PROJECT FINAL REPORT

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4.1 Final publishable summary report

4.1.1. Executive summary

The COPEWELL project has run for 54 months involving 17 partners from 10 countries has achieved it's main objective: to construct a knowledge base for a new integrative framework for the study of fish welfare based on the concepts of allostasis, appraisal, and coping styles. The knowledge generated can already be found in 21 published papers so far, and we expect this to double over the next year.

In WP1 COPING STYLES, we have developed and validated methods to characterize stress coping styles in Gilthead seabream, European seabass and Atlantic salmon. Robust behavioural and physiological correlates have been identified and we have highlighted both common and species-specific screening methods for diverse traits. Further, the identification of molecular biomarkers for stress coping styles in each species and across all 3 species has been a landmark. Molecular analyses of risk-taking behaviour across species has led to the identification of a genetic framework for this behaviour conserved through evolutionary time. Finally, we have built neuroanatomical maps with histological and neurotransmitter markers. We have shown for the first time that fish with differing coping styles have clear differences in forebrain area activation. These results confirm our hypothesis that structural mechanisms and neural plasticity are essential to contrasting stress coping styles and underpin the analysis of cognitive functions.

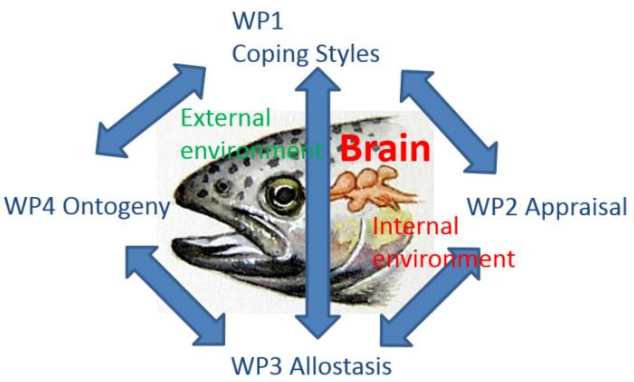
In WP2 APPRAISAL we have tested the occurrence of cognitive appraisal in teleost fish. Using the zebrafish as a model we have shown that what triggers a physiological and genomic response to environmental stimuli is the subjects' assessment of the stimulus, rather than the intrinsic characteristics of the stimulus per se. How individuals of the three target species evaluate both aversive and appetitive stimuli were characterized, and the role of predictability and controllability as modulators of appraisal was evaluated. As predicted, both predictability and controllability affected the way fish perceive the same stimulus. Our results show that appraisal, a cognitive ability classically considered as complex, is also present in a fish and that relies on particular neural circuits.

Within WP3 ALLOSTASIS, knowledge on fish stress physiology within the context of allostasis has been significantly advanced. Key components of the allostasis concept including the effect of predictability on the outcome of the stress response were evaluated showing how fish to reset and fine-tune their internal set points and equilibria. Furthermore for the first time (non-REM) sleep was demonstrated by EEG-analysis in seabass and seabream. Although fundamental in nature, the knowledge provided provides novel physiological insights to further develop hypotheses and concepts with respect to stress physiology and animal welfare as well as aquaculture industries to develop new protocols that can promote animal welfare.

In WP4 ONTOGENY the ontogeny of neuroendocrine and physiological processes mediating allostasis was characterised and the long-term effects of chronic stressors at early developmental stages on the functions of allostatic mediators, coping styles, and performance later in life the ontogeny evaluated. Seabass and seabream are able to respond to noxius stimuli as early as around first feeding, and HPI axis is fully mature around flexion. Stress exposure at early ontogeny affects the whole transcriptome, brain architecture and performance traits at subsequent stages of development. Moreover salmon parr with highly reduced growth rates caused by a chronic stress phase show growth compensation after stress, differences in brain monoaminergic levels, and a downregulated stress response. Our results interestingly indicate that stress exposure during early life may prepare individuals to perform better in aquaculture.

