the median. Whiskers represent the minimum and maximum values ($n = 64$). Different letters indicate significantly different means.

The dispersion of the data, as indicated by the % coefficient of variation, ranged between 22.54 % and 33.50 % in the four consecutive samplings, being highest in S2 and lowest in S4 (**Table 4.1**). When each family was examined separately, this variability was substantially reduced, apart from family F5 which showed high variability (**Table 4.1**).

Table 4.1. Descriptive analysis, expressed as mean, SD and CV %, of plasma cortisol concentrations in all samplings. Results are presented as data from the whole population (All; n = 64) and as data from each family separately (F1 to F6; n = 10-12). Family means with different letters differ significantly from one another ($P < 0.05$).

Sampling	Dataset	Mean	SD	CV%
S1	All	359.6	101.9	28.4
	F1	322.2[a]	46.1	14.3
	F2	389.6[ab]	43.9	11.3
	F3	362.7[a]	56.0	15.4
	F4	426.3[b]	49.0	11.5
	F5	221.9[c]	97.4	43.9
	F6	450.8[b]	83.7	18.6
S2	All	361.2	121.0	33.5
	F1	395.3[ab]	93.7	23.7
	F2	475.6[b]	102.3	21.5
	F3	320.6[ac]	73.5	22.9
	F4	375.7[ab]	90.0	24.0
	F5	232.8[c]	105.0	45.1
	F6	400.0[ab]	110.6	27.7
S3	All	325.1	93.9	28.9
	F1	291.8[ac]	39.0	13.4
	F2	414.9[b]	74.7	18.0
	F3	362.6[ab]	76.5	21.1
	F4	343.9[ab]	80.7	23.5
	F5	233.9[c]	98.2	42.0
	F6	327.0[abc]	78.9	24.1
S4	All	346.2	78.0	22.5
	F1	333.3[ab]	58.6	17.6
	F2	411.4[a]	57.1	13.9
	F3	384.7[a]	61.9	16.1
	F4	381.7[a]	47.8	12.5
	F5	290.3[b]	79.3	27.3
	F6	293.6[b]	76.0	25.9

When the effects of *family* were examined by repeated-measures ANOVA, a significant interaction between *family* and *sampling* was observed by the Huynh-Feldt F-test ($F_{14.21,164.945} = 6.578$; $P<0.001$) (**Fig. 4.2**).

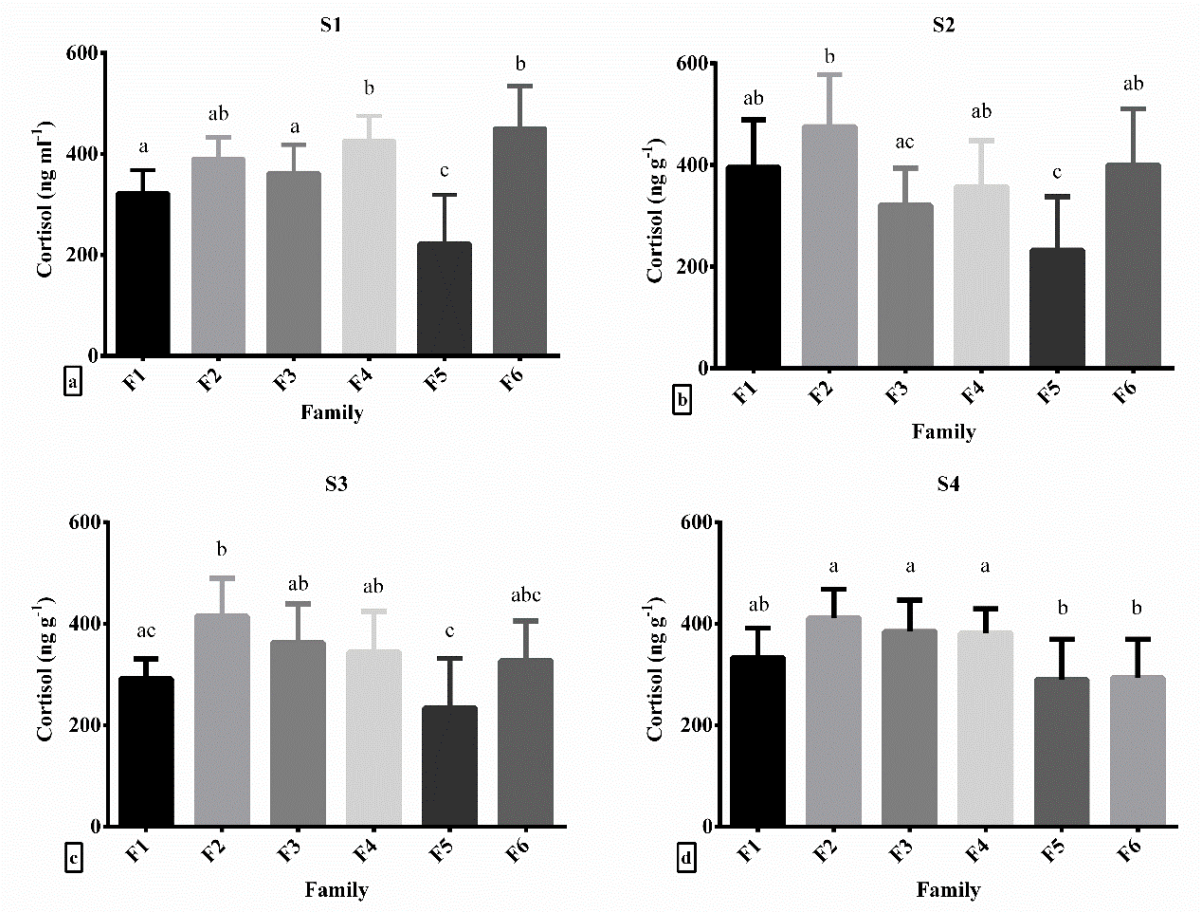


Fig. 4.2. Mean post-stress plasma cortisol concentrations of the 6 families in the four monthly samplings (a to d). Values are presented as means + standard deviation (n = 10 - 12). Means with different letters differ significantly from one another ($P < 0.05$).

In addition, ordination analysis, performed by non-metric Multidimensional Scaling (MDS) ordinated the different families separately (**Fig. 4.3**), with a statistically significant support ($P < 0.001$). This analyses showed that each family, except F3 and F4, was grouped separately from the other families.

Moreover, variance components analysis revealed that the *family* factor had a significant impact on cortisol responsiveness, contributing 28.54 % of the total variance observed in this trait, while an additional 12.04 % was attributed to the interaction between the factors *family* and *sampling*. The error term contributed a relatively high amount of the variance (29.81 %), followed by the *individual* within *family* factor (29.60 %).

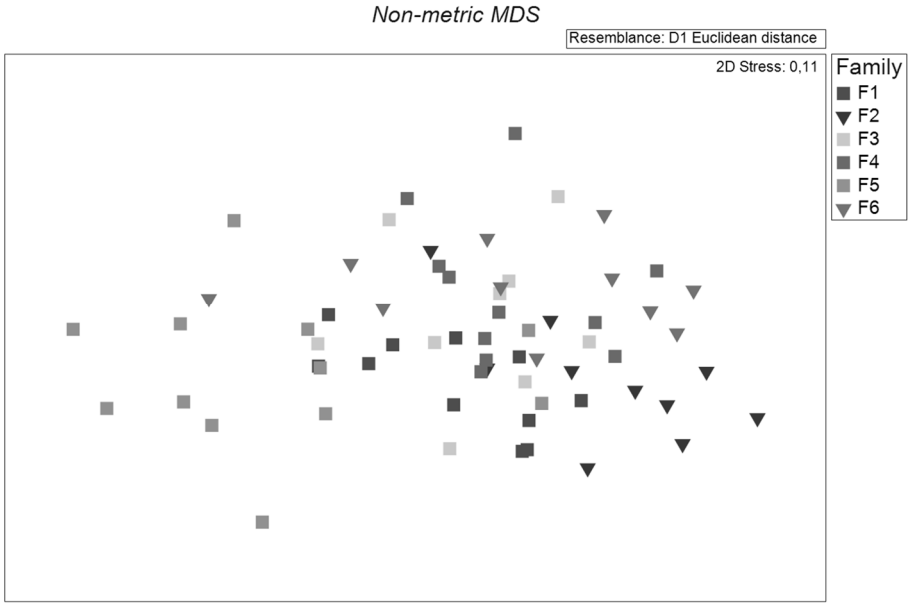


Fig. 4.3. Multidimensional scaling of the cortisol data of the individuals according to the families they belong. There was a statistically significant separation of families, apart from F3 and F4.

4.3.2. Repeatability of cortisol response

As indicated above, the factor *individual* within *family* contributed to 29.60 % of the total variance. Subsequently, a repeatability analysis was performed in order to quantify how much of the observed variance in the *individual* factor was contributed to the among-individual differences compared to the total variance (among- plus within-individual differences).

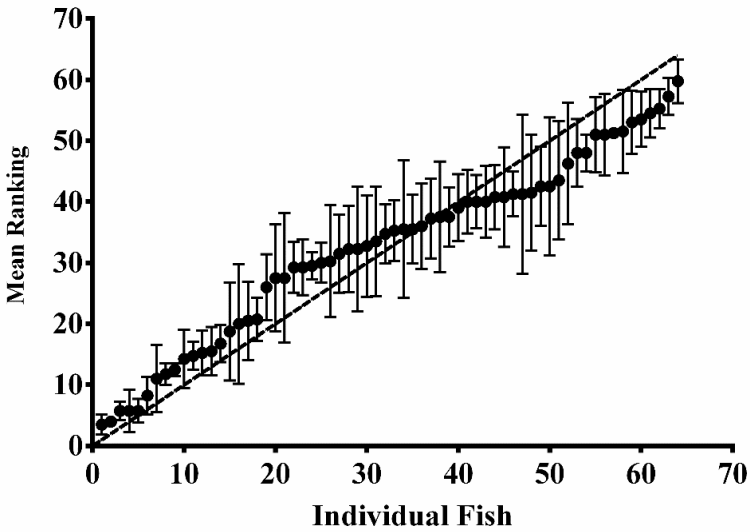


Fig. 4.4. Repeatability of ranked post-stress cortisol concentrations in E. sea bass (n = 64). Each point refers to the mean ranking of each fish ± S.E.M. The dotted line represents perfect repeatability, where one fish should have identical ranks across all trials.

Analysis of the post-stress plasma cortisol concentrations showed that cortisol responsiveness was a repeatable trait in E. sea bass individuals, as estimated by the nested ANOVA ($r = 0.389$; $F_{58,129} = 3.542$; $P < 0.001$) (**Fig. 4.4**).

4.3.3. LR and HR individuals

Based on the *z*-scores analysis, it was possible to distinguish between fish that showed a consistently low or high acute stress cortisol response. These two groups of fish (LR and HR; $n = 16$ per group) showed different post-stress plasma cortisol concentrations in all samplings ($F_{1,30} = 224.46$; $P < 0.001$; **Fig. 4.5**), while there were no significant effects of sampling ($F_{2.57,77.01} = 0.611$; $P = 0.585$) or their interaction ($F_{2.57,77.01} = 1.670$; $P = 0.187$), as shown by the Huynh-Feldt correction F-test. Specifically, lower cortisol concentrations were observed in LR than HR fish in all samplings (**Fig. 4.5**).

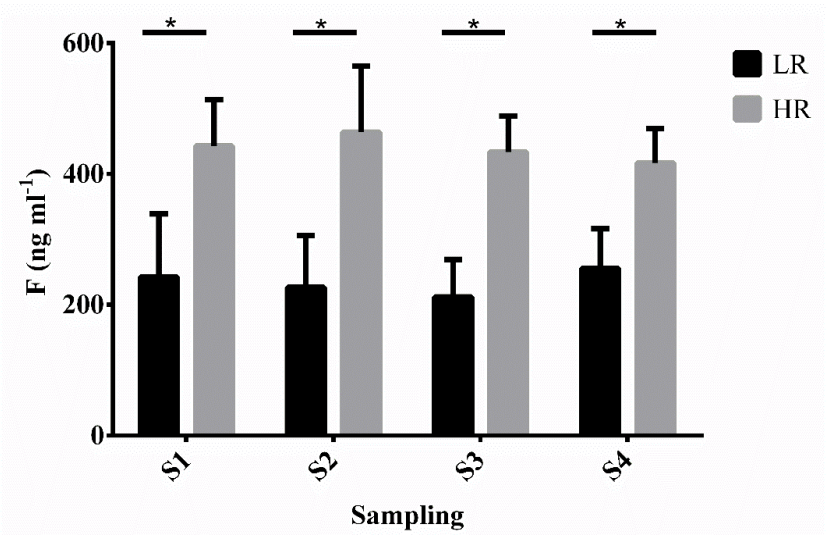


Fig. 4.5. Mean post-stress plasma cortisol concentrations in all samplings for fish identified as LR and HR. Values are presented as means ± standard deviation ($n = 16$ per group). An asterisk (*) indicates statistically significant differences between the two groups within each sampling point (S1 to S4) ($P < 0.05$).

A subsample of tested LR and HR fish from the S4 sampling were also checked for the concentration of free cortisol and total ACTH in their plasma ($n = 10$). LR fish showed lower levels of both total ($t_8 = 7.48$; $P < 0.001$) and free ($t_8 = 3.38$; $P = 0.01$) cortisol concentration; however, the percentage of free over total cortisol (% Free) showed no difference between LR and HR fish ($t_8 = 0.26$; $P = 0.80$) (**Table 4.2**). ACTH showed no differences between LR and HR fish ($t_8 = 1.405$; $P = 0.198$) (**Table 4.2**).

Table 4.2. Total, Free and % Free cortisol in a subsample of LR and HR fish from the S4 sampling (n = 10 per group). Different letters indicate statistically significant differences between the two groups ($P < 0.05$).

	LR	HR
Total Cortisol (ng ml^{-1})	217.2 ± 58.6^{a}	439.2 ± 31.1^{b}
Free Cortisol (ng ml^{-1})	129.1 ± 43.9^{a}	247.0 ± 85.1^{b}
% Free	59.4 ± 13.4	61.8 ± 15.8
ACTH (pg ml^{-1})	81.9 ± 9.1	90.4 ± 10.0

The contribution of the 6 families to the LR and HR fish groups was unequal (**Fig. 4.6**). There were families that included exclusively either LR or HR fish, and others that had both LR and HR fish. Specifically, families F1 and F5 had some fish that were characterized as LR and none as HR. On the contrary, families F2 and F4 contributed some individuals to the HR group and none to the LR. There were also two families, F3 and F6 that had both LR and HR fish.

Out of the four families that had individuals characterized as LR there was one family (F5) monopolizing the contribution. Specifically, ten out the sixteen LR fish belonged to F5, followed by F6, F1 and F3 with three, two, and one individuals, respectively. On the other hand, the four families with HR fish contributed more equally to the HR-fish group, with F2 contributing six out of sixteen fish, followed by families F4 and F6 with four individuals, and F3 with two individuals (**Fig. 4.6**).

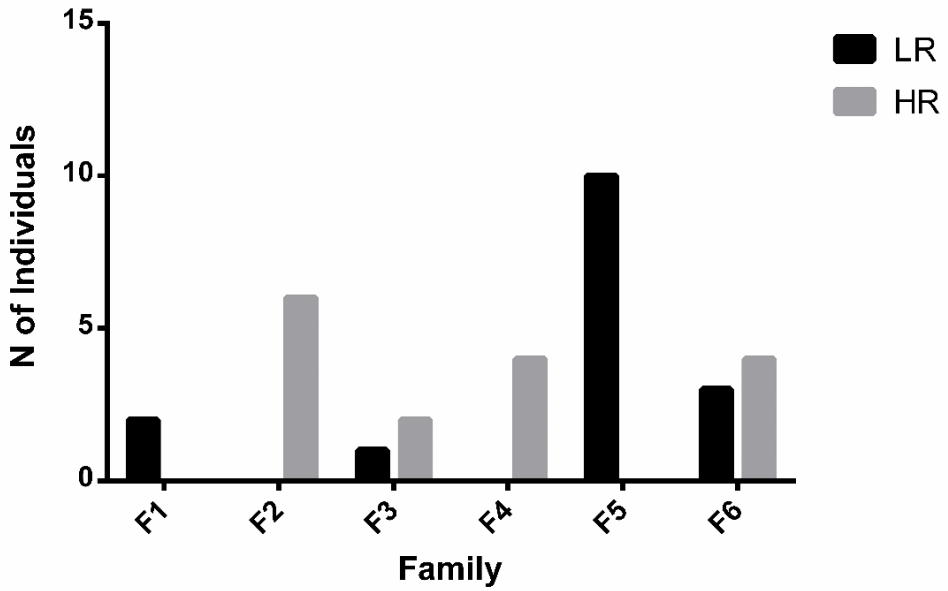


Fig. 4.6. Number of fish from each family (F1 to F6) identified as LR or HR (n = 16 per group).

4.3.4. Resting cortisol concentrations

When fish were sampled without the application of acute stress (S5), the variability of the whole population examined was high, with a CV % calculated at 62.5 % (**Table 4.3**). When the families were examined separately, the dispersion of the data within each family ranged between 29.5 % to 66.3 %, being lowest in F3 and highest in F5 (**Table 4.3**). Moreover, there were significant differences between families ($F_{5,58} = 11.292$; $P < 0.001$), with the families that contributed only to the LR fish group, *i.e.* F1 and F5, showing significantly lower mean resting cortisol concentrations than the rest of the families (**Table 4.3**).

Table. 4.3. Descriptive analysis, expressed as mean, SD and CV %, of the resting cortisol concentrations (S5). Results are presented as data from the whole population (n = 64) and as data from each family separately (n = 10 - 12). Family means with different letters differ significantly from one another ($P < 0.05$).

Sampling	Dataset	Mean	SD	CV%
S5	All	155.3	96.7	62.3
	F1	59.9^{a}	31.1	51.9
	F2	228.0^{b}	95.9	42.1
	F3	182.9^{b}	53.9	29.5
	F4	199.3^{b}	90.9	45.6
	F5	81.7^{a}	54.2	66.3
	F6	222.8^{b}	79.1	35.5

Differences in resting cortisol concentrations were also observed between LR and HR fish. In particular, at the final sampling (S5), individuals identified as LR showed more than two times lower resting cortisol levels (93.4 ± 63.4, n = 16) than HR fish (203.3 ± 121.1, n = 16) ($t_{30} = 3.216$; $P = 0.003$).

4.3.5. Transcriptome analysis

Transcriptome analysis showed that 169 transcripts were exclusively transcribed in LR, and 161 in HR fish. These transcripts were blasted against the European sea bass genome (Tine et al., 2014) and categorized by the linkage groups they belonged to (**Fig. 4.7**).

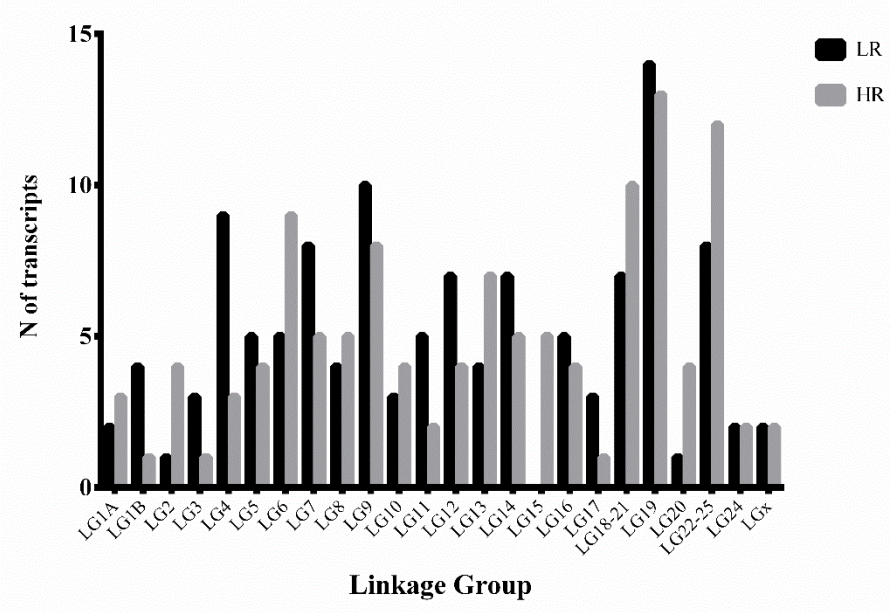


Fig. 4.7. Transcript distribution in E. sea bass linkage groups for the transcripts exclusively expressed in LR or HR fish. Sequences were blasted against the species genome, published by Tine et al. (2014). Only the transcripts that were successfully attributed to a linkage group were counted.

Gene ontology analysis showed that transcripts regulated in the LR fish were annotated to 326 GOs, while those in the HR fish to 289 GOs, 147 of which were shared between the two groups of fish (**Fig. 4.8**).

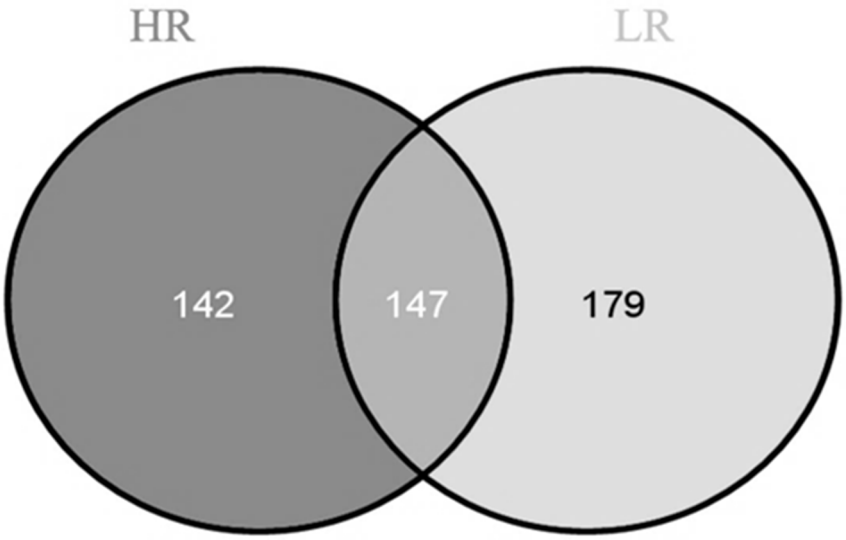


Fig. 4.8. Venn diagram of the GOs expressed in LR and HR fish and those shared between them.