In summary the COPEWELL project has given us a deeper understanding of how individual fish cope with their environment and the importance of the brain and the individual's interpretation of stressors and sensory signals. The results indicate that we can improve both selection, stress tolerance and performance of farmed fish by implementing hardening or training methods at the hatchery stage. This knowledge, fundamental in nature, provides both the research community and aquaculture industry with an integrative framework that will be highly valuable as we move forward toward understanding fish welfare from a biological, operational and ethical perspective. The work accomplished throughout this project has significantly enhanced our understanding of fish welfare, put EU-funded animal welfare research at the forefront and will serve as a robust foundation to meet the challenges of developing a sustainable aquaculture industry that contributes to EU food security

4.1.2 Project context and objectives



Farmed fish have a high risk of mortality and poor welfare, showing that current farming methods do not fulfil the welfare needs all fish. There is still a lack of knowledge about the underpinning mechanisms behind the variation in coping ability between individual fish. Why do some fish die while others thrive under the same conditions? The overall aim of COPEWELL has been to establish, evaluate, and further develop, a new scientific framework for the understanding and application of the concept of animal welfare in farmed fish derived from the evolutionary based concepts of allostasis, allostatic load, and overload (Korte et al., 2007). Allostasis is defined as the adaptive process for actively maintaining stability through change (Sterling and Eyer, 1988), and involves central neurobiological mediators that change the controlled physiological variables by predicting what level will be needed to meet anticipated demand. COPEWELL have been searching for novel discoveries and knowledge about a range of underpinning mechanisms: Evolutionary explanations imply the presence of individual variation, thus the project has addressed the presence of individual stress coping styles in farmed fish, including its basis in brain structure and function, gene expression, behaviour, and their plasticity and consistency of specific trait constellations during changing conditions. The emotional brain plays a central role in allostasis by controlling all the mechanisms simultaneously, involving experience, memories, and re-evaluation of needs in anticipation of physiological requirements (Korte et al., 2007). In this framework the ability to experience ***welfare*** is an emergent quality in animals with advanced central nervous systems, and a part of the animals evolved motivational and survival mechanisms. By focussing on cognition and emotion as evolved properties COPEWELL initiated a novel hypothesis-driven scientific approach to animal welfare leading to new discoveries and knowledge about how teleost fish experience their world.

According to the homeostatic-based definition, which postulates a negative linear relationship between stress and welfare, stability and no threats to homeostasis means the best welfare. The **new concept of welfare based on allostasis** suggests an inverted U-shaped relationship, where both too little (hypostimulation) or too much stress (allostatic load) gives poor welfare (Korte et al., 2007). We have put forward that the allostasis concept holds particular value as a model to discriminate between normal adaptive stress responses and situations of potential consequence to animal welfare. In this framework, good animal welfare is characterized by a broad predictive physiological, cognitive, and behavioural capacity to anticipate and respond to environmental challenges in a way that matches the environmental demands. Reduction of such capacities will lead to a mismatch between 1) the response required by the actual conditions and events and 2) the actual responses mounted by the individual, limiting the animal's ability to experience rewards and good welfare. In COPEWELL we have studied how the mediators of allostasis and coping ability are affected by stress level, ranging from hypo- to hyper-stimulation, as well as the effects of cognitive anticipation/prediction of the stressor based on earlier experiences. In this way, we have tested if fish have memory-based mechanisms as a basis for plasticity and efficiency to deal with environmental challenges. Finally, we have studied the ontogeny of neuroendocrine and physiological processes mediating allostasis, and the long-term effects of chronic stressors at early developmental stages on the functions of allostatic mediators, coping styles, and performance later in life. The most important aquaculture species in Europe, Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) have been the main species used in the project. In addition zebrafish (*Danio rerio*) and genetically high and low stress responsive rainbow trout (*Oncorhyncus mykiss*) have been used in selected tasks where these models were especially and justifiably suited for purpose.

The project has had a hypothesis- driven multidisciplinary approach, where a range of hypotheses have be tested. The COPEWELL project has through four scientific work packages focused upon underpinning mechanisms in four essential welfare relevant concepts: COPING STYLES, APPRAISAL, ALLOSTASIS and ONTOGENY. Through the selection of aquaculture relevant stressors, and by conducting experiments at different scales, at different life-stages and in different rearing systems, this has provided new insights on the interrelations between different relevant husbandry practices, fish performance and welfare.

PROJECT OBJECTIVES

WP1 COPING STYLES

Overall objective: To provide methods and tools to analyse coping styles, with specific reference to the presence, variability and consistency of individually distinct trait correlations relevant to the welfare of European finfish. The overall objective was met by a series of experimental studies using Atlantic salmon, seabass, seabream, and genetically selected lines of rainbow trout, with the specific objectives:

- To establish non-invasive methods for reliable identification of contrasting stress coping styles in farmed fish
- To demonstrate and quantify the presence and consistency of individual variation in coping style in Atlantic salmon, Gilthead seabream, and European seabass using a combination of behavioural and physiological measures
- To provide a causal mechanism for the presence of welfare relevant trait correlations in farmed fish, by addressing the molecular genetic regulation of coping styles at the level of the transcriptome
- To establish a link between stress coping ability and brain function by investigating differences in telencephalic structure and function between fish displaying divergent coping styles, with emphasis on localised expression of genes affecting learning, memory, and neuronal plasticity

WP2 APPRAISAL

Overall objective: To demonstrate the concept of appraisal in fish and to investigate its role in individual variation in coping styles. Specific objectives beyond state of the art:

- To test the occurrence of cognitive appraisal in fish and to understand how fish experience appetitive and aversive stimuli by assessing the behavioural, neurophysiological and genomic profiles of fish exposed to stimuli with positive or negative valence.
- To provide tools and methods to measure appraisal in fish.
- To investigate how appraisal differs between species and individuals with different coping styles.
- To explore how predictability and controllability modulate appraisal and coping ability in fish.

WP3 ALLOSTASIS

Overall objective: To demonstrate allostasis (constancy through change) as a fundamental regulatory process to explain variation in the stress responsiveness of salmon, seabass and seabream.

Specific objectives beyond state of the art:

- To test how chronic exposure to different levels of allostatic load, ranging from hypo-stimulation to high stress load, activate the various components of the stress axis, and how this affects the ability to deal with an additional acute stress test.
- To test whether individuals assigned to different stress coping styles by non-invasive markers respond differently to set degrees of allostatic load in chronic and acute stress experiments.
- To test if different degrees of allostatic load affect appraisal in fish, including fear and anticipatory responses as well as learning rate.

WP4 ONTOGENY

- To study the ontogeny of neuronal function and neuroendocrine stress responses in Atlantic salmon, Gilthead seabream and European seabass,
- To investigate the impact of early-life stress experiences upon the ontogenesis and performance of operant conditioning, allostatic mechanisms and on fish coping ability later in life (production cycle).
- To identify whether and how the rearing environment and husbandry practices during early life stages, impact the plasticity of the brain function associated with coping and the performance of fish at later stages of development

4.1.3 Main S&T results/foregrounds

WP1 COPING STYLES

The first concept focus area was the existence and mechanisms behind individual variation in **coping styles** (Kolhaas et al., 1999), with the aim to provide methods and tools to analyse coping styles, with specific reference to the presence, variability and consistency of individual trait correlations relevant to fish welfare. By using an evolutionary approach, this work package aims to integrate and explore the **links between brain function, behaviour and adaptive physiology**, especially aiming to explore individual variability in how fish species like Atlantic salmon, European seabass and Gilthead seabream develop and maintain their ability to cope with stressors. Studies were designed to explore whether individual phenotypes were clustered in groups displaying different coping styles (i.e. proactive/reactive, "hawk/doves", bold/shy). Underlying mechanisms were addressed by localising key elements of the stress-responsive serotonergic and neural plasticity systems in the telencephalon. For the first time we have analysed rates of brain cell proliferation, neurogenesis, and the expression of genes controlling other aspects of brain function, such as learning and memory, in fish with different coping style. We have studied whether these systems were differentially activated due to coping style in salmon, seabass and seabream. Due to the differences in basal stress responses and coping styles, these comparative approaches have helped us to understand the interaction between coping style, stress and regional memory formation.