Blast2Go analysis on the annotations of these transcripts showed that their putative functions included various metabolic processes such as single-organism, protein, and nitrogen compound metabolic processes, as well as signal transduction (**Fig. 4.9 a**). Unique metabolic processes observed only in LR individuals included protein metabolic processes, whereas immune system processes and nitrogen compound metabolic processes were only seen in HR fish and consisted of more than 50 % of the biological processes.

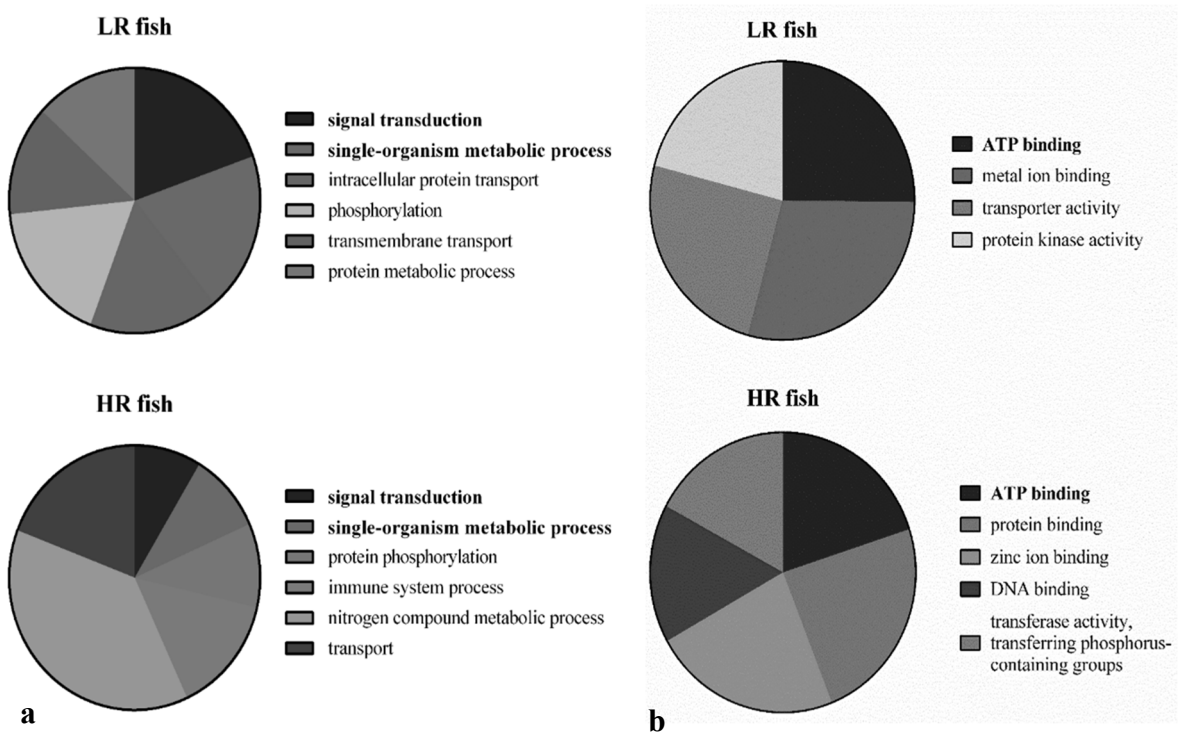


Fig. 4.9. Multi-level profile of the Biological Processes (a) and Molecular Functions (b) Gene Ontology terms in LR and HR fish liver. GO terms with a node score below 5 were filtered out. Bold lettering indicates that these terms were shared between LR and HR fish.

In addition, they included several molecular functions, such as ATP binding, DNA, protein, metal ion and zinc ion binding, as well as transporter, transferase and protein kinase activity (**Fig. 4.9 b**).

The Fisher's exact test showed that 26 gene ontologies were significantly over-expressed in LR fish (**Table 4.4**) and 29 GOs in HR fish (**Table 4.5**).

Table 4.4. Gene Ontology terms significantly enriched in LR fish.

GO ID	GO Term	p-value	Genes
GO:0004030	aldehyde dehydrogenase [NAD(P)+] activity	1.49E-02	*aldh3a2*
GO:0051895	negative regulation of focal adhesion assembly	3.11E-02	*aldh3a2*
GO:0006081	cellular aldehyde metabolic process	3.27E-02	*aldh3a2*
GO:1900016	negative regulation of cytokine production involved in inflammatory response	2.63E-02	*apod*
GO:0071638	negative regulation of monocyte chemotactic protein-1 production	2.63E-02	*apod*
GO:0060588	negative regulation of lipoprotein lipid oxidation	2.63E-02	*apod*
GO:0002088	lens development in camera-type eye	2.63E-02	*apod*
GO:0010642	negative regulation of platelet-derived growth factor receptor signaling pathway	2.63E-02	*apod*
GO:2000098	negative regulation of smooth muscle cell-matrix adhesion	2.63E-02	*apod*
GO:0015485	cholesterol binding	2.79E-02	*apod*
GO:0042308	negative regulation of protein import into nucleus	3.27E-02	*apod*
GO:0048662	negative regulation of smooth muscle cell proliferation	4.40E-02	*apod*
GO:0000302	response to reactive oxygen species	4.83E-02	*apod*
GO:0030791	arsenite methyltransferase activity	1.49E-02	*as3mt*
GO:2000405	negative regulation of T cell migration	2.63E-02	*cdipt*
GO:0090344	negative regulation of cell aging	2.63E-02	*cdipt*
GO:0010842	retina layer formation	3.44E-02	*cdipt*
GO:0060219	camera-type eye photoreceptor cell differentiation	3.59E-02	*cdipt*
GO:0005758	mitochondrial intermembrane space	1.49E-02	*cycs*
GO:0004714	transmembrane receptor protein tyrosine kinase activity	5.70E-03	*fgfr1a*
GO:0090140	regulation of mitochondrial fission	2.63E-02	*march5*
GO:0051865	protein autoubiquitination	4.40E-02	*march5*
GO:0042981	regulation of apoptotic process	3.59E-02	*nod1*
GO:0004594	pantothenate kinase activity	1.98E-02	*pank1*
GO:0015937	coenzyme A biosynthetic process	2.47E-02	*pank1*
GO:0004252	serine-type endopeptidase activity	3.06E-02	*tmprss6*

Table 4.5. Gene Ontology terms significantly enriched in HR fish.

GO ID	GO Term	p-value	Genes
GO:0003993	acid phosphatase activity	2.22E-02	*acp5*
GO:0000276	mitochondrial proton-transporting ATP synthase complex, coupling factor F(o)	2.95E-02	*atp5a1*
GO:2000021	regulation of ion homeostasis	1.86E-02	*ca5a*
GO:0004089	carbonate dehydratase activity	2.22E-04	*ca5a*
GO:0006730	one-carbon metabolic process	3.49E-03	*ca5a*
GO:0009264	deoxyribonucleotide catabolic process	1.12E-02	*dera*
GO:0004139	deoxyribose-phosphate aldolase activity	1.12E-02	*dera*
GO:0004359	glutaminase activity	1.49E-02	*gls*
GO:0006486	protein glycosylation	4.28E-02	*mgat1*
GO:0008375	acetylglucosaminyltransferase activity	7.17E-03	*mgat1*
GO:0005739	mitochondrion	1.16E-02	*pdk2* *ca5a* *atp5a1*
GO:0072332	intrinsic apoptotic signaling pathway by p53 class mediator	1.49E-02	*pdk2*
GO:0008286	insulin receptor signalling pathway	2.04E-02	*pdk2*
GO:0042593	glucose homeostasis	2.22E-02	*pdk2*
GO:0031670	cellular response to nutrient	2.22E-02	*pdk2*
GO:0034614	cellular response to reactive oxygen species	2.41E-02	*pdk2*
GO:0006885	regulation of pH	3.32E-02	*pdk2*
GO:0005967	mitochondrial pyruvate dehydrogenase complex	7.46E-03	*pdk2*
GO:0006111	regulation of gluconeogenesis	7.46E-03	*pdk2*
GO:0010510	regulation of acetyl-CoA biosynthetic process from pyruvate	7.46E-03	*pdk2*
GO:0004740	pyruvate dehydrogenase (acetyl-transferring) kinase activity	7.46E-03	*pdk2*
GO:1902494	catalytic complex	2.77E-02	*pdk2* *prkar1a*
GO:0005952	cAMP-dependent protein kinase complex	3.52E-04	*prkar1a*
GO:0001932	regulation of protein phosphorylation	3.72E-02	*prkar1a*
GO:0008603	cAMP-dependent protein kinase regulator activity	4.54E-04	*prkar1a*
GO:0019885	antigen processing and presentation of endogenous peptide antigen via MHC class I	7.46E-03	*tapbp*
GO:0031625	ubiquitin protein ligase binding	4.40E-02	*ube2w*
GO:0000139	golgi membrane	4.68E-02	*ube2w*
GO:0071218	cellular response to misfolded protein	7.46E-03	*ube2w*

Subsequently 72 transcripts related to the immune responses, carbon, protein, lipid, and energy metabolism that were differentially transcribed between LR and HR fish were selected and subjected to heatmap analysis (**Fig. 4.10**). Each row of the heat map represents the pattern of one transcript's expression, while columns represent the HR and LR fish. This analysis grouped the LR and HR fish separately (**Fig. 4.10**).

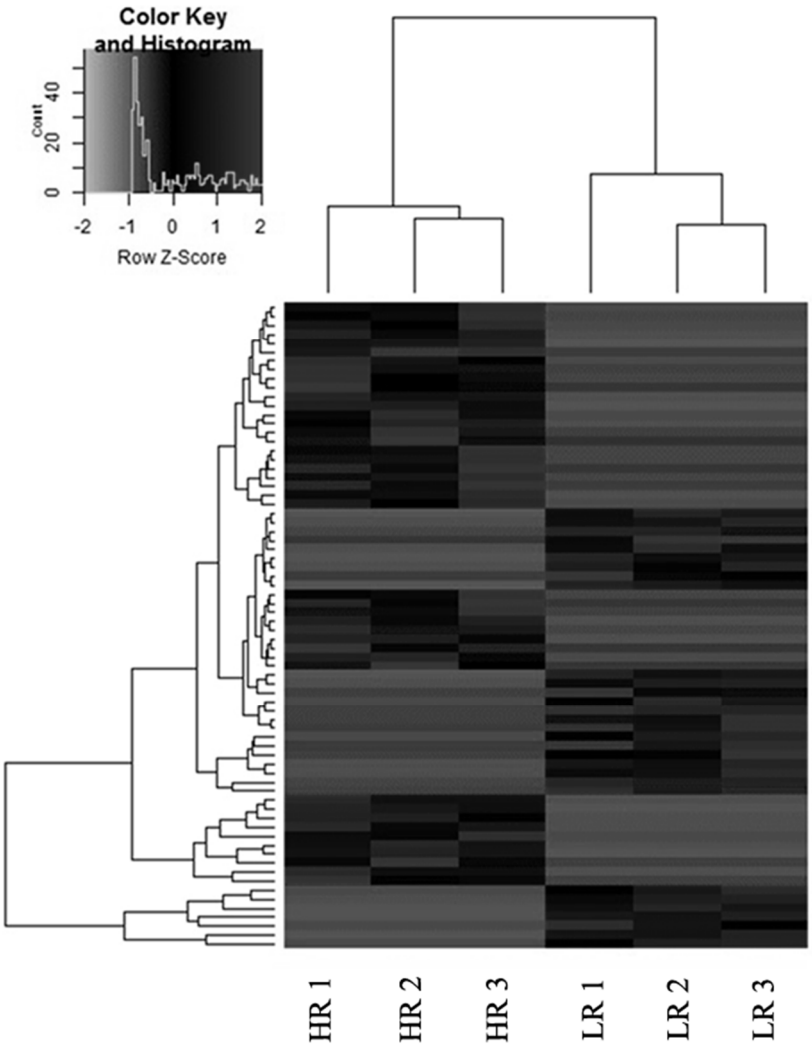


Fig. 4.10. Heatmap of differentially expressed transcripts between HR and LR fish, as indicated under each column of clusters. Each row represents the expression of one transcript in relation to the other, while shades of red represent upregulation and shades of green represent downregulation.

4.4. Discussion

The present study demonstrates for the first time the existence of both family- and individual-based differences in cortisol stress responses in E. sea bass. Cortisol responsiveness was shown to be a repeatable trait among individuals, and fish showing constantly low or high

responsiveness were identified and characterized as LR and HR. These fish also differed in their resting (*i.e.* without stress) cortisol levels, as well as the amount of free cortisol, which is the biologically active form of the hormone (Breuner & Orchimik, 2002). Finally, differences existed in the liver transcriptome profile between LR and HR fish, depicting differences in the regulation of metabolic and immune functions. Such results suggest that intra-specific differences should be considered when studying the stress responses in E. sea bass, and could in turn explain the high variability observed in cortisol responsiveness. Moreover, knowledge of this aspect of stress physiology could assist in optimizing husbandry and welfare, as well as introduce cortisol responsiveness as a new potential selection criterion in genetic selection programs. It should, however, be kept in mind that selection for cortisol responsiveness could potentially be correlated or co-selected with other traits of interest such as growth (Fevolden et al., 2002), lysozyme response (Fevolden et al., 1999; 2002), or not yet studied traits, which could exert positive, neutral or negative effects on fish performance.

The post-stress cortisol values of the whole population under study showed high variability, as indicated by the standard deviation and the coefficient of variation. This variance could be attributed to family-related differences at a fraction of approximately 1/3 of the total variation (28.54 % was attributed to the factor *family* and 12.04 % to the interaction between factors *family* and *sampling*). It was thus observed that some families, such as F5, had lower mean concentrations of cortisol than others, like F2. Contrary to the present results, no significant differences in the post-stress cortisol levels between different genetic groups were observed in a recent study by Vandeputte et al., (2016). It should be noticed, however, that in that study the genetic groups were defined merely by the origin of the sire (*i.e.* male parent) and not that of the dam (*i.e.* female parent). Specifically, eggs of wild-type females were mixed together and fertilized with sperm coming from all the males of the population, resulting in four genetic groups according to the genetic background of the male. Moreover, the sires that were used were obtained from the same G0 wild base population upon selection and domestication (Vandeputte et al., 2009; 2016), lowering in that way the genetic variability of the population. Still, however, an intermediate heritability ($h^2 = 0.33$) of the post-stress cortisol concentrations was estimated (Vandeputte et al., 2016). In the present dissertation, the heritability of cortisol responsiveness was not estimated due to the small number of families used, yet, as discussed above, it was shown that the genetic background (*i.e.* family) had a significant impact on this trait.

In terms of individuals, it was shown that individual variation accounted for 29.6 % of the total variance, and differences in cortisol responsiveness existed between fish. Cortisol

responsiveness was shown to be a repeatable trait, and given its repeatability, LR and HR individuals were identified based on the *z*-scores analysis, with the former showing constantly lower cortisol levels than the latter. Although LR and HR fish have been characterized in other fish species, such as rainbow trout (Pottinger et al., 1992; Fevolden et al., 2002; Weil, et al., 2004; Weber, 2008), Atlantic cod (Hori et al., 2012a), striped bass (Wang et al., 2004), and gilthead sea bream (Tort et al., 2001), it had previously not been feasible to identify LR and HR individuals in E. sea bass (Fanouraki, 2010), and cortisol response had been considered to show low to moderate heritability ($h^2 = 0.08$; Volckaert et al., 2012; $h^2 = 0.33$; Vandeputte et al., 2016).

Interestingly, this difference in cortisol was not accompanied by differences in the ACTH concentration in a subsample of LR and HR fish. This is the first time that ACTH has been estimated in this species, and no knowledge on the pattern of response of this hormone after stress is therefore available. ACTH response in salmonid fish species, however, indicates that ACTH reaches high levels at 30 min post-stress (Sumpter et al., 1986; Pottinger & Carrick, 2001), the time point where fish in the present study were sampled. It should be kept in mind, however, that species-specific differences could exist in the ACTH response, as it happens with other stress-related parameters, like cortisol and glucose (Fanouraki et al., 2011). In accordance with the present results, Pottinger & Carrick (2001) showed that no differences existed in the ACTH response to stress between LR and HR trout.

The resting cortisol concentrations of the individuals were also quantified. To the author's best knowledge this is the first time that resting cortisol concentrations of different families were analyzed in this species. Significant differences existed between families, with F1 and F5 having lower mean cortisol levels than the rest of the families, showing in that way that not only stress responsiveness, but the resting circulating levels of cortisol can also be affected by the genetic background, given that the husbandry conditions and feeding between all families were the same. Moreover, differently responding individuals seemed to differ in the resting concentrations of cortisol, with HR fish showing higher levels of circulating cortisol in their circulation. To the best of our knowledge, there are only two studies showing direct differences in the resting cortisol concentration between progeny of selected LR and HR families; in rainbow trout (Pottinger & Carrick, 2001) and in gilthead sea bream (Rotllant et al., 2003a). Significant positive correlations between family average post-stress and basal cortisol levels have also been shown between LR and HR families in rainbow trout (Weber & Silverstein, 2007). The results of the present and other studies in this species (Roche & Bogé 1996; Rotllant et al., 2003b; Varsamos et al., 2006) indicate that resting cortisol concentrations

can show high variation, as well as differ substantially between different studies (reviewed by Ellis et al., 2012), and genetic background can be a factor affecting this variability. The fact that divergent cortisol responsiveness is accompanied by divergent resting circulating cortisol concentrations is indicative of a connection between these two traits, and might open new insights in the stress physiology of E. sea bass, and how this is related to the overall performance of the fish.

This is also the first report on the concentration of free (*i.e.* unbound to proteins) cortisol in E. sea bass. Free cortisol is the biologically active fraction of the hormone (Breuner & Orchinik, 2002). The concentration of free cortisol was higher in HR than LR fish, indicating that these fish indeed show higher levels of biological active cortisol and no regulatory mechanism takes place to diminish the differences observed in the total concentration. Nevertheless, even though the amount of free cortisol differed, the percentage of free in respect to the total concentration of cortisol showed no differences. Although in general the concentration of free cortisol was higher in E. sea bass when compared to other fish species, the percentage observed did not seem to differ from that in gadoids (Hosoya et al., 2007; Hori et al., 2012a; b), but was slightly higher than Atlantic salmon, *Salmo salar* (Fast et al., 2008), and tiger pufferfish, *Takifugu rubripes* (Hosoya et al., 2008). In line with the present study, no differences in the fraction of free cortisol were observed between LR and HR in Atlantic cod (Hori et al., 2012a; b). These differences in the free concentration of cortisol, both between and within species, may reflect differences in the effects that cortisol could have on their performance, like immune function, metabolism and growth. It should however be noted that there are also other mechanisms regulating the actions of cortisol, like the abundance and affinity of the glucocorticoid receptors (Pottinger et al., 2000).

Liver is an important organ that regulates many functions of the organism, including metabolism, immune functions and the stress-induced secondary responses (De Boeck et al., 2001; Pemmasani, et al., 2011; Cortés et al., 2013; Teles et al., 2013;). In an attempt to associate the LR and HR phenotypes to the above-mentioned functions of the liver, a subset of LR and HR fish was subjected to hepatic transcriptome analysis. This analysis focused on transcripts that were exclusively transcribed in either LR or HR fish in order to identify the main crossing points between them. Specifically, 169 transcripts were transcribed only in LR fish, while 161 transcripts were exclusively transcribed in HR fish. These transcripts were annotated to Gene Ontology (GO) terms, some of which were shared between LR and HR fish, such as the single-organism metabolic process, and signal transduction, and others like protein, and nitrogen compound metabolic processes that were presented exclusively in LR or HR fish. In particular,

intracellular protein transport, phosphorylation, transmembrane transport, and protein metabolic processes were unique to LR fish. On the other hand, nitrogen compound metabolic processes, transport, and protein phosphorylation were unique to HR fish.

Out of all the biological processes exclusively annotated in the multi-level analysis in HR fish, nitrogen compound metabolic processes accounted for 37.7%. Moreover, enrichment analysis showed that glutaminase activity was significantly over-represented in HR fish, mediated by the transcription of *gls* transcript in HR fish only. The enzyme encoded by this transcript catalyzes the first reaction of the glutamate catabolism, and is therefore related to the metabolism of nitrogen and formation of glucose (Watford et al., 1993). This enzyme is also involved in other amino acid metabolic processes, as shown by the KEGG pathways of alanine, aspartate and glutamate metabolism, as well as arginine and proline metabolism (Reference Pathway map00250 and map00330 respectively; KEGG PATHWAY Database; Kanehisa Laboratories; http://www.genome.jp/kegg/pathway.html). Increased cortisol concentrations have been shown to stimulate nitrogen metabolism, in the sea raven, *Hemitripterus americanus* (Vijayan et al., 1996), while nitrogen excretion tended to be unaffected by chronic cortisol stimulation in rainbow trout (De Boeck et al., 2001).