Consistency over time and across contexts variation in how individuals react to positive and negative situations is often referred to as coping style (proactive vs. reactive), or animal personalities. These coping styles are the result of how individuals perceive their environment and how they process this information in order to decide on an appropriate response to cope with changing environments. The relationships between behavioural and physiological traits are complex, and proactive individuals are generally regarded as low cortisol responders with a suite of individual behavioural traits related to activity, aggressiveness, exploration, risk taking and sociability. Coping styles have implications across diverse fields of biological research (from ecology to aquaculture), so the development of appropriate screening methods and the understanding of their genomic and neurologic correlates appears to be the most effective way to extend our knowledge and to incorporate behavioural responses into common practices for example selection-based breeding programmes, to improve the welfare of farmed fish (for review see Castanheira et al 2015).

WP1 COPING STYLES consisted of four tasks covering seabream, seabass and Atlantic salmon. Four main hypotheses were formulated at the start of the project related each to one of the four tasks listed hereafter:

- In task 1, BREAMBASSCOPE, our hypothesis was that seabream and seabass show a suite of correlated behavioural, physiological and endocrine traits that are consistent over time and across situation, representing different coping styles.

- In task 2, SALMONCOPE, our hypothesis was that (1) there exist strong relationships between coping styles and behavioural traits in Atlantic salmon, and these traits will be consistent over time, ranging from non-flexible tactics in proactive and reactive individuals, to more flexible state-dependent behavioural traits in intermediate fish. (2) Proactive, low cortisol responders will have high number and density of dark eumelanin spots. (3) Experiments using the HR/LR trout model indicate that fish with contrasting coping styles may be sorted according to the avoidance reaction to hypoxic conditions.

- In task 3, TRANSCOPE, our hypothesis was that natural variations in levels of specific mRNA transcripts are directly related to the coping style of the individual, and thus coping style is an essential resolving variable for the interpretation of gene expression data.

- In task 4, TELCOPE, our hypothesis was that the telencephalic structure and function related to coping style influence perception, learning and memory ability, thus ability to cope under stressful situations.

To test theses hypotheses we developed and finalized three major objectives that were:

i) to provide methods and tools to analyse coping styles in Gilthead seabream, European seabass and Atlantic salmon to reach behavioural and physiological categorisation while developing non-invasive methods;

ii) to identify relevant genes across species in order to use transcriptome analysis to probe the mechanism underlying individual variation in coping style and measure regulation of gene expression in alternate coping styles;

and iii) to build neuroanatomical maps with histological and neurotransmitter markers to provide the consortium with the telencephalic description and micro-dissection protocols for gene expression and neurotransmitters with the final aim of exploring telencephalic basis for coping through function, plasticity and memory.

A common approach consisting of behavioural and physiological tests, molecular and neuroanatomical protocols has been used for all species to achieve integration within the project and allow for comparative conclusions regarding the objectives. The tasks were linked to research activities in other WPs including the coping styles sorting methods developed.

TASK 1.1 BREAMBASSCOPE - Characterization of coping styles in seabream and seabass

This task had the objective to validate screening tests to identify fish with opposite coping styles in Gilthead seabream and European seabass, and to further characterize behaviour and physiological characteristics of each species.

Methods: The tests were designed to measure fish behaviour and physiological responses under determined circumstances. The five tests depicted in Figure 1, are: 1) Feeding recovery in a novel environment, 2) Reaction to a novel environment or novel object, 3) Escape attempts during a net restraining, 4) Escape capacity from hypoxia exposure (hypoxia sorting) and 5) Risk-taking in an unknown environment. The first 3 tests were performed using isolated fish, while the last two measured individual coping style within groups. Behavioural results from these tests have also been related with physiological traits such as a marker of stress (blood cortisol levels), sperm quality and oxygen consumption under confinement (the two last for seabream only) and heritability of coping style has been estimated in seabass.

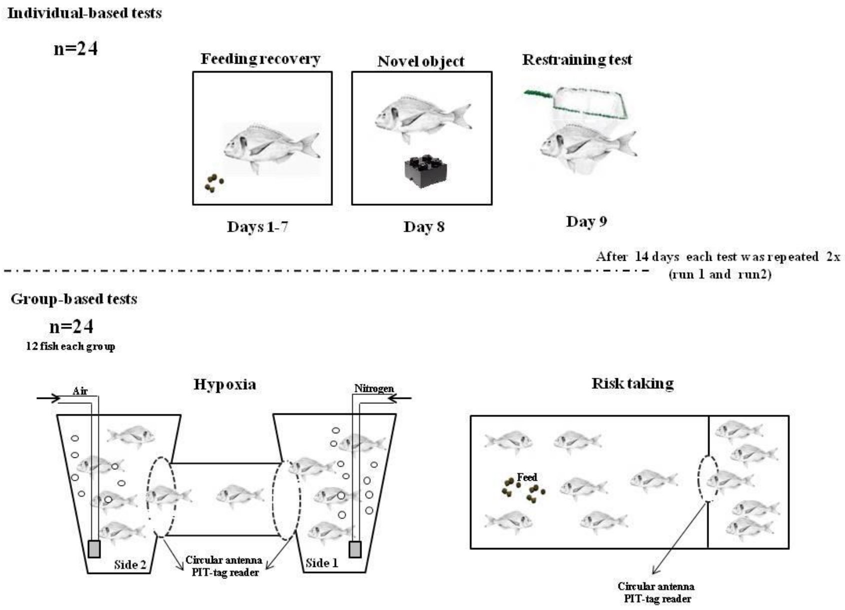


Fig 1.1. Overview of individual- and grouped-based tests used to screen coping styles in Gilthead seabream (*Sparus aurata*) and in European seabass (*Dicentrarchus labrax*).

Results and discussion in seabream, the results from these tests showed that individuals that escaped faster from hypoxia, tried to escape more in a restraining test, were risk-takers and took longer to recover feed intake while in isolation, they were classified as proactive (Castanheira et al 2013 a, b). The fish presenting the opposite behaviour characteristics were classified as reactive. Risk taking and escape behaviours during restraining were shown to be significantly consistent over time over a short-term period. However, after sexual maturity, it was no longer possible to find consistency in time in behavioural responses. Social context also seemed to influence fish personality traits since homogenous groups of proactive and reactive seabream did not exhibit consistent behavioural responses, contrary to intermediate and mixed groups. Finally, for physiological traits the cortisol response was not significantly correlated with behaviours measured during the restraining test, and was not consistent over time, and thus did not prove to be a solid physiological trait to inform for coping styles in the seabream. However, individual differences in cortisol responsiveness after the retraining test were predictive of aggressive behaviour (low cortisol was linked to high aggression, a proactive trait, Castanheira et al 2013b). As to sperm quality assessment, no significant differences were found in sperm motility related with the behavioural responses during the restraining test. Finally, a link between risk taking and oxygen consumption under confinement stress has been determined for this species (Herrera et al 2014): latency to take risks was negatively correlated to both movement and oxygen consumption rates, indicating that risk-avoiders (long latency, reactive) were less active and, hence, did not consume as much oxygen as risk-takers (proactive).

Results and discussion in seabass, we tested juveniles at least twice in individual-based tests (feeding recovery in novel environment and in isolation, aggressiveness, exploration in a T-maze and net restraining) and in group-based tests (risk-taking and hypoxia sorting), to assess coping style consistency in the short and long term and between tests. The results of individual-based tests were inconsistent over time and between tests in our set-up: the time between repeat tests, learning and species-specific behavioural responses appeared to have a major impact (Ferrari et al. 2015). By contrast, the results of group-based tests, such as risk-taking and hypoxia sorting, appeared to be consistent (both in the short and long term). These tests are the most relevant for the characterisation of coping style in European seabass. Furthermore, the results of these tests were also predictive of the cortisol stress response and were independent of the influence of social context. Fish identified as reactive in those experiments were also demonstrated to be those activating a self-feeder to distribute feed ration to the group (Ferrari et al 2014). Growth recovery post chronic stress exposure was better in intermediate fish than in proactive or reactive individuals. These tests are simple to perform and can be used to screen large numbers of fish, the first step in selection programmes including behavioural profiles. As a final step in the project, an experiment was conducted to understand the genetic basis of personality traits and reactions to a stressful or challenging situation in European seabass. To this aim, families from a full factorial mating were reared in common garden and individually tagged at an early age (95 days post hatching, dph) using microtags. Parentage assignment was performed using 12 microsatellite markers, resulting in 1308 uniquely assigned fish. The coping style of the animals was characterized through behavior-based tests at four different ages, categorizing fish into proactive or reactive: a hypoxia avoidance test and 3 risk-taking test sessions. We observed significant heritability of the coping style, higher for the average of risk-taking scores than for the hypoxia test. A mild genetic correlation between hypoxia avoidance and the average risk-taking score suggested that hypoxia and risk-taking tests do not address exactly the same behavioral and physiological responses. In addition, significant genetic correlations were observed between coping styles and phenotypic traits, particularly between hypoxia avoidance and thermal growth coefficients, showing that reactive fish have a higher growth than proactive fish under our experimental conditions. This study strongly suggests that the use of coping style characterization could represent an additional tool to improve the domestication process, selecting individuals better adapted to particular farming conditions, but also showing higher growth performances.