Apart from metabolism, liver is also an important organ in the immune response of fish, synthesizing various immune-related proteins (Martin et al., 2010). In this study, the GO term related to immune responses was annotated in the metabolic processes pie chart of HR fish (**Fig. 4.9**). In detail, the GO term of antigen processing and presentation of endogenous peptide antigen via MHC class I was enriched in HR fish, due to the high transcription of the *tapbp* transcript. Tapasin, a member of the MHC class Ia antigen-loading complexes, mediates the interaction between MHC class Ia molecules and the transporter associated with antigen presentation (Landis et al., 2006). Apart from *tapbp*, other MHC class Ia related transcripts were transcribed in HR fish, like the *mhc class ia antigen* and *major histocompatibilty complex I-related gene*. Unlike the present results, in rainbow trout high levels of cortisol were related to a down-regulation of the MHC class II antigen beta chain and the MHC class II antigen associated invariant chain (Teles et al., 2013). In LR fish, on the other hand, processes regarding the negative regulation of T-cell migration and cytokine production involved in inflammatory response were significantly enriched.

Differences between LR and HR fish were also present in the expression of transcripts encoding for proteins involved in the KEGG pathway of complement and coagulation cascades. Specifically, in LR fish, the liver transcripts encoding for complement C3 and Factor H (HF) proteins were transcribed when compared to HR fish. C3 protein plays a central role in the

activation of the complement system, which after its spontaneous split to C3a and C3b, forms the serine protease C3-convertase upon binding to factor B (Holland & Lampris, 2002). C3-convertase subsequently exerts a positive feedback to further splicing of C. The H Factor, on the other hand, functions to accelerate the decay of the C3-convertase (Holland & Lampris, 2002). In HR fish, a different, when compared to the LR fish, component of the complement system was transcribed, namely the *c7*, which is an important component of the Membrane Attack Complex (MAC) (Holland & Lampris, 2002). Finally, transcription of transcripts related to the coagulation of blood was observed in HR fish; specifically, *f5* and *f11*.

The above results indicate possible differences in the regulation of the immune functions between the LR and HR phenotypes. It is not, however, yet clear if these differences are of importance to the overall performance of fish. Future disease resistance experiments could shed light in this aspect. Furthermore, although cortisol is known to affect the immune function of organisms, it is unclear whether cortisol per se is responsible for these differences, or selection for cortisol responsiveness is correlated with aspects of the immune system, such as lysozyme (Fevolden et al., 1999; 2002), which is an important enzyme in the innate immune functions. In this context, although no data exists for the expression of these genes in LR and HR fish, when rainbow trout was faced with a chronic cortisol increase, via slow-releasing cortisol implants, a down-regulation in liver *c3* and H factor (*cfh*) genes, as well as B factor (*Bf*) was observed (Cortés et al., 2013), which is in line with the current results that show no expression of these transcripts in the HR fish.

A series of carbohydrate metabolism-related transcripts were transcribed in HR fish. Processes mediated by the mitochondrial pyruvate dehydrogenase complex were significantly enriched in these fish as a result of the high transcription of *pdk2*, which encodes for pyruvate dehydrogenase kinase 2 (PDK2). There are four known isozymes of this enzyme in mammals, namely PDK1 to PDK4, with PDK2 and PDK4 being the most widely distributed and highly expressed in heart, liver and kidneys (Zhang et al., 2014). This kinase phosphorylates the active form of the enzyme pyruvate dehydrogenase (PDH_a) to its inactive form (PDH_b), inhibiting in this way its activity (Richards et al., 2002). PDH is the first component enzyme of pyruvate dehydrogenase complex (PDC) which controls the conversion of pyruvate, Coenzyme A (CoA) and NAD^+ into acetyl-CoA, NADH and CO_2 (Sugden & Holness, 1994). By this action PDC links fatty acid metabolism, glucose metabolism and tricarboxylic acid (TCA) cycle. Specifically, when PDC is in its active form, the CoA-activated two-carbon unit produced by the catabolism of pyruvate can be condensed with oxaloacetate in the first reaction of the TCA cycle, or used for fatty acid and cholesterol synthesis (Strumiło, 2005). On the other hand,

inactivation of PDC by PDK2, results in conservation of pyruvate for gluconeogenesis in liver and kidney (Huang et al., 2002). Therefore, it seems that the expression of *pdk2* in HR fish works in favor of pyruvate conservation due to the inhibition over PDC activity. This inhibition leads to the conservation of three-carbon compounds for gluconeogenesis, instead of metabolite flux through fatty acid and cholesterol synthesis, and the tricarboxylic acid cycle (Walton & Cowey, 1982). It should however be kept in mind that these results are based solely on transcription data, and not on direct estimations of the enzymes abundance and activity.

In LR fish, on the other hand, the biosynthesis of coenzyme A process was significantly enriched. An excess of CoA in the cell can inhibit the activity of PDK in mammals, favoring in this way the oxidation of pyruvate through the TCA cycle for the production of energy, or fatty acids and cholesterol (Strumiło, 2005). Additionally, *glut2*, which encodes for a protein that is likely responsible for glucose transport and uptake by the liver, was transcribed in LR fish (Sugden & Holness, 1994). In mammals, the expression of this gene increases under high glucose concentrations to enhance the insulin secretory response to glucose (Sugden & Holness, 1994).

From all the above, it could be suggested that HR fish showed signs of energy and gluconeogenetic substrates conservation, contrary to a tendency towards substrate oxidation and glucose uptake in LR fish. This could be indicative of increased energy demands or reduced feed consumption and/or accumulation in HR fish, that might have resulted from reduced appetite, given that all tanks were feed *ad libitum*. These differences may in turn affect the overall performance of fish.

Processes involved in protein metabolism and modification showed some differential transcription between LR and HR fish. In general, cortisol is believed to exert proteolytic actions in fish (Wiseman et al., 2007), and elevated transcripts of ubiquitin after acute stress have been observed in rainbow trout (Wiseman et al., 2007). The ubiquitin-proteasome proteolytic pathway is of major importance in the degradation of proteins. In detail, different aspects of the ubiquitin-proteasome pathway were regulated in LR and HR fish. Specifically, in LR fish, protein autoubiquitination was significantly enriched while in HR fish the process of ubiquitin ligase protein binding was enriched. Additionally, in LR fish, the serine-type endopeptidase activity process, which is involved in protein degradation, was also enriched. The energy and amino acids released by this proteolysis are subsequently used in protein synthesis (Glickman & Ciechanover, 2002). Transcripts related to protein modification processes were also enriched in HR fish, involving important processes like the regulation of protein phosphorylation and glycosylation, as well as c-AMP dependent kinase regulation.

In conclusion, the present study showed for the first time that family-based differences in cortisol resting and post-stress levels exist in E. sea bass. Moreover, cortisol stress response was shown to be a repeatable trait, and subsequently LR and HR individuals were identified. These fish differed in the concentration of both total and free post-stress, as well as resting cortisol concentrations. Better understanding of these differences could be useful in genetic selection programs by introducing new potential selection criteria, given the important impacts that cortisol responsiveness may have on the performance and fitness of the fish. Specifically, these types of fish showed differences in their hepatic transcription profile, with transcripts being solely transcribed in LR or HR fish. Enrichment analysis of those transcripts, revealed many processes that were differentially expressed between these groups of fish. In short, nitrogen metabolism, and some aspects of the immune system were significantly more expressed in HR than LR fish, which highlights the need for further study of their ability to resist exposure to pathogens. HR fish also expressed transcripts related to the conservation of energy and glucose in contrast to LR fish that expressed transcripts involved in the flux of three-carbon molecules towards oxidation, suggesting in that way the possibility for a higher energy demand or reduced feed consumption by the HR fish, which could have important aquaculture applications.

Chapter V. Head kidney activity and sensitivity to ACTH regulate the differences in cortisol between LR and HR fish

5.1. Introduction

Cortisol is considered the main stress hormone in marine teleost fish, and its production, secretion and actions are being under multi-leveled control (see Mommsen et al., 1999 for review). In mammals, cortisol in synthetized in the adrenal gland, yet fish do not have a discrete adrenal gland, and the cells that produce the steroid, called interrenal cells, are distributed in the head kidney region (Mommsen et al., 1999). Although divergence in the cortisol responsiveness within the same species has been long described in fish (Pottinger et al., 1992) and other tetrapods (Benus et al., 1991; Sgoifo et al., 1996), the underlying mechanism regulating these differences has not yet been extensively described. In general, the main sites of regulation for the production of cortisol in the Hypothalamus – Pituitary – Interrenal (HPI) axis are the *nucleus preopticus* (NPO) in the hypothalamus, which produces corticotrophin-releasing hormone (CRH), the pituitary, which produces ACTH, and the interrenal cells in the head kidney, that produce cortisol (Flik et al., 2006).

Hypothalamic CRH-producing cells play an important role in stress response, stimulating the pituitary *pars distalis* to release ACTH. The signal for the production of cortisol from the head kidneys comes from this pituitary-derived hormone, which acts upon binding to the melanocortin 2 receptor (MC2R; Metz et al., 2005; Aluru & Vijayan, 2008; Agulleiro et al., 2013). This receptor is highly expressed in the head kidneys of teleost fish (Metz et al., 2005; Aluru & Vijayan, 2008), including E. sea bass (Agulleiro et al., 2013), and requires the melanocortin 2 receptor accessory protein (MPAR) for its functional expression. MC2R binds specifically ACTH and not other POMC-derived peptides, such as α-MSH (Aluru & Vijayan, 2008). Upon binding of ACTH to MC2R a cascade of enzymatic reaction to produce cortisol initiates. The expression of *mc2r* gene is crucial for the regulation of cortisol biosynthesis from the interrenal cells (Aluru & Vijayan, 2008; Agulleiro et al., 2013). The regulation of its expression is not yet fully described, but factors such as cortisol (Agulleiro et al., 2013) or ACTH administration (Aluru & Vijayan, 2008), and chronic stress (Metz et al., 2005) seem to be important in this regard.

For the production of cortisol, and steroids in general, cholesterol is used as a precursor, after its transfer across the mitochondrial membrane by the steroidogenic acute regulatory

protein (StAR; Clark et al., 1994). The first step in the biosynthesis reaction is the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage enzyme (P450ssc) in the inner mitochondrial membrane (Alsop & Vijayan, 2008). Pregnenolone subsequently becomes hydroxylated by cytochrome P450 17α-hydroxylase (CYP17A1), dehydrogenated by 3β-hydroxysteroid dehyodrogenase (HSD3B2) and further hydroxylated by cytochrome P450 21 hydroxylase (CYP21A2) to produced 11-deoxyxortisol (Mommsen et al., 1999). The conversion of this product to cortisol is catalyzed by 11β-hydroxylase (CYP11C1) (Alsop & Vijayan, 2008), while cortisol is inactivated by its conversion to corticosterone by 11β-hydroxysteroid dehydrogenase 2 (11β-hsd2; Draper & Stewart, 2005; Alsop & Vijayan, 2008).

Acute stress, chronic stress and ACTH administration can stimulate the mRNA expression of genes involved in cortisol biosynthesis in the head kidney, such as *StAR* (Gelsin & Auperin, 2004; Hagen et al., 2006; Aluru & Vijayan, 2008; Castillo et al., 2008; Fierro-Castro et al., 2015), *P450ssc* (Geslin & Auperin, 2004; Aluru & Vijayan, 2008; Nematollahi et al., 2009; Fierro-Castro et al., 2015) and *11β-hydroxylase* (Gelsin & Auperin, 2004; Hagen et al., 2006; Aluru & Vijayan, 2008; Fierro-Castro et al., 2015). An up-regulation of the expression of *11β-hsd2* after stress has also been observed (Nematollahi et al., 2009; 2012), most probably to reduce the circulating levels of cortisol.

Although there are several sites of regulation of cortisol production and secretion throughout the HPI axis, knowledge regarding the mechanisms regulating the differences in cortisol responsiveness between LR and HR individuals is scarce. Brain is one major site of regulation in the HPI axis, and specifically, the *nucleus preopticus* in the hypothalamus, producing CRH and the pituitary, especially the *pars distalis* which includes ACTH producing cells (Flik et al., 2006). CRH, produced at the higher level of regulation of the HPI axis, seems to play a crucial role in the differences observed in cortisol release between LR and HR fish in striped bass (Wang et al., 2004). Additionally, reduced cortisol responsiveness in rainbow trout is accompanied by increased gene expression of the mineralocorticoid receptor (*mr*), but not glucocorticoid receptors (*gr1* & *gr2*) in the telencephalon, hypothalamus, optic tectum and cerebellum of LR fish, suggesting divergent receptor regulation between differently stress responsive individuals (Johansen et al., 2011). In contrast, the concentration of ACTH, which stimulates the production of cortisol from the interrenal tissue, seems not to be the critical factor regulating the response in rainbow trout (Pottinger & Carrick, 2001). The head kidney, and particularly the interrenal tissue, is the third major site of regulation in the HPI axis (Flik et al., 2006). In rainbow trout, administration of the same concentration of external ACTH in dexamethasone treated LR and HR fish, showed that divergence in cortisol responsiveness

arises from interrenal and post-interrenal mechanisms (Pottinger & Carrick, 2001). *In vitro* interrenal stimulation with ACTH in another fish species, the gilthead sea bream, did not result in differentiated cortisol release between LR and HR fish (Rotllant et al., 2003a) leading the authors to suggest that other factors than the ACTH sensitivity affect the increased cortisol levels in HR fish. In other words, the interrenal tissue seems to play a crucial role in the regulation of cortisol responsiveness, where differences in the interrenal responsiveness to ACTH or the steroidogenic capacity of the interrenal tissue seem to cause the differences in cortisol responsiveness between LR and HR fish (Pottinger & Carrick, 2001). Indeed, it was recently shown that the expression of *mc2r* in the head kidney was higher in HR than LR rainbow trout (Khan et al., 2016), which could explain the increased ACTH responsiveness observed in these fish (Pottinger & Carrick, 2001). Additionally, a higher mRNA expression of genes encoding for enzymes regulating the synthesis of cortisol (StAR, P450scc, 3βHSD) from the interrenal tissue was observed in Atlantic cod HR fish, outlining a possible site of regulation concerning the differences in cortisol response (Hori et al., 2012b).

The aims of the present study were to examine whether differences in the resting cortisol concentrations between LR and HR fish existed after 1.5 year of their characterization, and if differences were also present in the ACTH circulating levels. Moreover, it was aimed to quantify the cortisol production capacity of the head kidney between LR and HR fish upon stimulation with the same concentration of ACTH, and the mRNA expression of genes involved in the regulation of cortisol production in the head kidney between LR and HR fish.

5.2. Materials and Methods

5.2.1. Fish and husbandry conditions

Low (LR) and High (HR) response fish identified as described in Chapter IV were placed together in a 2.5 m^3 circulating tank at Nireus S.A. research facilities (Pyrgoulaki, Euboea). In total, there were 72 fish, which were fed twice per day, for 6 days a week, using a commercial diet (Blue Line 45:20 3.5 mm, Feedus S.A., Greece). Throughout the experimental period, photoperiod was set at 12L:12D, water temperature was 18.20 ± 0.03°C, and salinity 27. Oxygen and pH ranged between 6 – 10 mg L^{-1} and 7.20 – 7.40, respectively. The experiment was carried out at Nireus S.A. research facilities.

In order to perform the superfusion experiment, fish were sampled randomly from the tank by the use of a baited-hook. This was performed to minimize disturbance to the remaining

fish, which were to be sampled during the next days of the experiment. No bias in terms of number of fish that bit the bait was observed, since in total the same number of LR and HR fish were caught, while in each sampling day both LR and HR fish were sampled.

In total, 4 fish were sampled each day of the experiment, and immediately euthanatized in high dose of 2-phenoxyethanol (500 ppm). Blood was then immediately drawn from the caudal vein via heparinized syringes, and fish were dissected to collect the head kidneys, which were placed in the superfusion medium. Specifically, both head kidneys were dissected from each fish, minced into fragments of approximately ~1 mm^3, and placed in the superfusion medium in order to remove the excess of blood. The medium was refreshed 4 times, until no signs of blood were observed. Afterwards, approximately 200 mg of tissue from each fish were placed in the superfusion chambers, while the rest was snap frozen in dry ice for mRNA molecular analysis.

5.2.2. Superfusion

Head kidneys were superfused with carbogen saturated (95% O_2/5% CO_2) 15 mM HEPES/Tris buffer (pH 7.4) containing 171 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 0.25% (w/v) glucose, 0.03% (w/v) bovine serum albumin and 0.1 mM ascorbic acid. The medium was delivered to the superfusion chambers at a constant rate of 30 µl min^{-1} via a peristaltic pump (Minipuls 3; Gilson Inc., WI, USA) (**Fig. 5.1**).

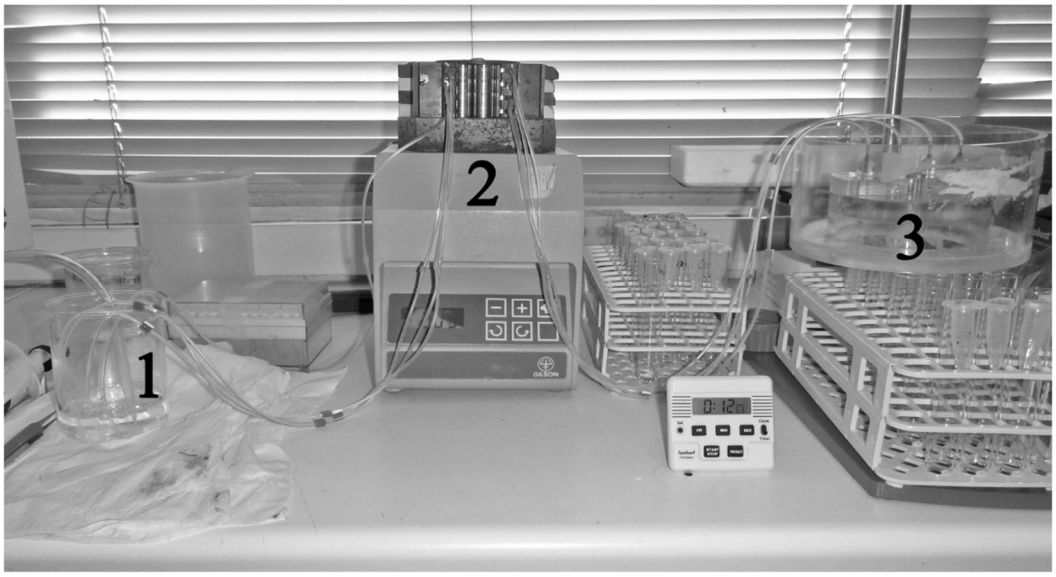


Fig. 5.1. The superfusion system used in this study. The carbogen-saturated (95% O_2 / 5% CO_2) superfusion medium (1) flows through the peristaltic pump (2) to the superfusion chamber device (3) where the head kidneys are placed.

Superfused medium dribbled from the chamber at the same rate, and was collected every 30 min, before the hormone treatment, and over 5- or 15-min intervals after the hormone addition, and stored at -20°C until analysis. After 150 min of superfusion, when the release rate has reached a baseline release (as seen by the superfusion of head kidneys without the addition of hormone; **Fig. 5.3**), the head kidneys were superfused with 10^{-7} M of human $ACTH_{1-39}$ (Sigma-Aldrich® A042, USA) dissolved in the superfusion medium.

5.2.3. Analytical procedures

Plasma cortisol and cortisol released in the superfusion medium by the head kidney were measured using a commercial enzyme-linked immunoassay kit (DRG® Cortisol ELISA, DRG® International Inc, Germany). Plasma ACTH concentrations were estimated by a commercial, specialized for fish, enzyme-linked immunoassay kit (CUSABIO® Fish Adrenocorticotropic hormone ELISA kit, CUSABIO BIOTECH Co. Ltd, China), as described in Chapter IV.

5.2.4. RNA purification and cDNA synthesis

Head kidney samples (20-30 mg) were disrupted and homogenized using the TissueRuptor (Qiagen, Hilden, Germany) for 20 s in 350 µl LBP lysis buffer (NucleoSpin® RNA Plus, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Total RNA was isolated using the NucleoSpin® RNA Plus kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), according to manufacturers' instructions. RNA yield and purity were determined by measuring the absorbance at 260 and 280 nm, using Nanodrop® ND-1000 UV-Vis spectrophotometer (Peqlab, Erlangen, Germany), while its integrity was tested by 1% agarose gel electrophoresis. Reverse transcription (RT) was performed using 1 µg RNA with the QuantiTect Reverse transcription kit (Qiagen, Valencia, USA), following manufacturers instructions.

5.2.5. Quantitative real-time PCR (qPCR)

The mRNA expression of genes encoding for *gr1*, *gr2*, *mr*, *11β-hydroxylase*, *11β-hsd2* and *mc2r* was determined with quantitative polymerase chain reaction (qPCR) assays using the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Willmington, USA). Oligonucleotides used

in the qPCR analysis are shown in **Table 5.1** (Tsalafouta et al., 2014; Tsalafouta unpublished data). Reactions were cycled and the resulting fluorescence was detected with MJ Mini Thermal Cycler (Bio-Rad) under the following parameters: (1) 95˚C for 3 min (HotStarTaq DNA Polymerase activation step), (2) 94˚C for 15 s (denaturation step), (3) 60˚C for 30 s (annealing step), (4) 72˚C for 20 s (extension step), cycling steps (2) to (4) for 40 cycles. Levels of *gr1*, *gr2*, *mr*, *11β-hx* and *11β-hsd2* mRNA were normalized based on the reference genes *β-actin*, *eEF1α*, and *18s*. A relative standard curve was constructed for each gene, using 4 sequential dilutions (1:5) of a pool of all the cDNA samples. geNORM analysis was also performed in order to validate which were the most suitable reference genes to serve as internal control and showed that *18s*, and *eEF1α* were the most appropriate.

Table 5.1. Primer sequences used in qPCR.