TASK 1.2 SALMONCOPE - Individual variation and consistency in coping styles, and the development of non-invasive screening methods for Atlantic salmon

Earlier studies in salmonids indicate the occurrence of individual differences separating a fish population in distinct sub-group and referred to as coping styles. Validated experimental data on large fish groups are however lacking, and the methods to sort fish are not validated in large-scale experiments. In this study the groups were separated in terms of behaviour (e.g. shy versus bold), or physiology (e.g. proactive versus reactive) and we focused on the development of coping styles in juvenile fish from hatching to the smolt stage, using approx. 500 unsorted fish in order to maintain as much variability as possible. The aim of the study was to correlate the behavioural and physiological responses of individual fish, including the morphology of dermal eumelanin spots, and use this as a basis for neurobiological and genetic samplings.

Methods: Atlantic salmon were reared at 10°C water temperature, continuous light and surplus feeding, and the fish population was randomly divided into eight groups of 60 fish. The fish were individually tagged and weighed at irregular intervals throughout the growth period. Each fish were photographed during the sampling, in order to quantify the dark eumelanin spots. The fish were tested 10 months after hatching. The test system to measure the escape capacity from hypoxia exposure consisted of two custom-made 200 L circular tanks (denoted as the hypoxia and normoxia tanks), connected at the water surface level with a tube integrated with a custom-made spool antenna. The inlet in the hypoxia tank was connected to a gas exchanger deoxygenating the incoming water. The end point for each experiment was set to 2.5 mg O_2 L^{-1}, corresponding to 25% saturation of oxygen. The swimming activity of the fish was monitored using three infrared sensors in each tank. The fish leaving hypoxia were classified as the *leavers*, while the ones staying in the hypoxia tank during the decline in oxygen concentration represent the *stayers*. After the hypoxia experiment, a confinement stress test was conducted with fish from five randomly chosen fish tanks, and the physiological stress response measured as changes in blood cortisol level. Plasma chloride concentration was measured in a separate seawater challenge test, in order to measure smolt status.

Result and discussion in Atlantic salmon: During the growth period the fish groups developed a bimodal growth pattern, with one group of lower modal (LM) parr and one group of upper modal (UM) smolts, confirmed by the seawater challenge test. Such variability in life history development is common in nature, and demonstrated that we succeeded in keeping the variability within our experimental group. Prior to the decline in oxygen in the hypoxia sorting tests the mean swimming depths were between 20 and 30 cm over the tank bottom. After the onset the hypoxia tank had a nearly linear reduction of about 0.05-0.10 mg O_2 $minute^{-1}$ (Figure 2), while the normoxia tank was not affected.

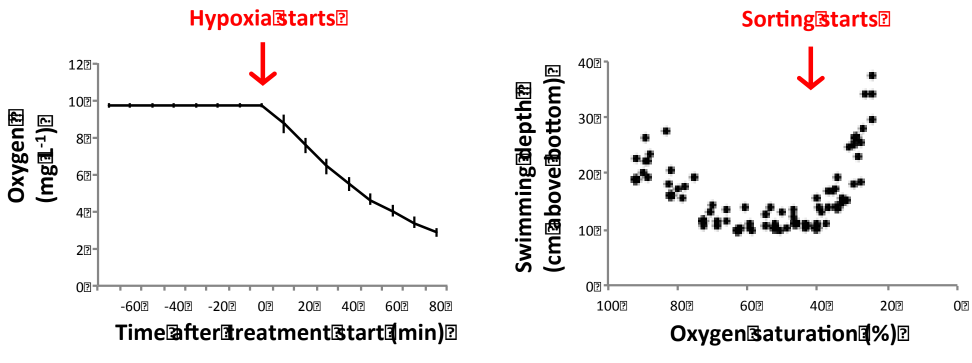


Figure 1.2. Left panel; Oxygen level 80 min before and after the onset of the oxygen decline. Right panel; Swimming depth as a function of oxygen saturation.