Gene	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
β-actin	CGCGACCTCACAGACTACCT	AACCTCTCATTGCCGATG
eEF1α	GCCAGATCAACGCAGGTTACG	GAAGCGACCGAGGGGAGG
18s	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCACA
gr1	GAGATTTGGCAAGACCTTGACC	ACCACACCAGGCGTACTGA
gr2	GACGCAGACCTCCACTACATTC	GCCGTTCATACTCTAACCAC
mr	CCTGTCTCCTCATGAATGG	AATCTGGTAATGGAATGAATGTC
11β-hsd2	CACCCAGCCACAGCAGGT	ACCAAGCCCCACAGACC
11β-hx	GGAGGAGGATTGCTGAGAACG	AGAGGACGACACGCTGAGA
mc2r	CATCTACGCCTTCCGCATTG	ATGAGCACCGCCTCCATT

5.2.6. Statistical analysis

Statistical analysis was performed using the SPSS v22.0 (IBM Statistics for Windows; IBM Corp., Armonk, NY, USA), while the figures were created using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Prior to analysis data were tested for normality using the Kolmogorov-Smirnov & the Shapiro-Wilk tests. Plasma cortisol and ACTH were analysed using t-tests. A two-way repeated measures Analysis of Variance (ANOVA) was performed, using a mixed design with one between factor (*type of response*, *i.e.* LR or HR) and one within factor (*superfusion time*), which consisted the repeated measured factor. Data were tested for sphericity using Mauchly's test, while the most stringent Greenhouse-Geisser correction F-test was used to determine significant effect of *superfusion time*, *type of response* and their interaction. When significant differences existed, Tukey's post-hoc tests were used

to determine which means differed significantly from each other. All tests were performed with $P < 0.05$ set as the levels of significance.

In order to estimate the magnitude of cortisol response after superfusion of the head kidneys with ACTH, the Area Under the Curve (AUC) was calculated based on the trapezoidal method, using the GraphPad Prism 6.0 (GraphPad Software, USA). Moreover, since each sample had a different baseline cortisol release rate (t = 150 min), the AUC with respect to each specific baseline value was calculated as described by Fekedulgen et al. (2007). Differences between the mean AUC of LR against HR fish were tested using t-test.

5.3. Results

5.3.1. Plasma cortisol and ACTH

Resting levels of cortisol of LR and HR fish differed significantly, being higher in HR fish ($t_{18} = 2.913$; $P = 0.009$) (**Fig. 5.2 a**). Plasma ACTH concentrations, on the other hand, showed no statistically significant differences between LR and HR fish ($t_{18} = 1.505$; $P = 0.150$) (**Fig. 5.2 b**).

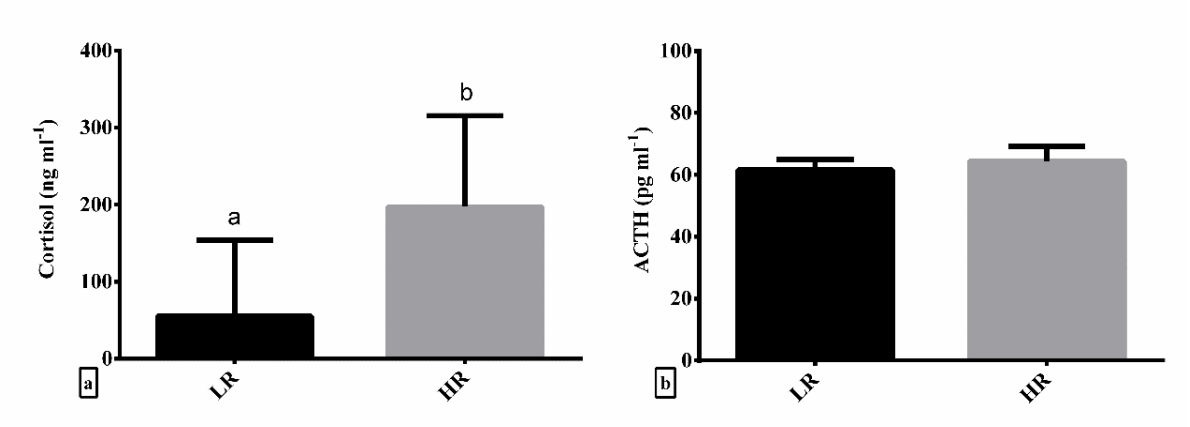


Fig. 5.2. Mean resting cortisol (a) and ACTH (b) concentrations for the LR and HR E. sea bass fish. Values are presented as means + standard deviation ($n = 10$). Means with different letters differ significantly from one another ($P < 0.05$).

5.3.2. Superfusion

Superfusion of head kidneys without stimulation with ACTH showed that cortisol release rate was declining from the beginning of the superfusion (first measurement at 30 min) reaching an equilibrium at 150 min (**Fig. 5.3**). From 150 min until 300 min of superfusion,

cortisol release fluctuated between 85.1 % (at 240 min) and 117.9 % (at 210 min) of the rate at 150 min (**Fig. 5.3 b**). For that reason, this time point (150 min) was chosen as the starting point of ACTH stimulation for the main experiment. In order to exclude the possibility of non-proper superfusion of the head kidneys, an excess of K^+ (60 mM) was dissolved in the superfusion medium at the end of the 300-min period, and delivered to the tissues over an extra period of 60 min. This was performed to depolarize the tissue and release cortisol aspecifically.

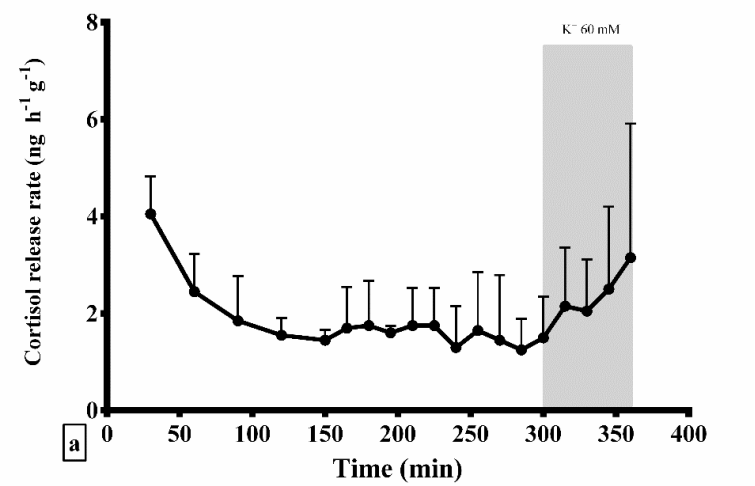


Fig. 5.3. *In-vitro* release of cortisol from unstimulated superfused head kidneys expressed as cortisol release rate (ng min^{-1} g^{-1}).

Cortisol release rate expressed as the amount of cortisol released (ng) in respect to time (min) and to the weight of tissue used (g) showed an increase after ACTH stimulation in both LR and HR fish (**Fig. 5.4**). When the response curves were compared by analysis of variance no statistically significant differences existed between LR and HR fish ($F_{1,10}$ = 2.142; P = 0.174), although the release in HR fish was more than 2-times higher than the LR.

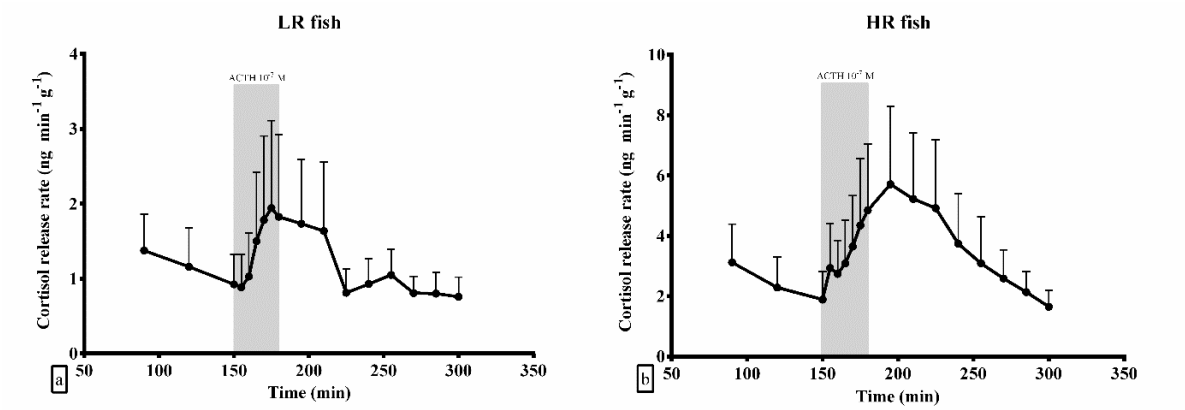


Fig. 5.4. *In-vitro* release of cortisol from superfused head kidneys of LR (a) and HR (b) fish stimulated with ACTH, expressed as cortisol release rate (ng min^{-1} g^{-1}). Values are presented as means + standard error (n = 6). Note that the y-axis is in different scale.

However, when the mean Areas Under the Curve (AUC) of the response (*i.e.* after the administration of ACTH) of LR and HR fish were compared, significant differences existed, showing a higher response in the HR fish (T = 4.000; $P = 0.025$) (**Table 5.2**).

Table 5.2 Basal and maximum cortisol release rate, time to reach the maximum release rate, and mean Area Under the Curve for the LR and HR fish. Values are presented as means ± SD (n = 6). Means with different letters differ significantly from one another ($P < 0.05$).

	LR	HR
Basal release rate (ng min^{-1} g^{-1})	0.91 ± 0.99	1.89 ± 2.27
Maximum release rate (ng min^{-1} g^{-1})	2.14 ± 2.86	5.71 ± 6.32
Time of maximum release rate (min)	175	195
AUC (ng g^{-1})	55.75 ± 65.04[a]	282.67 ± 250.62[b]

When the relative stimulation of cortisol secretion was analysed, estimated by manually adjusting cortisol release prior to ACTH administration (*i.e.* 150 min) to 100% and calculating the other times point in respect to that, significant stimulation of cortisol secretion was observed, as shown by the Greenhouse-Geisser F-test ($F_{3.78,37.77} = 4.473$; $P = 0.005$) (**Fig. 5.5**). Post-hoc analysis showed that the stimulation in HR fish was fast, becoming statistically significant at 155 min and remaining significantly higher than basal until to 270 min post-ACTH stimulation (**Fig. 5.5**). In LR fish significant stimulation was observed at 165 min and remained significant until 210 min of superfusion (**Fig. 5.5**). Comparing the response curves between LR and HR fish it was shown that ACTH stimulation caused a significantly higher response in the HR than LR fish ($F_{1,10} = 5.280$; $P = 0.044$), (**Fig. 5.5**).

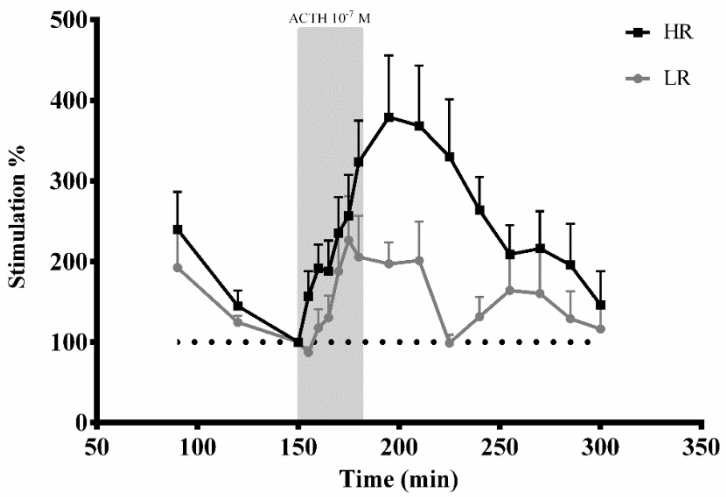


Fig. 5.4. *In-vitro* release of cortisol from superfused head kidneys of LR and HR fish stimulated with ACTH, expressed as percental secretion relative to basal. Values are presented as means + standard error ($n = 6$).

5.3.3. Gene expression in the head kidney

Molecular mRNA gene expression analysis revealed no differences in the expression of the glucocorticoid receptors, *gr1*, and *gr2* (*gr1*: $t_8 = 1.329$; $P = 0.221$; *gr2*: $t_8 = 1.547$; $P = 0.160$) (**Fig. 5.5 a & b**), as well as the mineralocorticoid receptor, *mr* ($t_8 = 0.049$; $P = 0.962$) (**Fig. 5.5 c**) between the head kidneys of LR and HR fish. The ratio between the expression of the glucocorticoid receptors and the mineralocorticoid receptor also did not differ between LR and HR fish (*gr1/mr*: $t_8 = 1.846$; $P = 0.102$; *gr2/mr*: $t_8 = 2.052$; $P = 0.074$) (**Fig. 5.5 d & e**).

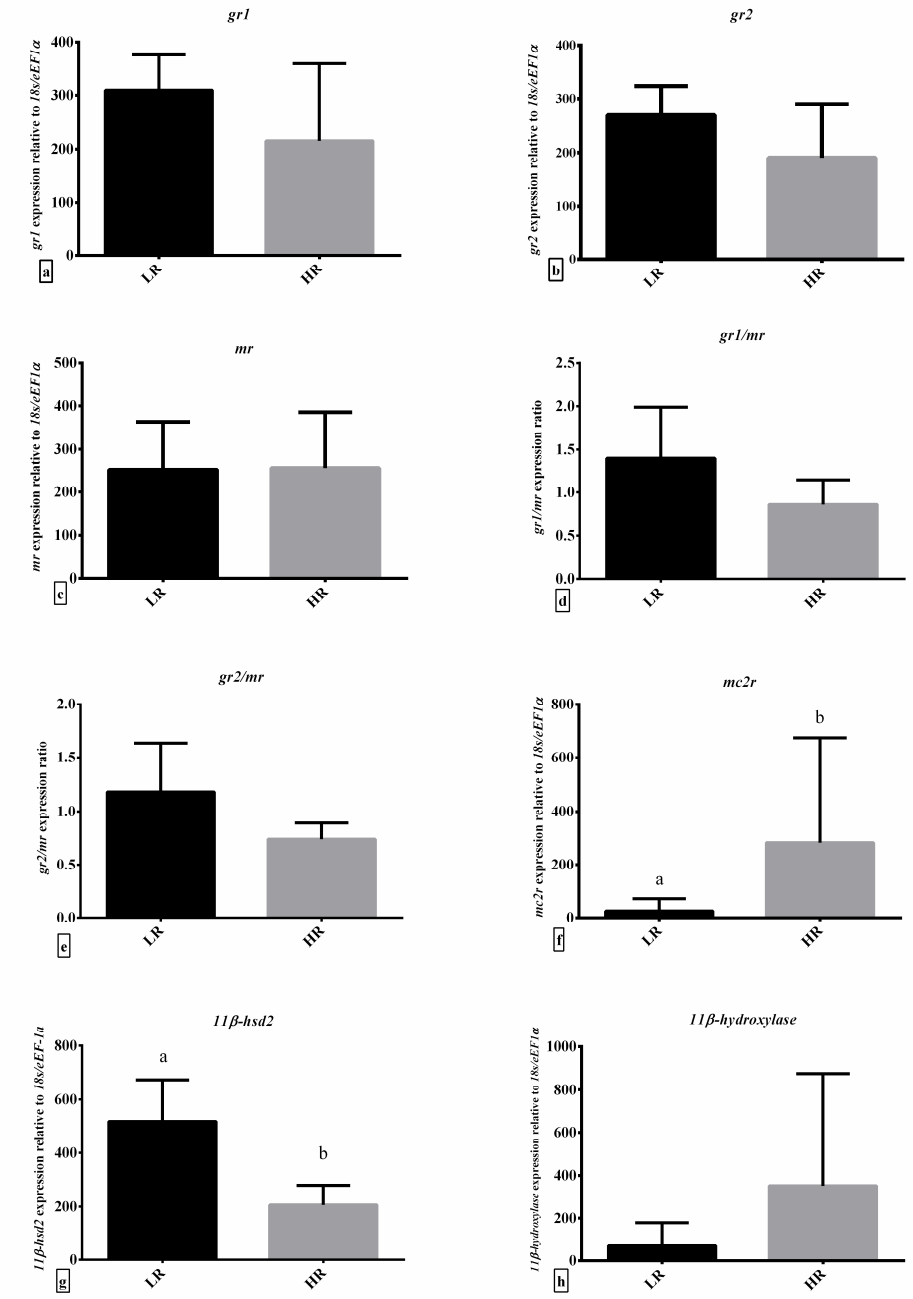


Fig. 5.5. mRNA transcript levels of *gr1*, *gr2*, *mr*, *gr1/mr*, *gr2/mr*, *mc-2r*, *11β-hydroxylase* and *11β-hsd2* in the head kidneys of LR and HR fish. Values are presented as means + standard deviation ($n = 6$). Means with different letters differ significantly from one another ($P < 0.05$).

On the other hand, the type of response seemed to affect the expression of the gene that encodes for the receptor of ACTH, *i.e.* the *mc2r*, which was significantly up-regulated in HR compared to LR fish (T = 4.500; P = 0.032) (**Fig. 5.5 f**). The opposite pattern was observed for the gene encoding for a protein which oxidizes cortisol to the inactive cortisone, *i.e. 11β-hsd2*, being significantly higher in LR than HR fish (t_8 = 4.028; P = 0.038) (**Fig. 5.5 g**). Finally, no significant differences were observed in the expression of *11β-hydroxylase*, an enzyme that is involved in cortisol synthesis (T = 6.000; P = 0.222) (**Fig. 5.5 h**), although there was a 2.3-fold increase in HR compared to LR fish.

5.4. Discussion

Results of the present study demonstrate that even 19 months after the identification of LR and HR fish, and when in co-culture, the differences in the resting cortisol levels between them remain. On the other hand, no differences existed in the resting plasma ACTH concentrations, while, as discussed in the previous chapter (Chapter IV), there were also no differences in the post-stress concentration of ACTH as well. Moreover, when the head kidneys of E. sea bass LR and HR fish were stimulated with the same concentration of ACTH in an *in vitro* superfusion system, HR fish showed greater overall outcome of cortisol production and higher relative-to-basal production compared to LR. These results indicate that ACTH concentration seems not to be the determinant factor regulating cortisol differences between LR and HR fish, but point out that the biosynthetic capacity and sensitivity of the interrenal tissue to ACTH might be the regulatory factor behind divergent cortisol responsiveness.

Studies in LR and HR rainbow trout also question the role that the concentration of ACTH plays in the regulation of cortisol responsiveness (Pottinger & Carrick, 2001). Specifically, no differences between LR and HR trout in both resting and post-stress ACTH levels in the circulation have been observed (Pottinger & Carrick, 2001). Additionally, the biosynthetic capacity of interrenal tissue in LR and HR trout has also been studied *in vivo*, by exogenous ACTH administration in fish previously treated with dexamethasone in order to block the endogenous ACTH release. Results showed a significantly higher plasma cortisol concentration in the HR than the LR fish, although fish had been injected with the same amount of ACTH (Pottinger & Carrick, 2001). Controversial results, on the other hand, were derived from an *in vitro* superfusion experiment in gilthead sea bream (Rotllant et al., 2003a), since

although HR fish showed higher unstimulated cortisol production than LR fish, when ACTH was administered no differences between LR and HR fish were observed.

These results suggest that differences in cortisol responsiveness between LR and HR fish could most probably be regulated at the level of the interrenal tissue. Specifically, this differential regulation in the head kidney could result from divergent sensitivity to ACTH stimulation through its receptors and/or different process of this signal through the subsequent steps of cortisol synthesis and degradation. Differences in the adrenocorticotric capacity and sensitivity to ACTH between strains with divergent cortisol responsiveness have also been described in birds such as domestic fowl (Carsia & Weber, 1986), and Japanese quail (Carsia et al., 1988), and mammals, such as rats (Gómez et al., 1996) and pigs (Kanitz et al., 1999).

For that reason, head kidneys from LR and HR E. sea bass were analyzed for mRNA expression of genes related to the regulation of action (*gr1, gr2* and *mr*), biosynthesis (*11β-hydroxylase*), and degradation (*11β-hsd2*) of cortisol, as well as that of the receptor of ACTH, the *mc2r*.

Results showed that indeed the head kidney seemed to be a major site of regulation of cortisol responsiveness, since ACTH binding, as well as biosynthetic capacity for cortisol were regulated in a different way between LR and HR fish. Specifically, *mc2r* was significantly over-expressed in HR than LR fish, while there was a higher than 2-fold increase in the expression of *11β-hydroxylase* in HR fish, though not statistically significant.

In line with the present results, an overexpression of the *mc2r* gene has also been reported in HR rainbow trout head kidney (Khan et al., 2016), leading the authors to suggest that this could explain the increased cortisol response to ACTH in these fish. Additionally, a differential regulation of the gene expression of enzymes involved in cortisol biosynthesis has also been observed between LR and HR Atlantic cod (*Gadus morhua*) (Hori et al., 2012b). Specifically, the genes of the enzymes StAR, P450scc, and 3βHSD were significantly upregulated in HR compared to LR fish. Therefore, it seems that both the sensitivity to ACTH and the biosynthetic capacity of the interrenal tissue is higher in HR than LR fish, explaining partly the observed differences in the resting and post-stress cortisol concentrations between these phenotypes.

In the present study, the expression of the enzyme *11β-hsd2*, which encodes for the enzyme that inactivates cortisol to cortisone, was also examined, and showed significantly reduced expression in HR compared to LR fish. To the author's best knowledge, this is the first time that significant differences in the expression of this cortisol-catabolizing gene have been reported between LR and HR fish or tetrapod species. This enzyme can exert intracrine and endocrine effects. The former mainly concern animals that produce aldosterone and cortisol at

the same tissue, where *11β-hsd2* is essential in inactivating cortisol, a potent MR ligand, in order to avoid unnecessary MR activation (Pruner et al., 2006; Chapman et al., 2013). In terms of endocrine effects, this enzyme can alter circulating levels of cortisol, and it has been suggested that it inactivates approximately 30-40% of the total daily production (Chapman et al., 2013). Inhibition of *11β-hsd2* has led to increased cortisol levels in zebrafish (Alderman & Vijayan, 2012) and corticosterone in rats (Musajo et al., 1996), reflecting thus its capacity to regulate the activity of the HPI axis. It seems, therefore, that not only LR fish tend to show reduced perception of the ACTH signal and lower capacity for cortisol production, but also higher regulation of cortisol catabolizing enzymes in the head kidney.

Interestingly, in mammals, ACTH exerts inhibitory effects on *11β-hsd2*, with the mechanism not being yet fully understood (Walker et al., 1992; Walker et al., 1994; Musajo et al., 1996; Morita et al., 1997). All studies suggest an indirect effect of ACTH on *11β-hsd2*, proposing a yet unknown ACTH-dependent corticosteroid inhibition of the enzyme (Walker et al., 1992; Musajo et al., 1996), acting via the ACTH receptor (Morita et al., 1997). It is therefore tempting to speculate that the reduced expression of *11β-hsd2* in HR fish could be the regulated by actions of ACTH as reflected by the increased *mc2r* expression.

As a result, this higher expression of *11b-hsd2* in LR fish could also possibly indicate an ability of these fish for faster recovery after stress, or after an *in vitro* stimulation with ACTH, as was observed in the present study. Faster recovery after acute stress has also been observed in LR rainbow trout fish when compared to HR fish (Weil et al., 2001). The current data, however, do not suffice to elucidate the role of *11β-hsd2* in the post-stress cortisol recovery rate in LR and HR fish, and experiments estimating the expression of this gene after stress could assist in getting a better insight in this regard.

To summarize, it seems that the differences in the circulating cortisol and the production and secretion of cortisol by the interrenal tissue are mainly regulated at the level of the interrenal tissue, and not by the levels of ACTH in the circulation. This regulation is carried out by a combination of increased sensitivity of the interrenal tissue to ACTH, upregulation of enzymes involved in the biosynthesis of cortisol and downregulation in the expression of genes regulating degradation of cortisol in HR fish.

Chapter VI. Summary & Conclusions

1. Resting and post-stress cortisol concentration in E. sea bass show high variability that can be affected and/or regulated by environmental, physiological, and genetic factors.

2. Environmental water temperature is a major factor affecting circulating and post-stress cortisol, as well as its release rate into the water. Specifically, higher resting cortisol levels were observed with increasing temperature. In terms of the response, the highest overall outcome was observed at 15°C, while the fastest response was seen at 25°C. Cortisol release rate in the water was also faster and more intense at higher temperatures, which could, at least in part, explained the differences in plasma cortisol response pattern. These patterns should be considered when evaluating the stress status in E. sea bass.

3. Determination of the allostatic load is of prime importance for the concept of allostasis. In this regard, a protocol of repeated predictable stress of various intensities was developed in order to define allostatic load in E. sea bass fish.

4. Different allostatic loads exerted on fish significantly affected performance and cortisol levels in E. sea bass, both before and after an additional acute stressor. Specifically, allostatic load resulted in decreased feed consumption and growth in E. sea bass. Fish exposed to the highest allostatic load exhibited high basal cortisol levels and an inability to further respond to acute stress. Gene expression in the liver showed that fish in the high stress group had lower transcripts of the cortisol-inactivating enzyme *11β-hsd2* than lower allostatic load groups. Finally, gene expression in the head kidney revealed that both the ACTH receptor, *mc2r*, and an enzyme involved in cortisol biosynthesis, *11β-hydroxylase*, were up-regulated in chronically stressed fish when compared to controls.

5. Genetic background is another important factor explaining variability in basal cortisol levels and cortisol response to stress. Specifically, family-based differences existed in both resting and post-response cortisol levels. These differences accounted for 28.54% of the total variance observed. Moreover, cortisol responsiveness was shown to be a repeatable trait (repeatability calculated at 38.9%), and therefore Low (LR) and High (HR) fish were identified. These fish differed in their resting, post-stress and free cortisol

levels, being higher in HR, while no differences were observed in post-stress plasma ACTH.

6. LR and HR fish showed differences in the hepatic transcription profile. In details, there were 169 transcripts expressed exclusively in LR and 161 in HR fish. Enrichment analysis of those transcripts revealed many processes differentially expressed between these groups of fish. In short, nitrogen metabolism, and some aspects of the immune system were significantly more expressed in HR than LR fish. HR fish also expressed transcripts related to the conservation of energy and glucose in contrast to LR fish that expressed transcripts involved in the flux of three-carbon molecules towards oxidation, suggesting in that way the possibility for a higher energy demand or reduced feed consumption by the HR fish.

7. Differences in resting cortisol concentrations between LR and HR fish were maintained even after more than 1.5 years from the selection. These differences were not accompanied by differences in circulating ACTH, but the biosynthetic capacity of the head kidney was higher in HR than LR fish when stimulated *in vitro* with the same concentration of ACTH. Moreover, LR fish showed a shorter, in terms of duration, response to ACTH.

8. LR and HR fish showed differences in the expression of genes involved in cortisol synthesis from the head kidney. In details, the expression of the gene encoding for ACTH receptor, *mc2r*, was significantly over-expressed in HR fish, while *11β-hydroxylase*, an enzyme involved in cortisol synthesis, showed a 2.3-fold increase in these fish, though this difference was not statistically significant. Finally, the expression of *11β-hsd2*, encoding for an enzyme that inactivates cortisol, was significantly higher in LR fish. It seems, therefore, that the differences in cortisol levels between LR and HR are mainly regulated at the level of the interrenal tissue, and not by the levels of circulating ACTH. This is achieved by an increased sensitivity of the interrenal tissue to ACTH, the upregulation of enzymes involved in cortisol biosynthesis, and downregulation in the expression of genes regulating degradation of cortisol in HR fish.

9. Overall, the present thesis showed for the first time a strong genetic component in cortisol response, identified LR and HR individuals and characterized them at the molecular and endocrine level.

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Scientific Papers in Peer-Reviewed Journals

PAPER I

SCIENTIFIC REPORTS

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Repeatability of cortisol stress response in the European sea bass (*Dicentrarchus labrax*) and transcription differences between individuals with divergent responses

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Understanding the stress responses of organisms is of importance in the performance and welfare of farmed animals, including fish. Especially fish in aquaculture commonly face stressors, and better knowledge of their responses may assist in proper husbandry and selection of breeding stocks. European sea bass (*Dicentrarchus labrax*), a species with high cortisol concentrations, is of major importance in this respect. The main objectives of the present study were to assess the repeatability and consistency of cortisol stress response and to identify differences in liver transcription profiles of European sea bass individuals, showing a consistent low (LR) or high (HR) cortisol response. The progeny of six full sib families was used, and sampled for plasma cortisol after an acute stress challenge once per month, for four consecutive months. Results suggest that cortisol responsiveness was a repeatable trait with LR and HR fish showing low or high resting, free and post-stress cortisol concentrations respectively. Finally, the liver transcription profiles of LR and HR fish showed some important differences, indicating differential hepatic regulation between these divergent phenotypes. These transcription differences were related to various metabolic and immunological processes, with 169 transcripts being transcribed exclusively in LR fish and 161 exclusively in HR fish.

Knowledge of the stress physiology of animals, and especially of the mechanisms regulating the physiological stress responses, are of importance in order to understand how animals respond to stressors and explain the biological significance of the intra-specific variation observed in these responses. This is also important for the welfare of farmed animals, since it could help in optimizing their husbandry and lead to a more appropriate selection of the breeding stock, especially in genetic selection breeding programs. In general, the effects of a stressor depend heavily on how an animal perceives and processes the stressful stimuli and how it can cope with it[1]. In this context, much attention has been given during the last decades to the fact that individuals of the same species may show consistently divergent physiological and behavioral responses to a stimuli or stressor. These sets of responses have been described as coping styles[1], behavioral syndromes, personalities or temperaments, with a more or less synonymous use[2]. Two main categories of coping styles have been identified in vertebrates, namely the proactive and reactive[1], that differ in many aspects of their behavior and physiology. In specific, proactive animals seem more aggressive, display the fight-flight response and show low behavioral flexibility, in contrast to reactive animals that tend to be non-aggressive and cautious, adopt the freezing response strategy and are behaviorally flexible. In terms of physiology, proactive animals have been linked to low stress axis output, regulated by low mRNA expression of CRH in the hypothalamus, and low glucocorticoid (cortisol or corticosterone depending on the

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taxa) production. Moreover, these animals show low parasympathetic and high sympathetic activity compared to reactive individuals which show the reverse pattern[3]. Therefore, identification of such intra-specific differences in cortisol responsiveness and better understanding of their impact in the animal performance and fitness would be beneficial towards better husbandry and selection of breeding stocks in genetic selection programs.

Divergence in the cortisol response has been described in many tetrapod vertebrates, including fish[2,4], amphibians[5], reptiles[4], birds[6], and mammals[7,8]. Fish, in particular, are among the most widely studied taxa in this aspect, and consistent differences in the cortisol response between individuals of the same species have been described[9–14]. Consequently, low (LR) and high (HR) responding fish have been identified in respect to the intensity and consistency of their cortisol response after exposure to acute stressors. Cortisol responsiveness has also shown moderate to high heritability in rainbow trout (h^2 ranging between 0.22 to 0.56[15–18]), with strains of low and high responding individuals having been established[16] though still merely used for research purposes. This divergence in cortisol responsiveness has also been associated with behavioral differences and coping styles[19,20], as well as animal performance and fitness. Specifically, differences in growth[9,21,22], plasma metabolites[12,23], hepatic carbohydrates reserves and enzymatic activity[23] and differential expression in many genes involved in physiological functions including metabolic and immune functions[24] have been observed between LR and HR individuals.

Still however, individual divergence in cortisol responsiveness has not been identified in the European sea bass (*Dicentrarchus labrax*), a species of major economic importance in the Mediterranean aquaculture. This species shows an intense stress response[25] and is considered susceptible to stress by inducing reproductive dysfunctions and disease outbreaks[26], which, in turn, hamper production. Moreover, cortisol concentrations are characterized by high variability in both basal and response levels, so between studies[27] as between individuals of the same population[28]. Consequently, it is of great importance to gain better knowledge on the stress physiology of this species by investigating the divergence of the response among individuals, since stress responsiveness can affect their performance[9,21]. Previous studies have shown low ($h^2 = 0.08$[29]) to moderate ($h^2 = 0.34$[30]) heritability of post-stress cortisol concentrations in this species, while LR and HR fish have not been identified. Yet, three suggestive Quantitative Trait Loci (QTL) for cortisol stress response have been identified[31], indicating the existence of a genetic background regulating this response. It could be therefore suggested that an important confounding aspect in the studies showing low heritability[29] or no consistency in the cortisol response[32] in European sea bass might have been that the progeny used was produced from a breeding population with a small effective size and hence low genetic variability.

In this context, the main objectives of this study were to investigate the consistency of the cortisol stress response in European sea bass, to characterize possible differences in free cortisol concentrations among LR and HR individuals, and to get a better insight into the liver transcription profile of LR and HR fish. Such results could provide better knowledge of the stress physiology of the species, which could also constitute a potentially new selection trait to consider in selection programs for the species in aquaculture. Finally, analysis of the hepatic transcriptome between LR and HR fish could provide better insight to the metabolic and immune mechanisms that accompany the LR and HR phenotypes.

Results

Cortisol repeatability and identification of LR and HR fish. Analysis of the post-stress plasma cortisol concentrations of the fish used in this study showed that this was a repeatable trait in European sea bass individuals, as estimated by the nested ANOVA ($r = 0.389$; $F_{58,129} = 3.542$; $P < 0.001$) (Fig. 1a). The measurement error of the repeated cortisol measures was estimated at 69.13 ng ml^{-1}.

Moreover, based on the Z-scores analysis, it was possible to distinguish between fish that showed a consistently low or high acute stress cortisol response (Fig. 1). These two groups of fish (LR and HR; n = 16 per group) showed constantly different post-stress plasma cortisol concentrations in all samplings, with lower cortisol concentrations in LR than HR fish ($F_{1,30} = 224.49$; $P < 0.001$; Fig. 1b). The contribution of the 6 families to the LR and HR fish groups was unequal, while the family factor had a significant impact on cortisol responsiveness, explaining 28.54% of the total variance observed in this trait, as calculated by the variance components analysis.

Differences in resting cortisol concentrations were also observed between LR and HR fish sampled 15 days after the last exposure to the acute stress protocol. In particular, at the final sampling (S5), individuals identified as LR showed resting cortisol levels more than two times lower (93.4 ± 63.4, n = 16) than HR fish (203.3 ± 121.1, n = 16) ($t_{30} = 3.216$; $P = 0.003$).

A subsample of tested LR and HR fish (n = 10 per group) was also checked for the concentration of free cortisol in their plasma (Table 1). LR fish showed lower levels of both total ($t_8 = 7.48$; $P < 0.001$) and free ($t_8 = 3.38$; $P = 0.01$) cortisol concentration; however, the percentage of free over total cortisol (% Free) showed no difference between LR and HR fish ($t_8 = 0.26$; $P = 0.80$; Table 1).

Transcriptome analysis. The analysis showed that 169 transcripts were only transcribed in LR fish, and 161 transcripts were exclusively transcribed in HR fish. The transcripts were blasted against the European sea bass genome[33] and categorized by the linkage groups they belonged to (Supplementary Table 1). Blast2Go analysis on the annotations of these transcripts showed that their putative functions included various metabolic processes, such as single-organism, protein, and nitrogen compound metabolic processes, as well as signal transduction (Fig. 2). In addition, they included several molecular functions, such as ATP binding, DNA, protein, metal ion and zinc ion binding, as well as transporter, transferase and protein kinase activity (Supplementary Figure 1). Unique metabolic processes observed only in LR individuals included protein metabolic processes, whereas immune system processes and nitrogen compound metabolic processes were only seen in HR fish, and consisted of more than 50% of the biological processes.

The Fisher's exact test showed that 26 gene ontologies were significantly over-expressed in LR fish (Table 2) and 29 GOs in HR fish (Table 3).

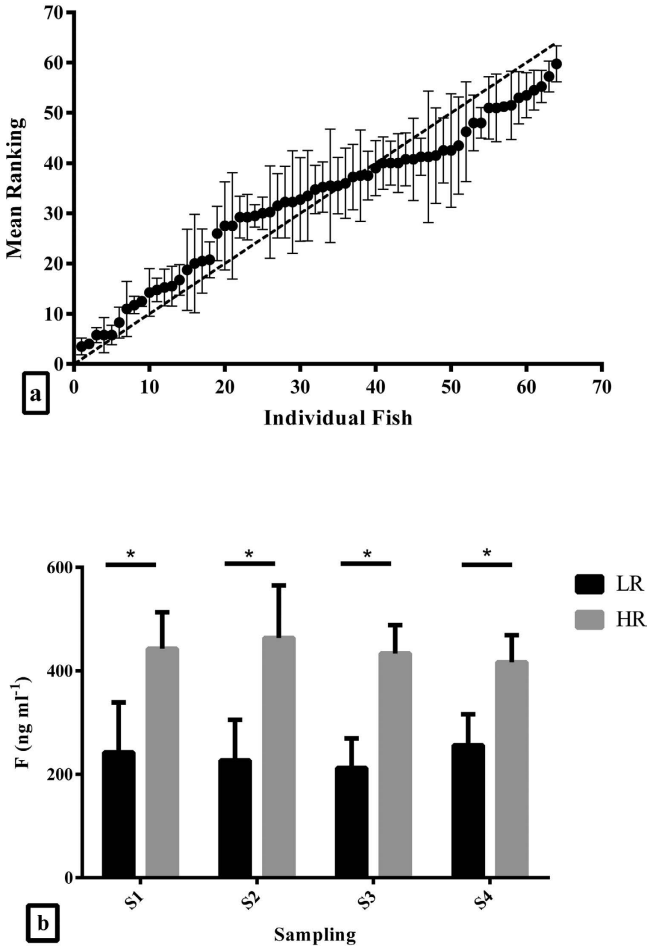

Figure 1. Cortisol concentration analysis. (**a**) Repeatability of ranked post-stress cortisol concentrations in E. sea bass (n = 64). Each point refers to the mean ranking of each fish ± S.E.M. The dotted line represents perfect repeatability, where one fish should have identical ranks across all trials. (**b**) Post-stress cortisol concentrations in all samplings for fish identified as LR and HR (n = 16 per group). An asterisk (*) indicates statistically significant differences between the two groups within each sampling point (S1 to S4) ($P < 0.05$).

	LR	HR
Total F (ng ml^{-1})	217.2 ± 58.6*	439.2 ± 31.1**
Free F (ng ml^{-1})	129.1 ± 43.9*	247.0 ± 85.1**
% Free	59.4 ± 13.4	61.8 ± 15.8

Table 1. Total, Free and % Free cortisol (F) in selected LR and HR fish from the S4 sampling (n = 10 per group). Asterisks indicate statistically significant differences between the two groups ($P < 0.05$).

Subsequently 72 transcripts related to the immune responses, carbon, protein, lipid, and energy metabolism that were differentially transcribed between LR and HR fish were selected and subjected to heatmap analysis (Fig. 3). Each row of the heat map represents the pattern of one transcript's expression, while columns represent the HR and LR fish. This analysis grouped the LR and HR fish separately (Fig. 3).

Discussion

The present study demonstrates for the first time the existence of individual-specific cortisol stress responses in European sea bass. Cortisol responsiveness was shown to be a repeatable trait, and fish showing constantly low or high responsiveness were identified and characterized as LR and HR fish. These fish also differed in their resting (*i.e.* without stress) cortisol levels, as well as the amount of free cortisol, which is the biologically active form of the hormone[34]. Finally, differences existed in the liver transcriptome profile between LR and HR fish, depicting differences in the regulation of metabolic and immune functions. Such results suggest that intra-specific differences should be taken into account when studying the stress responses in European sea bass, and could in turn explain the high variation observed in cortisol responsiveness. Moreover, knowledge of this aspect of stress physiology could assist in optimizing husbandry and welfare, as well as suggest cortisol responsiveness as a new

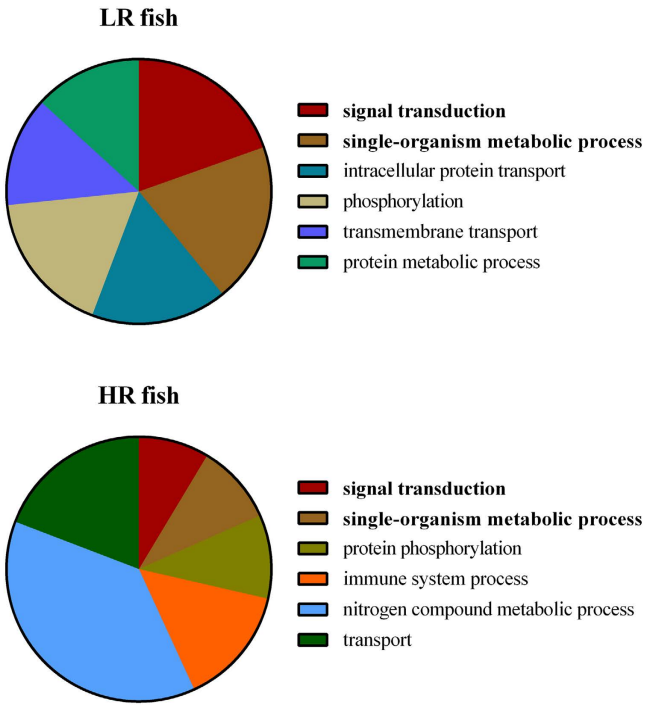

Figure 2. Multi-level profile of the Biological Processes Gene Ontology terms in (**a**) LR and (**b**) HR fish liver. GO terms with a node score below 5 were filtered out. Bold lettering indicates that these terms were shared between LR and HR fish.

potential selection criterion in genetic selection programs. It should, however, be kept in mind that selection for cortisol responsiveness could potentially correlate or be co-selected with other traits of interest such as growth[9], and lysozyme response[9,15], or not yet studied traits, which could exert positive, neutral or negative effects on fish performance.

LR and HR fish have been described in other fish species, such as rainbow trout[9,11,14,18], Atlantic cod[10], striped bass[13], and gilthead sea bream[12]. However, up until today the cortisol response of European sea bass had been considered to show low to moderate heritability[29,30]. Additionally, it has not been feasible to identify LR and HR individuals[32], possibly due to the low genetic variability of the population used, since fish were the progeny of few parents. Given the small number of families used in the present study, the heritability of this trait was not estimated, yet it was shown that the genetic background (*i.e.* family) had a significant impact on cortisol responsiveness. Whether the traits regulating resting cortisol as well as responsiveness to acute stressors are heritable and linked to specific QTLs in European sea bass or not is currently under investigation by our group.

Moreover, in the present study, differently responding fish seemed to differ in the resting concentrations of cortisol, with HR fish showing higher levels of circulating cortisol in their blood. To the best of our knowledge, there are only two studies showing direct differences in the resting (i.e. before stress) cortisol concentration between progeny of selected LR and HR families; in rainbow trout[35] and in gilthead sea bream[36]. Significant positive correlations between family average post-stress and basal cortisol levels have also been shown between LR and HR families in rainbow trout[22]. However, prior to selective breeding towards the selection of divergent cortisol responses in rainbow trout, there were no differences between the basal cortisol levels of LR and HR fish[11].

This is the first report on the concentration of free (*i.e.* unbound to proteins) cortisol in European sea bass. Free cortisol is the biologically active fraction of the hormone[34]. The concentration of free cortisol was higher in HR than LR fish, indicating that these fish indeed show higher levels of biological active cortisol and no regulatory mechanism takes place to diminish the differences observed in the total concentration. Nevertheless, even though the amount of free cortisol differed, the percentage of free in respect to the total concentration of cortisol showed no differences. Although in general the concentration of free cortisol was higher in the European sea bass when compared to others fish species, the percentage observed did not seem to differ from that in gadoids[10,37], but was slightly higher than Atlantic salmon, *Salmo salar*[38], and tiger pufferfish, *Takifugu rubripes*[39]. In line with the present study, no differences in the fraction of free cortisol were observed between LR and HR in Atlantic cod[10]. These differences in the free concentration of cortisol, both between species as well as within the same species between LR and HR fish, may reflect differences in the effects that cortisol could have on their performance, like immune function, metabolism and growth. It should however be noted that there are also other mechanisms regulating the actions of cortisol, like the abundance and affinity of the glucocorticoid receptors[40].

Liver is an important organ that regulates many functions of the organism, including metabolism, immune functions and the stress-induced secondary responses[24,41–43]. In an attempt to associate the LR and HR phenotypes to the above-mentioned functions of the liver, a subset of LR and HR fish was subjected to hepatic transcriptome analysis. This analysis focused on transcripts that were exclusively transcribed in either LR or HR fish in

GO ID	GO Term	*p*-value	Genes
GO:0004030	aldehyde dehydrogenase [NAD(P)+] activity	1.49E-02	*aldh3a2*
GO:0051895	negative regulation of focal adhesion assembly	3.11E-02	*aldh3a2*
GO:0006081	cellular aldehyde metabolic process	3.27E-02	*aldh3a2*
GO:1900016	negative regulation of cytokine production involved in inflammatory response	2.63E-02	*apod*
GO:0071638	negative regulation of monocyte chemotactic protein-1 production	2.63E-02	*apod*
GO:0060588	negative regulation of lipoprotein lipid oxidation	2.63E-02	*apod*
GO:0002088	lens development in camera-type eye	2.63E-02	*apod*
GO:0010642	negative regulation of platelet-derived growth factor receptor signaling pathway	2.63E-02	*apod*
GO:2000098	negative regulation of smooth muscle cell-matrix adhesion	2.63E-02	*apod*
GO:0015485	cholesterol binding	2.79E-02	*apod*
GO:0042308	negative regulation of protein import into nucleus	3.27E-02	*apod*
GO:0048662	negative regulation of smooth muscle cell proliferation	4.40E-02	*apod*
GO:0000302	response to reactive oxygen species	4.83E-02	*apod*
GO:0030791	arsenite methyltransferase activity	1.49E-02	*as3mt*
GO:2000405	negative regulation of T cell migration	2.63E-02	*cdipt*
GO:0090344	negative regulation of cell aging	2.63E-02	*cdipt*
GO:0010842	retina layer formation	3.44E-02	*cdipt*
GO:0060219	camera-type eye photoreceptor cell differentiation	3.59E-02	*cdipt*
GO:0005758	mitochondrial intermembrane space	1.49E-02	*cycs*
GO:0004714	transmembrane receptor protein tyrosine kinase activity	5.70E-03	*fgfr1a*
GO:0090140	regulation of mitochondrial fission	2.63E-02	*march5*
GO:0051865	protein autoubiquitination	4.40E-02	*march5*
GO:0042981	regulation of apoptotic process	3.59E-02	*nod1*
GO:0004594	pantothenate kinase activity	1.98E-02	*pank1*
GO:0015937	coenzyme A biosynthetic process	2.47E-02	*pank1*
GO:0004252	serine-type endopeptidase activity	3.06E-02	*tmprss6*

Table 2. Gene Ontology terms significantly enriched in LR fish.

order to identify the main crossing points between them. Specifically, 169 transcripts were transcribed only in LR fish, while 161 transcripts were exclusively transcribed in HR fish. These transcripts were annotated in metabolic processes that were shared between LR and HR fish, such as single-organism metabolic process (*i.e.* chemical reactions and pathways, including anabolism and catabolism, by which living organisms transform chemical substances and which involve a single organism) and signal transduction, and others like protein, and nitrogen compound metabolic processes that were included in LR or HR fish exclusively. In particular, intracellular protein transport, phosphorylation, transmembrane transport, and protein metabolic processes were unique to LR fish. On the other hand, nitrogen compound metabolic processes, transport, immune responses, and protein phosphorylation were unique to HR fish.

Out of all the biological processes exclusively annotated in the multi-level analysis in HR fish, nitrogen compound metabolic processes accounted for 37.7%. Moreover, enrichment analysis showed that glutaminase activity was significantly over-represented in HR fish, mediated by the transcription of *gls* transcript in HR fish only. The enzyme encoded by this transcript catalyzes the first reaction of the glutamate catabolism, and is therefore related to the metabolism of nitrogen and formation of glucose[44]. This enzyme is also involved in other amino acid metabolism, as shown by the KEGG pathways of alanine, aspartate and glutamate metabolism, as well as arginine and proline metabolism (Reference Pathway map00250 and map00330 respectively; KEGG PATHWAY Database; Kanehisa Laboratories; http://www.genome.jp/kegg/pathway.html). Increased cortisol concentrations have been shown to stimulate nitrogen metabolism in the sea raven, *Hemitripterus americanus*[45], while nitrogen excretion tended to be unaffected by chronic cortisol stimulation in rainbow trout[43].

Apart from metabolism, liver is also an important organ in the immune response of fish, synthesizing various immune-related proteins[46]. In this study, the GO term related to immune responses was annotated in the metabolic processes pie chart of HR fish (Fig. 2). In detail, the GO term of antigen processing and presentation of endogenous peptide antigen via MHC class I was enriched in HR fish, due to the high transcription of the *tapbp* transcript. Tapasin, a member of the MHC class Ia antigen-loading complexes, mediates the interaction between MHC class Ia molecules and the transporter associated with antigen presentation[47]. Apart from *tapbp*, other MHC class Ia related transcripts were transcribed in HR fish, like the *mhc class ia antigen* and *major histocompatibilty complex I-related gene*. Unlike the present results, in rainbow trout high levels of cortisol were related to a down-regulation of the MHC class II antigen beta chain and the MHC class II antigen associated invariant chain[41]. In LR fish, on the other hand, processes regarding the negative regulation of T-cell migration and cytokine production involved in inflammatory response were significantly enriched.

Differences between LR and HR fish were also present in the expression of transcripts encoding for proteins involved in the pathway of complement and coagulation cascades (Fig. 4). Specifically, in LR fish, the liver transcripts encoding for complement C3 and Factor H (HF) proteins were transcribed when compared to HR fish

GO ID	GO Term	*p*-value	Genes
GO:0003993	acid phosphatase activity	2.22E-02	*acp5*
GO:0000276	mitochondrial proton-transporting ATP synthase complex, coupling factor F(o)	2.95E-02	*atp5a1*
GO:0004089	carbonate dehydratase activity	2.22E-04	*ca5a*
GO:0006730	one-carbon metabolic process	3.49E-03	*ca5a*
GO:2000021	regulation of ion homeostasis	1.86E-02	*ca5a*
GO:0009264	deoxyribonucleotide catabolic process	1.12E-02	*dera*
GO:0004139	deoxyribose-phosphate aldolase activity	1.12E-02	*dera*
GO:0004359	glutaminase activity	1.49E-02	*gls*
GO:0008375	acetylglucosaminyltransferase activity	7.17E-03	*mgat1*
GO:0006486	protein glycosylation	4.28E-02	*mgat1*
GO:0005967	mitochondrial pyruvate dehydrogenase complex	7.46E-03	*pdk2*
GO:0006111	regulation of gluconeogenesis	7.46E-03	*pdk2*
GO:0010510	regulation of acetyl-CoA biosynthetic process from pyruvate	7.46E-03	*pdk2*
GO:0004740	pyruvate dehydrogenase (acetyl-transferring) kinase activity	7.46E-03	*pdk2*
GO:0005739	mitochondrion	1.16E-02	*pdk2 ca5a atp5a1*
GO:0072332	intrinsic apoptotic signaling pathway by p53 class mediator	1.49E-02	*pdk2*
GO:0008286	insulin receptor signaling pathway	2.04E-02	*pdk2*
GO:0042593	glucose homeostasis	2.22E-02	*pdk2*
GO:0031670	cellular response to nutrient	2.22E-02	*pdk2*
GO:0034614	cellular response to reactive oxygen species	2.41E-02	*pdk2*
GO:1902494	catalytic complex	2.77E-02	*pdk2 prkar1a*
GO:0006885	regulation of pH	3.32E-02	*pdk2*
GO:0005952	cAMP-dependent protein kinase complex	3.52E-04	*prkar1a*
GO:0008603	cAMP-dependent protein kinase regulator activity	4.54E-04	*prkar1a*
GO:0001932	regulation of protein phosphorylation	3.72E-02	*prkar1a*
GO:0019885	antigen processing and presentation of endogenous peptide antigen via MHC class I	7.46E-03	*tapbp*
GO:0031625	ubiquitin protein ligase binding	4.40E-02	*ube2w*
GO:0000139	golgi membrane	4.68E-02	*ube2w*
GO:0071218	cellular response to misfolded protein	7.46E-03	*ube2w*

Table 3. Gene Ontology terms significantly enriched in HR fish.

(Fig. 4). C3 protein plays a central role in the activation of the complement system, which also forms the serine protease C3-convertase upon binding to factor B[48]. The H Factor, on the other hand, functions to accelerate the decay of the C3-convertase[48]. In HR fish, on the other hand, a different component of the complement system was transcribed, namely the *c7* (Fig. 4), which is an important component of the Membrane Attack Complex (MAC)[48]. Finally, transcription of transcripts related to the coagulation of blood was observed in HR fish; specifically, *f5* and *f11* (Fig. 4).

The above results indicate possible differences in the regulation of the immune functions between the LR and HR phenotypes. It is not, however, yet clear if these differences are of importance to the overall performance of the fish. Future disease resistance experiments could shed light in this aspect. Furthermore, although cortisol is known to affect the immune function of organisms, it is unclear whether cortisol *per se* is responsible for these differences, or selection for cortisol responsiveness is correlated with aspects of the immune system, such as lysozyme[9,15] which is an important enzyme in the innate immune functions. In this context, although no data exists for the expression of these genes in LR and HR fish, when rainbow trout was faced with a chronic cortisol increase, via slow-releasing cortisol implants, a down-regulation in liver *c3* and *cfh* genes, as well as B factor (*Bf*) was observed[42], which is in line with the current results that show no expression of these transcripts in the HR fish.

A series of carbohydrate metabolism-related transcripts were transcribed in HR fish. Processes mediated by the mitochondrial pyruvate dehydrogenase complex were significantly enriched in these fish as a result of the high transcription of the *pdk2*. This kinase phosphorylates the active form of the enzyme pyruvate dehydrogenase (PDH_a) to its inactive form (PDH_b), inhibiting in this way its activity[49]. This inhibition leads to the conservation of three-carbon compounds for gluconeogenesis, instead of metabolite flux through fatty acid and cholesterol synthesis, and the tricarboxylic acid cycle[50].

In LR fish, on the other hand, the biosynthesis of coenzyme A process was significantly enriched. An excess of CoA in the cell can inhibit the activity of PDK in mammals, favoring in this way the oxidation of pyruvate through the TCA cycle for the production of energy, or fatty acids and cholesterol[51]. Additionally, *glut2*, which encodes for a protein that is likely responsible for glucose transport and uptake by the liver, was transcribed in LR fish[52]. In mammals, the expression of this gene increases under high glucose concentrations to enhance the insulin secretory response to glucose[52]. From all the above, it could be suggested that the conservation of energy

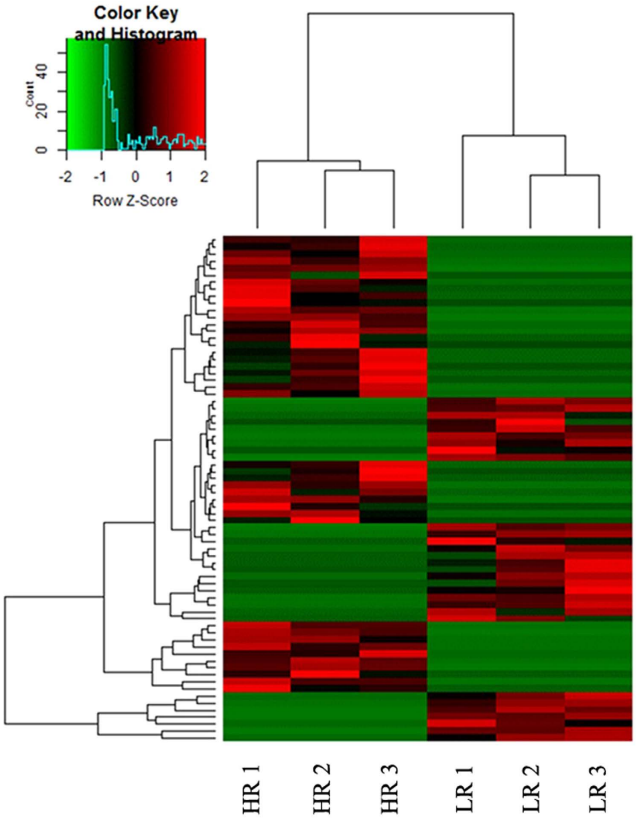

Figure 3. Heatmap of differentially expressed transcripts between HR and LR fish, as indicated under each column of clusters. Each row represents the expression of one transcript in relation to the other, while shades of red represent upregulation and shades of green represent downregulation.

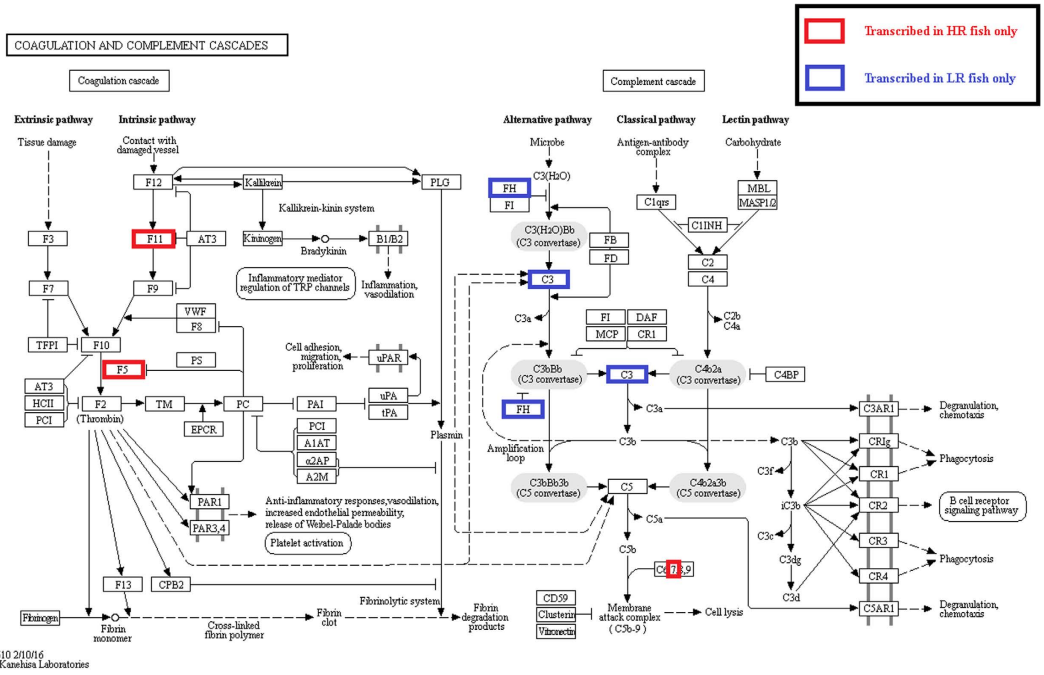

Figure 4. Annotated transcripts in the complement and coagulation cascades KEGG pathway. Blue and red boxes indicate annotated transcripts expressed in LR and HR fish exclusively.

and glucose synthesis in HR, in contrast to a tendency towards oxidation of pyruvate and uptake of glucose in the LR fish, could be indicative of increased energy demand or reduced appetite for food, given that all tanks were feed *ad libitum*, which may in turn affect the overall performance of the fish.

Processes involved in protein metabolism and modification showed some differential transcription between LR and HR fish. In general, cortisol is believed to exert proteolytic actions in fish[53], and elevated transcripts of ubiquitin after acute stress have been observed in rainbow trout[53]. The ubiquitin-proteasome proteolytic pathway is of major importance in the degradation of proteins. In detail, different aspects of the ubiquitin-proteasome pathway were regulated in LR and HR fish. Specifically, in LR fish, protein autoubiquitination was significantly enriched while in HR fish the process of ubiquitin ligase protein binding was enriched. Additionally, in LR fish, the serine-type endopeptidase activity process, which is involved in protein degradation, was also enriched. The energy and amino acids released by this proteolysis are subsequently used in protein synthesis[54]. Transcripts related to protein modification processes were also enriched in HR fish, involving important processes like the regulation of protein phosphorylation and glycosylation as well as c-AMP dependent kinase regulation.

In conclusion, the present study showed for the first time that the cortisol stress response is a repeatable feature in European sea bass, and therefore LR and HR individuals can be identified. These fish differ in the concentration of both total and free post-stress as well as resting cortisol concentrations. Better understanding of these differences could be useful in genetic selection programs by introducing new potential selection criteria, given the important impacts that cortisol responsiveness may have on the performance and fitness of the fish. Specifically, these types of fish showed differences in their hepatic transcription profile, with transcripts being solely transcribed in LR or HR fish. Enrichment analysis of those transcripts, revealed many processes that were differentially expressed between these groups of fish. In short, nitrogen metabolism, and some aspects of the immune system were significantly more expressed in HR than LR fish, which highlights the need for further study of their ability to resist exposure to pathogens. HR fish also expressed transcripts related to the conservation of energy and glucose in contrast to LR fish that expressed transcripts involved in the flux of three-carbon molecules towards oxidation, suggesting in that way the possibility for a higher energy demand or reduced feed consumption by the HR fish, which could have important aquaculture applications.

Materials and Methods

Fish and housing conditions. Six full sib families of European sea bass were randomly chosen out of 96 families from the Nireus S.A. (Greece) family-based breeding program. All families were created on the same day from different parents (6 females and 6 males). Each of the 6 families consisted of 12 immature fish (17 months-old at the beginning of the experiment), randomly chosen from the whole progeny of each respective family (mean weigh ($\pm$SD) of $93.2 \pm (20.5$ g)). Each family was reared separately in open circulation tanks at Nireus S.A. research facilities. Fish were fed twice per day, for 6 days a week, using a commercial diet (Blue Line 45:20 3.5 mm, Feedus S.A., Greece). Throughout the experimental period, the photoperiod was set at 12L:12D, the water temperature was 18.20 ± 0.03 °C, and the salinity 27. Oxygen and pH ranged between 6–10 mg L^{-1} and 7.20–7.40, respectively.

Sampling. In the first sampling, all fish per tank were confined by lowering the water of the tank and then chased with a net for 5 minutes. Fish were left confined for 30 minutes, and then carefully netted, immediately anaesthetized in ethylene glycol monophenyl ether (300 ppm; Merck; 807291; USA) and then weighed and tagged using unique glass PIT tags. Blood was immediately collected from the caudal vessel via heparanized syringes and centrifuged (2,000 g; 10 min), and the resulting plasma was stored at −20 °C until analyzed. The stress protocol was repeated once a month for four consecutive months, termed hereafter as S1 to S4.

Fifteen days following the completion of the last stress experiment (S4), a final sampling was performed to assess resting cortisol values and to collect liver samples for *mRNA* analysis (S5). Fish were immediately captured, euthanized with high doses of anaesthetic, and their blood was sampled as described previously. Liver samples collected for molecular genetic analysis were immediately frozen in liquid nitrogen and subsequently stored at −80 °C until analyzed.

Z-score calculation, repeatability statistic, and LR and HR fish identification. In order to correct for variations in the cortisol data between the samplings the Z-score was used instead of the raw data. By calculating Z-scores of the individual post-stress cortisol levels for each sampling, the cortisol concentrations of each individual fish were standardized in respect to the overall mean and standard deviation of each respective sampling[14].

The repeatability of the post-stress cortisol levels was assessed using the repeatability statistic, r[55]. The consistency of the response within an individual across the four samplings was tested by a nested ANOVA, with the factor Individual being nested in the factor Family. The null hypothesis was that individual Z-scores were inconsistent within individuals and hence the variable was not repeatable.

For the identification of LR and HR individuals, the sum of the Z-scores of all samplings was calculated for each individual fish. In this way, high responders should always have a positive Z-score, and therefore show a high sum of Z-scores. In contrast, low responders should always show lower cortisol concentrations than the overall mean, and have negative sum Z-scores. Finally, fish with either intermediate or no consistence response should have intermediate sum of Z-scores. In this manner, the Z-scores of all individuals were ranked, and fish belonging to the upper quartile of the distribution of Z-scores were identified as HR, and those belonging to the lower quartile as LR fish. This method ensures that both the intensity and the consistent of the cortisol response were taken into consideration when characterizing these types of responses.

Analytical measurements. Plasma total and free (*i.e.* unbound) cortisol were measured by the use of a commercial enzyme immunoassay kit (DRG® Cortisol ELISA, DRG® International Inc, Germany). The performance of the kit has been previously evaluated with linearity and recovery tests in E. sea bass plasma samples[28].

The separation of free and bound cortisol was performed by ultrafiltration of the plasma, as described by[37]. Briefly, 200 μl of plasma were loaded in centrifugal filter units (Centrifree®-MPS, Micropartition devices, Millipore Corp, USA) and centrifuged at 2,000 g for 30 min at 18 °C. Subsequently, the resulting filtrate with the free cortisol was stored at −20 °C until analyzed. Determination of free cortisol was performed in a subsample (n = 10) consisting of the LR and HR individuals with the lowest and highest sum of Z-scores.

RNA extraction. Liver tissue from the 3 LR and 3 HR fish with the lowest and highest sum of Z-scores, respectively, was subjected to RNA extraction. Disruption of the samples was performed in liquid nitrogen using a mortar and pestle. After adding lysate buffer, the lysate was homogenized by passing it through a 20G needle attached to a sterile plastic syringe 5 times. RNA was subsequently extracted using the RNA extraction Kit II of Machinery Nagel (Dueren, Germany), according to the manufacturers' instructions. RNA concentrations were determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington USA) and the quality was assessed by gel electrophoresis as well as by RNA Nano Bioanalysis chip (Agilent 2100 Bioanalyzer).

NGS Sequencing. Total RNA was submitted for Illumina 100 bp reads paired-end sequencing (RNA-Seq) to the Norwegian High Throughput Sequencing Centre. Tags reads were separated using a mulitplex identifier (MID). Evaluation of the reads was assessed using the freely available FastQC software program (version 0.10.0; http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Low quality reads as well as adaptors were removed by Trimmomatic software[56]. Read assembly was obtained by applying Trinity, version 2012-06-08[57]. Raw sequence data as well as metadata were submitted to the Short Read Archive (SRA) database of NCBI available under the accession number SRP064240.

Expression analysis. Paired end reads were mapped against the constructed reference transcriptome in order to assess expression abundances quantified by RSEM version 1.2.3[58]. Contigs with low read support were excluded from downstream analysis. Transcript expression profiles were assessed by the estimation of pairwise abundance of transcript between the two types of response using the R Bioconductor package DESeq[59]. The most stringent dataset of transcripts was used, by analyzing transcripts that were exclusively expressed in one condition (no transcripts identified in the other) and in all three replicates, with p-value < 0.05 and FDR-value < 0.05.

Functional annotations and gene ontology. All transcripts obtained were submitted against the non-redundant protein database (nr) as well as the non-redundant nucleotide database (nr/nt) using the standalone BLAST tools (version 2.2.25); threshold cut-off e-values of 10^{-6} and 10^{-10}, respectively, were chosen. Annotations and GO terms assignment were retrieved by Blast2Go software, while the reference pathway in Fig. 4 was obtained by KEGG database[60].

Statistical analysis. Statistical analysis was performed using the SigmaStat 3.1 statistical package (Systat Software, San Jose, CA, USA) and SPSS v.22 (IBM Corp., Armonk, NY, USA). Results are presented as means ± standard deviation. Two-way repeated measures analysis of Variance (ANOVA) tests were performed. The type of individual (LR vs HR) and sampling time (S1 to S4) were used as fixed factors, with their interaction being also checked, and the individuals as subjects. For the comparison of free cortisol in a subset of LR and HR samples, and of the resting cortisol levels (S5) between LR and HR individuals t-tests were used. In all statistical tests data were examined for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test prior to analysis. Variance components analysis was performed using the VARCOMP command in SPSS.

Ethical statement. All experiments were performed in accordance with relevant guidelines and regulations. Nireus S.A. research facilities are certified and have obtained the codes for the rearing and use of fish for scientific purposes (EL04-BIOexp-01). All procedures on fish used in this study were approved by the Departmental Animal Care Committee following the Three Rs principle, in accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals.

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Author Contributions

A.S., M.P. and L.P. co-designed the study, A.S., M.P., E.S. and A.D. wrote the manuscript. L.K. carried out the provision and husbandry of fish and participated in the samplings, M.P. and A.D., performed the experiment, A.S., M.P. and E.S. co-analyzed the data. A.S. prepared Figures 1, 2 and 4, and all the tables, E.S. prepared figure 3. All authors reviewed the manuscript.

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PAPER II (submitted)

Water temperature modifies the acute stress response of European sea bass, *Dicentrarchus labrax* L.

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Abstract

The effects of water temperature (15, 20 and 25°C) on the stress response of European sea bass, *Dicentrarchus labrax*, were studied. Blood and water samples were collected prior and at 0.5, 1, 2, 4 and 8 hours post-stress for hormonal and biochemical analysis. Water temperature affected the resting concentrations of all stress indicators examined, and the stress response of cortisol, glucose and osmolality, but not that of lactate. Cortisol showed the response with the highest outcome at 15°C, and the fastest at 25°C. Cortisol release rate in the water was also faster and more intense at higher temperatures. Glucose had both the fastest and higher in magnitude response at 25°C, while lactate responded similarly in all temperatures tested. Finally, osmolality responded only at the highest temperature. These result suggest that temperature should be taken into consideration when evaluating the stress status of E. sea bass in laboratory, rearing and field conditions. Moreover, it was shown that cortisol release rate in the water, a non-invasive stress indicator, can depict the effects of temperature on cortisol stress response.

Keywords: cortisol; cortisol release rate; glucose; European sea bass; temperature

1. Introduction

Temperature widely affects the physiology and performance of ectotherm organisms. Fluctuations of physiological parameters, such as hormones and metabolites, in relation to the annual cycle due to temperature, photoperiod and the physiological state of the fish have been described in many teleost species (Fregeneda-Grandes et al. 2013; Vargas-Chacoff et al. 2009b; Gómez-Milán et al. 2011), including European sea bass, *Dicentrarchus labrax*, (Pascoli et al. 2001; Gutiérrez et al. 1987). However, fish, and particularly eurythermal species, are capable of functioning adequately well in a wide range of temperatures by using a variety of different enzyme orthologs according to temperature (Somero 2004). Although fish can function in a spectrum of water temperatures, there is a thermal preferendum for each species, a range over which the performance of the animal is maximized (Beitinger & Fitzpatrick 1979). Since different aspects of the physiology and functions of an organism can have different thermal preferendums, the whole-animal optimum is determined by (a) the integration of various functions and systems preferendum of the organism, (b) the optimum of the most sensitive function, or (c) may be governed by the masking influence of other environmental factors (Beitinger & Fitzpatrick 1979).

In E. sea bass the critical minimum and maximum temperatures have been estimated to be around 4-6°C (Dügler et al. 2012), and 33-35°C (Dügler et al. 2012; Madeira et al. 2013), respectively. However, the thermal optimum has been suggested to be close to 25°C for growth, 25-29°C for feed intake, 19-25°C for feed efficiency ratio (Person-Le Ruyet et al. 2004), and metabolism (Claireaux & Lagardère 1999), from 19.3 to 29.6°C for swimming performance (Koumoundouros et al. 2002) and 22-28°C for the whole animal performance (Dügler et al. 2012). Spawning, on the other hand, is performed in much lower temperatures; specifically, between 9 and 18°C, with the optimal temperatures ranging between 13-15°C (Mañanós et al. 2009).

Water temperature has also been referred to change the rate of many biological processes of an organism, including mechanisms that regulate the stress response. Many physiological parameters commonly used as stress indicators, such as cortisol, glucose, lactate and osmolality, show a tendency for higher concentrations at high temperatures in several fish species (King et al. 2006; Lankford et al. 2003; Barton & Schreck 1987; Davis & Parker 1990; Sumpter et al, 1985; Vargas-Chacoff et al. 2009a; b). In addition, the pattern of the stress response is usually faster, in terms of both time to peak and to recover, and more intense in higher temperatures. However, there are fish species that deviate from this general pattern, such

as goldfish, *Carassius auratus* (Umminger & Gist 1973) and chinook salmon, *Oncorhynchus tshawytscha* (Barton & Schreck 1987) which show a lessened response in some of the examined physiological traits, like glucose and liver glycogen.

During the last years, non-invasive indicators of stress, such as cortisol released from the fish into water, have been developed (Ellis et al. 2004; Ellis et al. 2005; Scott & Ellis 2007) and evaluated in many fish species (Ellis et al. 2004; Fanouraki et al. 2011; Pavlidis et al. 2013). However, the effects of temperature on the release of cortisol have been poorly examined. To our best knowledge, there is only one study in this respect, which showed reduced release after an acute temperature drop in common carp, *Cyprinus carpio* (Jaxion-Harm & Ladich 2014). However, plasma cortisol was not quantified in that study, and therefore it cannot be concluded whether this reduced cortisol release was due to an impaired release or to lower plasma values. The aim of the present study was to examine the effects of acclimation temperature on the response of commonly used stress indicators, such as plasma and water-born cortisol, plasma glucose, lactate and osmolality, by applying a previously validated acute stress protocol and by keeping other environmental parameters such as photoperiod constant.

2. Materials and Methods

2.1. Fish and husbandry conditions

Immature E. sea bass individuals were hatched and raised in the Hellenic Centre for Marine Research (HCMR, Greece, Crete), and were transferred and maintained in 3 x 250 L aquaria at the installations of the Fish Physiology Laboratory, University of Crete. The aquaria were continuously aerated and water was recirculated through a biological and mechanical filter (Eheim external canister filter, EHEIM GmbH & Co. KG, Germany). Temperature was controlled by thermostat heaters (Resun®, China) and chillers (Sfiligoi, S.R.L., Italy) while the photoperiod was set at 12L:12D.

Initially, 252 fish (mean weight ± S.E.M.: 29.6 ± 0.6 g) were transferred to the experimental aquaria, and divided into 84 fish per aquarium, resulting in a density of 9.80 ± 0.06 kg m^{-3}, and were held at a water temperature of 20°C, similar to the holding and transportation temperature. After one week of acclimation to the novel environment, and after fish showed resumption to feeding, a protocol of mild temperature change was performed. In particular, in two of the aquaria the desired temperatures of 15°C and 25°C were reached by appropriately changing the temperature by 1°C every second day, with the whole procedure lasting for 10 days. In the

third aquarium the temperature was held constant at 20°C. Subsequently, fish were left for an extra period of two weeks to acclimate to the new conditions prior to sampling, with the mean temperatures in each aquarium during this time being 15.05 ± 0.03°C; 19.80 ± 0.08°C; 25.03 ± 0.36°C; termed hereafter as 15, 20 and 25 °C groups respectively.

Fish were fed daily a constant quantity (2% of their body weight) of a commercial dry pellet diet (Irida S.A., Greece) consisting of approximately 44% protein and 19% lipids. During the whole time water parameters were monitored daily (temperature, pH) or weekly (ammonia, nitrate, nitrite).

2.2.Experimental design

In order to study the resting and post-stress levels of stress indicators in fish acclimated to different temperatures 70 fish from each temperature treatment were used. Specifically, 10 fish from each aquarium were immediately captured (0h, unstressed fish), euthanatized in phenoxyethanol (500 ppm; Merck, 807291) and bled from the caudal vein via heparinized syringes. Additionally, two water samples were collected from each respective tank (0.5 L). The remaining fish were acutely stressed by lowering the water to approximately 1/3 of its initial volume and then chasing with a net for 5 minutes. After the completion of the stress protocol, fish were divided in 6 x 30 L tanks (10 individuals per tank), filled up with the water from the home aquarium, covered with a lid in order to keep fish in the shade and provided with aeration. Sampling was performed, as in the unstressed fish, at 0.5, 1, 2, 4 and 8 h post-stress by sampling all the fish from one tank at each time point. Fish sampled at 8 h post-stress were held in two tanks (10 individuals per tank) and were used for water sampling. At 8 h post-stress all fish from both tanks were anaesthetized and their body mass was estimated, while 5 fish per tank were randomly chosen for blood sampling.

Water samples (V = 0.5 L) were collected at the same time points as the blood samples from the duplicate tanks holding fish that were sampled at 8 h post-stress. By collecting successive water samples from the same tank it was possible to calculate the release rate of cortisol from the fish in that tank, which would not have been possible if the water samples were collected from different tanks at each sampling point. Care was taken when removing water in order to avoid disturbing and further stressing the fish. For that reason, water was collected by the use of a catheter tube without removing the cover of the tank or exposing fish to any disturbance. Immediately afterwards, the water that was removed during the sampling was replaced, using the same way, in order to maintain the same stocking density throughout the trial. During the

whole experimental period (8 h), fish were held in the same temperature as the one they had been acclimated to, by setting the environmental room temperature at the respective level. Following blood collection, blood was centrifuged (2,000 g; 10 minutes) and the resulting plasma was stored at -20°C until the analysis.

2.3. Analytical procedures

A series of parameters were measured in fish plasma. In particular, plasma and water-born cortisol were estimated by the use of a commercial enzyme immunoassay kit (DRG® Cortisol ELISA, DRG® International Inc, Germany). The performance of the kit has been previously evaluated with linearity and recovery tests in E. sea bass plasma samples (Samaras et al. 2015). Cortisol from water samples was extracted, as described by Ellis et al. (2004). Briefly, water samples were pumped with a peristaltic pump at ca. 10 ml min^{-1} through a pre-filter (0.45 µm pore-size, AcroCap™, Gelman Sciences, MI, USA) and then through an activated solid phase extraction cartridge (Sep-pak® Plus C18, Waters Ltd., UK). Subsequently, cortisol was eluted from the extraction cartridges with ethyl acetate, which was evaporated at 45°C under nitrogen gas and the residue was re-dissolved in 1 ml of phosphate buffer saline (which has shown no interference with the specific ELISA analysis). All samples were run in duplicate.

The amount of cortisol within each bucket was calculated at each sampling point using the estimated water cortisol concentration and taking into account the amount of cortisol removed by sampling (0.5 L) and renewed afterwards (0.5 L). 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.

Plasma glucose and lactate concentrations were measured by commercial enzymatic colorimetric kits (Biosis, Greece, for glucose; Spinreact, Spain, for lactate), whereas plasma osmolality was determined by the use of an osmometer (Osmomat 030, Gonotec GmbH, Germany). Due to technical issues, in all temperatures examined lactate was measured in 5 out of 10 individuals in each time group.

2.4. Statistical analysis

Statistical analysis was performed using the SigmaStat 3.1 statistical package. Results are presented as means ± standard deviation (SD). One-way Analysis of Variance (ANOVA) tests were used to check for significant effects of *temperature* on the resting concentration of the

examined parameters, and two-way Analysis of Variance tests to check for significant effects of *time post-stress*, *temperature*, and their interaction. When significant differences existed, at a level of $P < 0.05$, Tukey's post-hoc multiple comparison analysis were subsequently performed. Prior to analysis, data from the samples collected from the duplicate tanks at 8 h post-stress were compared using t-test, and since no significant differences were found in all parameters examined (*i.e.*, cortisol, glucose, lactate, osmolality) they were pooled together. Moreover, before the analysis data were checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test and, when necessary, data were log-transformed before analysis. In the case of the resting values of osmolality the one-way ANOVA assumptions were not met even after data transformation and therefore the non-parametric Kruskal-Wallis test was used followed by Dunn's post-hoc analysis.

In order to estimate the magnitude of the response of each physiological trait as a whole, the Area Under the Curve (AUC) was calculated based on the trapezoidal method, using the GraphPad Prism 6.0 software (GraphPad Software, USA). Specifically, the total AUC (AUC_t) was calculated by counting the total area between the ground (y = 0) and the curve. Additionally, since fish at different temperature conditions showed different baseline values, the AUC with respect to increase from the baseline levels (y = value of the physiological trait at 0 h) (AUC_i), was also calculated. In details, AUC_i calculates the area with respect to the baseline measurement subtracting the area that goes below that baseline, when such area exists, emphasizing in that way on the changes over time (Fekedulgen et al. 2007). In addition, the slope from baseline to peak (SBP), defined as the difference between the baseline and peak value divided by the time interval between these two points (in minutes) was calculated as a descriptive indicator of the speed of the response.

3. Results

3.1. Resting concentrations

One-way ANOVA analysis showed that the mean resting values for cortisol differed between fish reared at different temperatures ($F_{2,27} = 8.278$; $P = 0.002$; Table 1 & Fig. 1). Specifically, lower values were observed at 15°C compared to those at 20°C ($P = 0.048$) and 25°C ($P < 0.001$; Table 1 & Fig. 1).

Glucose resting levels also varied between rearing temperatures ($F_{2,27} = 7.300$; $P = 0.003$; Table 1 & Fig. 2), being lower at 15°C than at 20°C ($P = 0.005$) and 25°C ($P = 0.012$). Water

temperature affected resting lactate concentrations ($F_{2,13}$ = 4.685; P = 0.029), and specifically higher levels were observed at 20°C than 25°C (P = 0.030; Table 1 & Fig. 3). Resting concentrations of osmolality differed between temperatures (H_2 = 14.003; P < 0.001), with higher levels observed at 20°C and 25°C than at 15°C as shown by the subsequent Dunn's post-hoc analysis (Fig. 4).

3.2. Stress response

In all temperature conditions, a cortisol response was evoked after acute stress, yet however significant interactions between the factors temperature and time post-stress were presented ($F_{2,158}$ = 4.774; P < 0.001). Specifically, fish acclimated at 20°C and 25°C reached the highest mean concentrations of plasma cortisol at 0.5 h post-stress (Fig. 1), showing approximately 2.5 and 4 times higher slope to peak than 15°C, respectively (Table 2), and remained unaltered until 4 h post-stress in the former and 8 h post-stress in the latter conditions. In contrast, at 15°C a continuous increase was observed until 4 h post-stress, with signs of recovery afterwards (Fig. 1). Apart from the variations in the resting values, differences in cortisol concentration existed both at 0.5 h and 4 h post-stress, with lower and higher concentrations, respectively, in the 15°C group (P < 0.01 in all cases). Moreover, when comparing the mean peak concentrations between temperatures, it was observed that higher peak levels were achieved at 15°C compared to 20°C ($F_{2,29}$ = 3.588; P < 0.041). The magnitude of cortisol response, as depicted by the Area Under the Curve with respect to increase from the baseline value (AUC_i), was highest in 15°C group, followed by fish reared at 20°C and then 25°C (Table 2).

The rate of cortisol release into the water was affected by the interaction of temperature and time post-stress ($F_{8,15}$ = 3.215; P = 0.025; Fig. 5). In details, the 25°C group showed the highest maximum values of release rate (3.1 ± 1.2 ng g^{-1} h^{-1}) compared to the other two groups (2.5 ± 2.1 ng g^{-1} h^{-1} and 1.4 ± 0.2 ng g^{-1} h^{-1} at 20°C and 15°C, respectively). The pattern of cortisol release into the water also differed, with fish reared at 25°C showing an intense and fast release, being highest at the interval 0 to 0.5 h post-stress (P < 0.01), and declining to almost non traceable release after 2 h. On the other hand, fish acclimated at 20°C showed the highest release of cortisol into the water at the intervals between 1 and 4 h, showing no release during 4 to 8 h. Finally, at 15°C no differences were observed in the release rate of cortisol (P = 0.28), there was, however, a constant release of cortisol even after 8 h post-stress.

Significant interactions between temperature and time post-stress were also present in glucose ($F_{10,154} = 4.042$; $P < 0.001$). At 15°C an initial slight increase at 0.5 h ($P = 0.01$), was followed by a plateau between 0.5 and 4 h, until a further increase at 8 h, when maximum mean values were achieved (12.0 ± 5.5 mmol l^{-1}; Fig. 2). On the other hand, at 20°C a slow increase occurred, becoming statistically significant at 1h ($P = 0.006$), and maintaining high values for up to 8 h ($P = 0.006$). At 25°C the time course changes were more intense than both other conditions (Fig. 2), showing a faster and higher in magnitude stress response, as indicated by the higher SBP and AUC_i values (Table 2). The highest values were reached at 2 and 4 h (14.1 ± 5.5 mmol l^{-1} and 14.1 ± 4.3 mmol l^{-1}, respectively; Fig. 2), leading finally to decreased values at 8 h, though still higher than 0 h ($P = 0.044$).

Plasma lactate concentrations also showed to be affected by the interactions of temperature and time post-stress ($F_{10,70} = 4.539$; $P < 0.001$). The only significant difference in the post-stress concentrations was seen at 8 h post-stress, where 15°C had higher levels of lactate than both at 20°C and 25°C ($P < 0.001$ in both cases; Fig. 3). The intensity, pattern and speed of the response were similar in all examined rearing temperatures (Fig. 3; Table 2).

Significant interactions were also present in osmolality ($F_{10,150} = 3.457$; $P < 0.001$; Fig. 4). In details, no significant differences between time post-stress existed at 15°C, whereas increased values at 0.5 and 1 h post-stress compared to 8 h were observed at 20°C ($P = 0.015$ and $P = 0.004$, respectively). The response at 25°C was intense and fast, with higher than the resting values at 0.5, 1 and 4 h post-stress ($P = 0.002$; $P = 0.018$ and $P = 0.007$ respectively; Fig. 4). Moreover, the response in total was higher at 25°C, as shown by the high AUC_i, and much faster than the other temperatures as shown by the SBP (Table 2). Finally, since at the 20°C the values from 2 h and onwards were lower than the resting values a negative AUC_i was observed (Table 2).

4. Discussion

The results of the present study demonstrate that the environmental water temperature can modify the resting circulating levels of commonly used stress indicators as well as the timing, magnitude, and duration of the stress response in E. sea bass. Previous studies have shown that temperature can affect the stress response in a species-specific way (Barton & Schreck 1987; Davis & Parker 1990; King et al. 2006; Costas et al. 2012), depending mostly on the biology, thermal tolerance and thermal preference of each species. Apart from temperature, however, other environmental factors such as photoperiod (Pavlidis et al. 1999; Biswas et al. 2006) and

season (Pascoli et al. 2011; Maricchiolo et al. 2011; Danzmann et al. 2016) are also significant in regulating the physiological status of the fish. Specifically, the concentrations of cortisol and glucose have been closely correlated with photoperiod in E. sea bass (Pascoli et al. 2011; Kavadias et al. 2003). In addition, diel fluctuations in physiological traits due to the circadian rhythm also exist in fish (Pickering & Pottinger 1983; Fatira et al. 2014). In E. sea bass, a peak in cortisol concentration has been observed at dusk (*i.e.* when the lights turn off) (Fatira et al. 2014). For these reasons, in the present study apart from the differences in water temperature, the other environmental conditions in the tanks, such as photoperiod and water quality were held constant, while the tanks were covered with a lid in order to avoid diel fluctuations.

In the present study, resting cortisol levels were affected by temperature, being lower in 15°C than the two other temperature regimes. A positive relationship between cortisol and water temperature and photoperiod has been suggested in this species (Pascoli et al. 2011; Planas et al. 1990), which is in accordance with the present results. This pattern has been observed in other fish species as well, like the Atlantic cod, *Gadus morhua*, and haddock, *Melanogrammus aeglefinus*, (King et al. 2006) the striped bass, *Morone saxatilis*, (Davis & Parker 1990) and the Adriatic sturgeon *Acipenser naccarri* (Cataldi et al. 1998). On the other hand, the absence of temperature effects on cortisol levels has also been reported in some species, like chinook salmon, (Barton & Schreck, 1987) and green sturgeon, *Acipenser medirostris*, (Lankford et al., 2003). Finally, a more complex pattern was observed in Senegalese sole, *Solea senegalensis*, where higher cortisol values were achieved in either higher or lower than the control temperatures (Costas et al., 2012).

Moreover, a response in cortisol was observed in all acclimation temperatures, however with differences in both the rapidity and magnitude of the response. Specifically, at 25°C and 20°C the time to peak was shorter, accompanied by a larger slope to peak than at 15°C, where a prolonged response until 4 h post-stress was observed. This could be the result of a higher metabolic rate in the high temperatures (Claireaux & Lagardère 1999; Claireaux et al. 2006), or be caused by a possible temperature-derived effect on cortisol dynamics through altering the rate of cortisol synthesis and clearance (Barton & Schreck 1987). In general, a faster cortisol response at higher temperatures has been observed in many fish species (King et al. 2006; Lankford et al. 2003; Barton & Schreck 1987; Sumpter et al. 1985; Davis & Parker 1990), being in line with the present study in E. sea bass.

The response at 15°C, although delayed, was of a higher magnitude as described by the high AUC_i. Davis and Parker (1990) have suggested that in striped bass both the magnitude of the response and the rapidity of return to pre-stress conditions reflect the imbalance that a stressor

causes to the organism. Based on that, they have suggested that stressing striped bass at temperatures lower or higher than the optimum augments the response, which might also be the case when stressing E. sea bass at low temperatures, such as 15°C.

Apart from plasma cortisol, temperature also affected the release rate of cortisol into the water. The differences in the response of plasma cortisol were in accordance with the pattern of release rate of cortisol into the water. Specifically, fish reared at 25°C showed the fastest and most intense cortisol release rate, followed by those at 20°C and finally those at 15°C, which showed a constant and stable low rate of cortisol release. Reduced release rate at lower temperature has also been observed in a freshwater fish species, the common carp (Jaxion-Harm & Ladich 2014). However, a direct comparison cannot be made since in that study fish were not acclimated to the desired temperature, but faced an acute temperature reduction of 6°C through a time interval of 3.5 hours.

In general, cortisol release in the water is suggested to be a mainly passive procedure, with cortisol coming off the body through the gills due to the concentration difference of the hormone between blood and water (Ellis et al. 2004; Ellis et al. 2005; Scott & Ellis 2007). One possible explanation for the decreased release of cortisol in low temperatures could be the reduced gill permeability (Ellis et al. 2004). In rainbow trout, *Oncorhynchus mykiss*, gill arches cultured in vitro showed a negative effect of temperature on the permeability of the gills for non-electrolyte substances (Isaia 1979). Another important factor is the branchial blood flow and the ventilatory water flow, since in both cases higher flow could result in increased release of cortisol (Ellis et al. 2004; Scott & Ellis 2007). In this respect, cardiac output seemed to increase with temperature in rainbow trout (Farrell et al. 1996), as did the ventilatory flow in common carp (Klyszejko et al. 2003). In E. sea bass, an increase in temperature within the thermal preferendum of the species (from 18°C to 22°C), has led to increased heart beat rate, yet this increase was compensated by a lower maximum stroke volume, leading to the same maximum cardiac output (Farrell et al. 2007).

In the present study, E. sea bass showed higher concentrations of plasma glucose when acclimated to high temperatures. As feeding was the same in all thermal regimes, these increased concentrations could not have been a result of increased feeding at the higher temperatures. A possible explanation could be that energy demands set on fish are increased at high temperatures, as suggested by the increase of metabolic rate with temperature in this species (Claireaux & Lagardère, 1999; Claireaux et al. 2006). The effects of temperature on glucose levels are controversial between different fish species since fish reared at high temperatures have shown either higher (Costas et al. 2012; Barton & Schreck 1987; Vargas-

Chacoff et al. 2009a; b) unaltered (Cataldi et al. 1998; King et al. 2006; Lankford et al. 2003; Umminger & Gist 1973) or lower glucose concentrations (Davis & Parker 1990) compared to rearing at lower temperatures. In the present study, the post-stress increase was faster and more intense at the highest temperature. At 15°C a second increase in glucose was observed at 8 h post-stress, possibly following the increase of cortisol that occurred at 4 h post-stress. Until that time point, apart from the highest speed, the response at the highest temperature also showed the greater magnitude, as described by the AUC, a pattern observed in almost all of the species studied so far (King et al. 2006; Davis & Parker 1990; Lankford et al. 2003; Barton & Schreck 1987) apart from the goldfish (Umminger & Gist 1973).

Although temperature had a significant effect on the basal levels of plasma lactate, the pattern was different than that of cortisol and glucose, being highest at 20°C, followed by 25°C and finally 15°C. This contradicts the pattern observed in the same species under culture in sea cages (Samaras et al. 2015), where other factors such as photoperiod, feeding activity, seasonality, and available space for swimming may have played a crucial role. Moreover, in other fish species (Costas et al. 2012; Lankford et al. 2003; Vargas-Chacoff et al. 2009a, b) higher temperatures led to increased plasma lactate. In addition, the response was unaffected by temperature, with the exception of 15°C at 8 h post-stress, where a significant further increase was observed, probably due to the enhanced cortisol response at 4 h. This, however, was not the case in other species, such as rainbow trout, where blood lactate accumulation showed a higher magnitude at the highest temperature, although the pattern of response in respect to time was unaffected (Kieffer et al. 1994). In that study, the production of lactic acid in the white muscle was independent of temperature, and was therefore suggested that the observed differences in blood lactate could be attributed to a higher diffusion rate of lactate from the muscle and/or the blood perfusion on the muscle at higher temperatures. Such a mechanism could also, alongside with the cortisol increase at 4 h post-stress, explain the delayed recovery observed in low temperatures in E. sea bass.

Plasma osmolality was also affected by temperature, showing increased values at the high temperatures, which is in accordance with previous studies in E. sea bass reared in sea cages (Samaras et al. 2015), and gilthead sea bream, *Sparus auratus* (Vargas-Chacoff et al. 2009b). There are also fish species that show different patterns, such as turbot, *Scophthalmus maximus*, where the opposite pattern was observed (Imsland et al. 2003), Adriatic sturgeon, where no effects of temperature on plasma osmolality were observed (Cataldi et al. 1998), and common carp, a freshwater species, where both high and low temperatures outside of the optimum range led to increased plasma osmolality (Metz et al. 2003). When E. sea bass was acclimated at low

temperatures no response of osmolality to stress was observed, although a previous study in sea cages revealed a response at 0.5 h post-stress (Samaras et al. 2015). A slight response was observed at 20°C, while at 25°C the response was intense and fast, denoting a significant effect of temperature on the osmolality stress response.

In conclusion, this study clearly showed that acclimation temperature significantly affects the basal levels, timing, magnitude and duration of peak values of stress indicators in E. sea bass. The release rate of cortisol into the water was also significantly affected by temperature, being slower and of lower intensity in the low temperature examined. Therefore, apart from possible temperature-derived differences in the synthesis and/or clearance of cortisol, the prolonged and of high magnitude cortisol response at low temperatures could be explained by the reduced cortisol release in the water at such temperatures. The above mentioned results may provide a basis to understand the significant differences observed in stress intolerance, disease susceptibility, and performance of E. sea bass individuals, especially when reared in temperatures close to the lower and upper limits, in which they may be exposed in open sea cage rearing conditions or in the wild. Finally, these differences should be taken into consideration when evaluating the stress status of fish either in laboratory or field conditions.

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Ethical Approval

The Animal House Facility at the Department of Biology, University of Crete, is certified by the Veterinary Unit of the Region of Crete for the rearing (EC91-BIObr-09) and use of laboratory animals for scientific purposes (EL91-BIOexp-10). All procedures have been approved by the Departmental Animal Care Committee following the Three Rs principle, in accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals.

Conflict of Interest

The authors declare that they have no conflict of interest

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Legends to Figures

Fig. 1 Cortisol concentrations after acute stress in fish acclimated at 15°C (-●-), 20°C (-■-) and 25°C (-▲·). Values are given as mean + SD (n=10). Within each temperature treatment different letters indicate statistically significant differences, while different symbols ($^{*, \#, +}$) indicate differences between temperatures at each sampling point

Fig. 2 Glucose response after acute stress in fish acclimated at 15°C (-●-), 20°C (-■-) and 25°C (-▲·). Values are given as mean + SD (n=10). Within each temperature treatment different letters indicate statistically significant differences, while different symbols ($^{*, \#, +}$) indicate differences between temperatures at each sampling point

Fig. 3 Lactate response after acute stress in fish acclimated at 15°C (-●-), 20°C (-■-) and 25°C (-▲·). Values are given as mean + SD (n=5). Within each temperature treatment different letters indicate statistically significant differences, while different symbols ($^{*, \#, +}$) indicate differences between temperatures at each sampling point

Fig. 4 Osmolality response after acute stress in fish acclimated at 15°C (-●-), 20°C (-■-) and 25°C (-▲·). Values are given as mean + SD (n=10). Within each temperature treatment different letters indicate statistically significant differences, while different symbols ($^{*, \#, +}$) indicate differences between temperatures at each sampling point

Fig. 5 Cortisol release rate into the water after acute stress in fish acclimated at different temperatures. The time points (T1-T5) represent the intervals between two successive samplings (T1: 0 – 0.5 h; T2: 0.5 – 1 h; T3: 1 – 2h; T4: 2 – 4 h; T5: 4 – 8 h). Within each temperature treatment different letters indicate statistically significant differences, while different symbols ($^{*, \#, +}$) indicate differences between temperatures in each sampling point

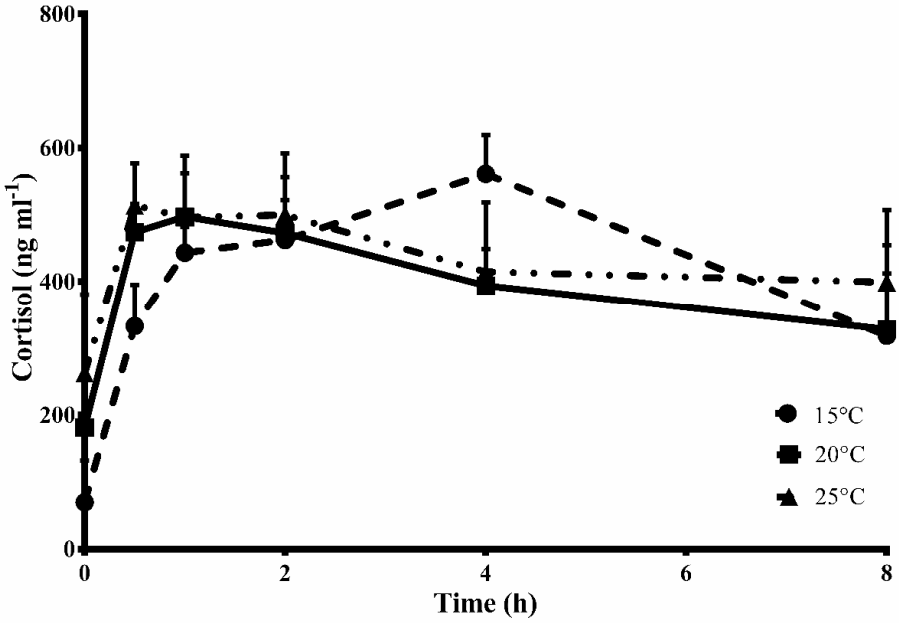
Table 1. Descriptive statistics of the resting levels of cortisol, glucose, osmolality and lactate of fish acclimated to different temperatures. Mean: average concentration of the group; SD: standard deviation; %CV: % coefficient of variance; N: number of animals. Different letters in the mean column indicate statistically significant differences between the groups ($P < 0.05$).

	Temperature	Mean	SD	%CV	N
Cortisol	*15°C*	69.92[a]	62.11	88.83	10
	20°C	189.08[b]	116.25	61.49	10
	25°C	262.37[b]	118.45	45.15	10
Glucose	*15°C*	3.86[a]	1.00	25.94	10
	20°C	6.51[b]	2.03	31.13	10
	25°C	6.25[b]	2.11	33.66	10
Lactate	*15°C*	3.79[ab]	0.82	21.69	5
	20°C	6.33[a]	2.89	45.66	5
	25°C	3.20[b]	0.48	14.89	5
Osmolality	*15°C*	339.70[a]	8.65	2.55	10
	20°C	376.63[b]	27.33	7.26	10
	25°C	402.80[b]	22.90	5.68	10

Table 2. Descriptive statistics of the stress response curve of cortisol, glucose, osmolality and lactate of fish acclimated to different temperatures. AUC_t: total Area Under the Curve; AUC_i: Area Under the Curve with respect to increase; SBP: slope from baseline to peak.

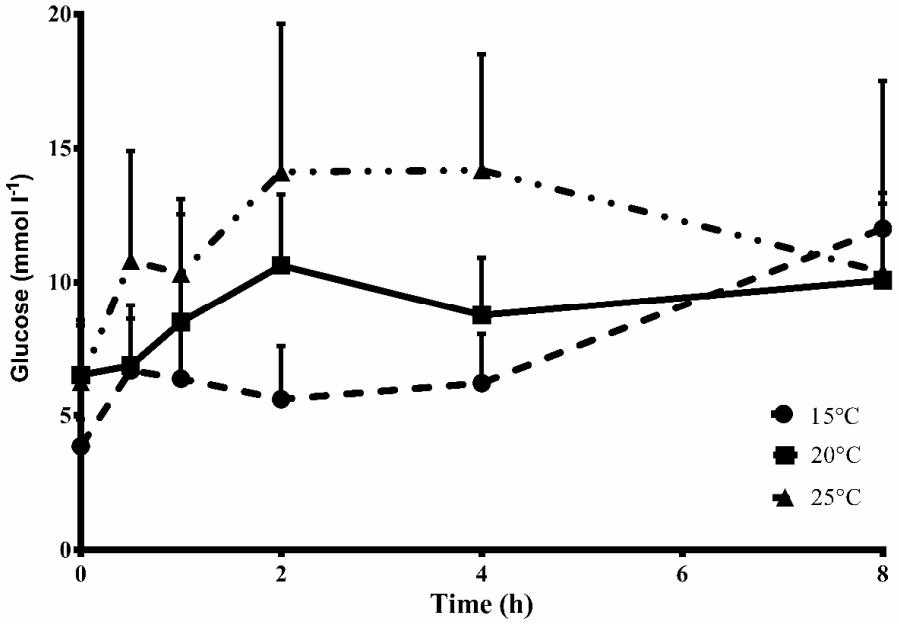
	Temperature	AUC_t	AUC_i	SBP
Cortisol	*15°C*	3531	2972	122.9
	20°C	3203	1751	315.9
	25°C	3488	1389	503.9
Glucose	*15°C*	60.13	29.25	1.0
	20°C	74.45	28.61	2.4
	25°C	99.18	49.18	3.9
Lactate	*15°C*	70.85	40.53	4.0
	20°C	73.13	22.52	3.9
	25°C	55.20	29.64	4.9
Osmolality	*15°C*	2877	159.4	-
	20°C	2833	-255.3	8.9
	25°C	3667	444.3	175.1

Figure 1.



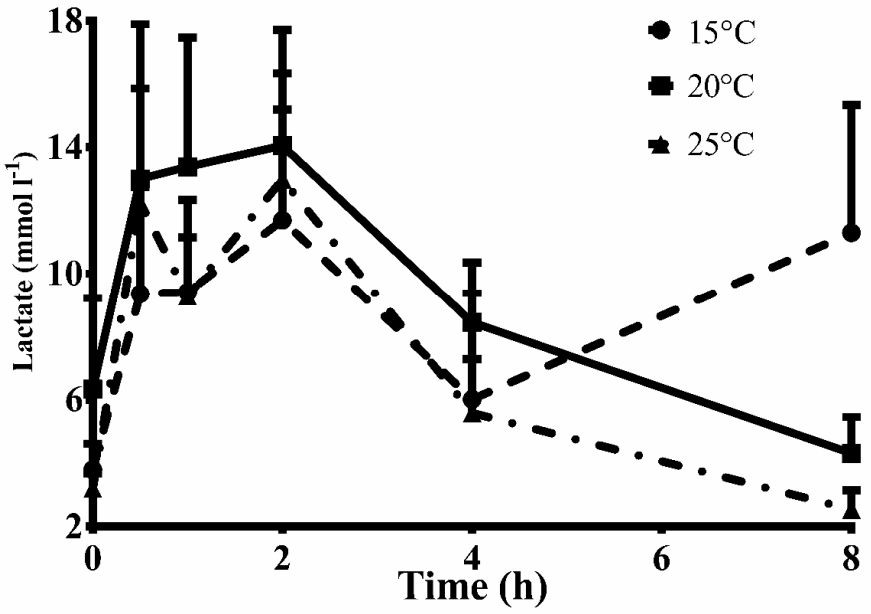
	0 h	0.5 h	1 h	2 h	4 h	8 h
15°C	a*	b*	bc	c	c*	b
20°C	a**	b**	b	b	bc**	c
25°C	a**	b**	b	b	b**	b

Figure 2.



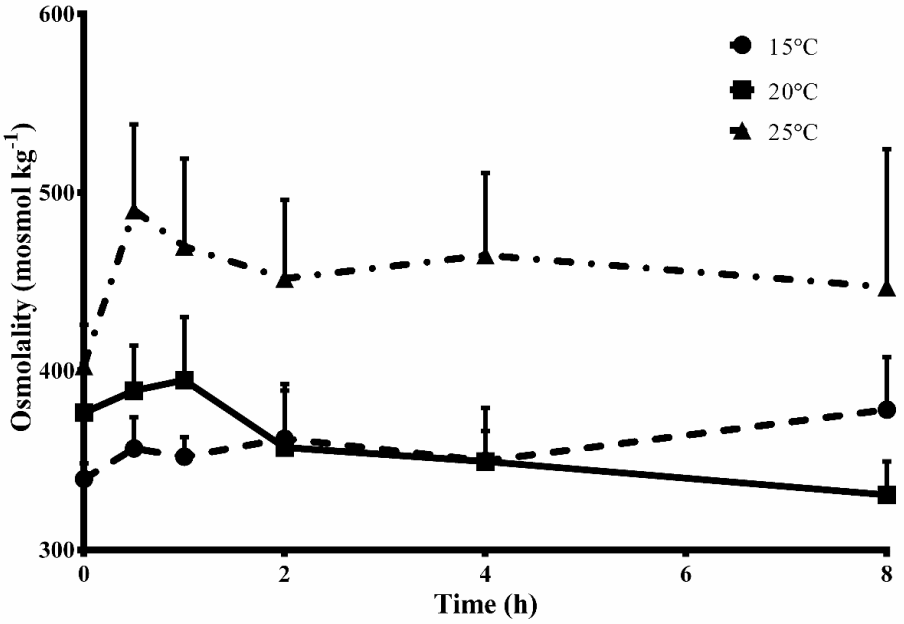
	0 h	0.5 h	1 h	2 h	4 h	8 h
15°C	a*	b*	b*	ab*	b*	c
20°C	a**	ab	b**	b**	b**	b
25°C	a**	b**	bc**	bc**	c***	bc

Figure 3.



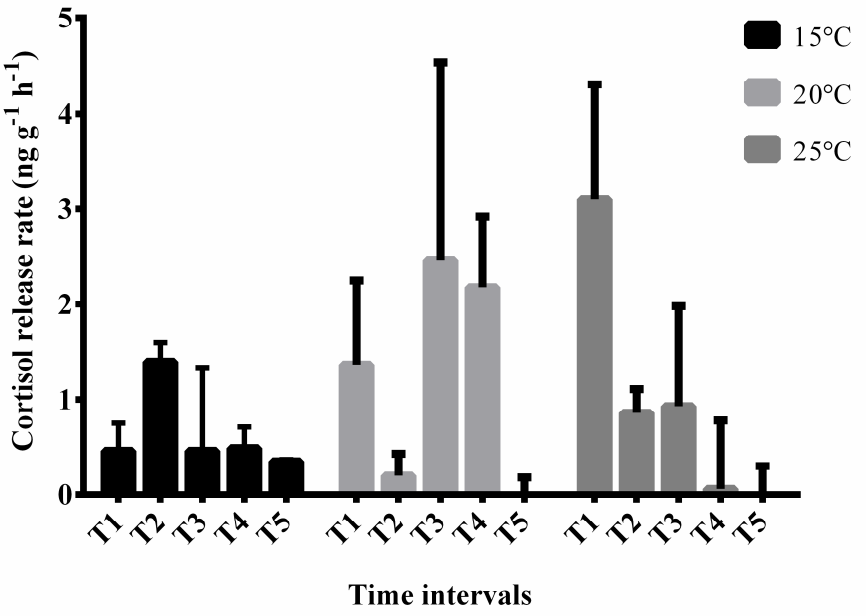
	0 h	0.5 h	1 h	2 h	4 h	8 h
15°C	a	bc	bc	c	ab	c*
20°C	a*	b	b	b	ab	a**
25°C	ac**	b	bc	b	c	a**

Figure 4.



	0 h	0.5 h	1 h	2 h	4 h	8 h
15°C	a*	a*	a*	a*	a*	a*
20°C	abc**	bc*	c**	abc*	ab*	a**
25°C	a**	b**	b***	ab**	b**	ab**

Figure 5.



	T1	T2	T3	T4	T5
15°C	a*	a	a	a	a
20°C	ab	ab	a*	ab	b
25°C	a**	ab	ab**	b	b