Within 20 minutes after the onset of the oxygen decline (100 - 70% oxygen), the fish became inactive close to the tank bottom. This behavioural response continued till about 50-60 minutes after the onset (<40% oxygen), whereas the fish became hyperactive in the tank and swam close to the water surface (Fig. 2). More than half of the fish were defined as *leavers*, and the *leavers* swam over to the normoxia tank on average after 69 minutes, with an oxygen level of 3.3 mg O_2 minute^{-1} (30.4% saturation). Most of the parr fish did not leave the hypoxia tank during the oxygen decline, while approximately half of the smolts were *leavers*.

The confinement stress tests significantly demonstrated the differences in stress responses between parr and smolts, and between the *stayers* and *leavers* (Figure 1.3). The smolts that left hypoxia had a significant lower cortisol response, compared to those that remained in hypoxia. The result showed that the *leavers* could be regarded as proactive fish, in contrast to earlier suggestions. The analysis of spots could not confirm that proactive, low cortisol responders have a higher number and density of dark eumelanin spots compared to reactive fish, probably due to poor visibility of spots during smoltification.

The basic behavioural responses to hypoxia are expected to be complex, as it is a trade-off between increasing oxygen demand and the need to avoid a suboptimal habitat. This may explain the individual differences in the response, and the experiments concluded that the hypoxia test would be a valid method for large-scale sorting of salmon according to coping styles.

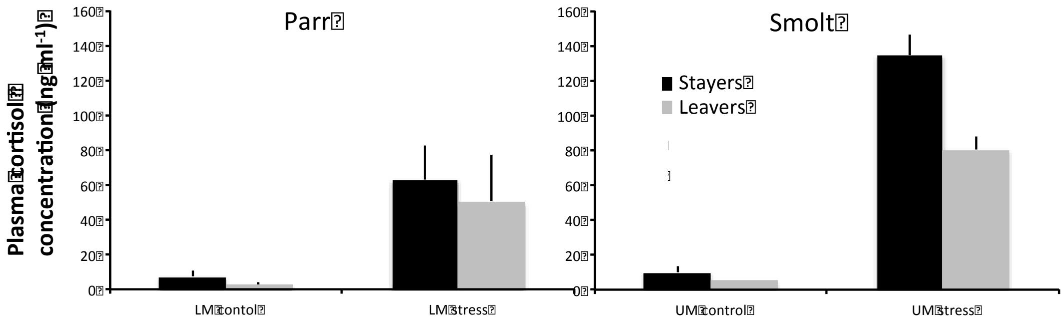


Fig 1.3 Cortisol concentration in stayers and leavers before and after a confinement stress. Left panel; LM parr salmon. Right panel; UM Smolts.

TASK 1.3 TRANSCOPE - Using transcriptome analysis to probe the mechanism underlying individual variation in coping style

The basis for these studies is that natural variation in gene expression within a population of fish is directly related to the stress coping style of the individual. Following this reasoning we predicted that the level of specific genes when expressed are representative of that stress coping style and therefore are biomarkers for these behavioural traits. In an initial study we identified a large set of potential genes whose products, mRNAs, were measured to have distinct differences in their abundance in the brains of Zebrafish (Rey et al. 2013). We carefully selected the best set of these mRNAs and tested their abundance in seabream, seabass and salmon brains.

Genetic signatures in the fish brain predict personality: In each of the species tested we were able to observe differences in the abundance of these stress coping style genetic signatures (Figure 1.4 as an example). These signatures were measured using the method of absolute quantitative polymerase chain reaction. In this method each individual mRNA is exactly measured.

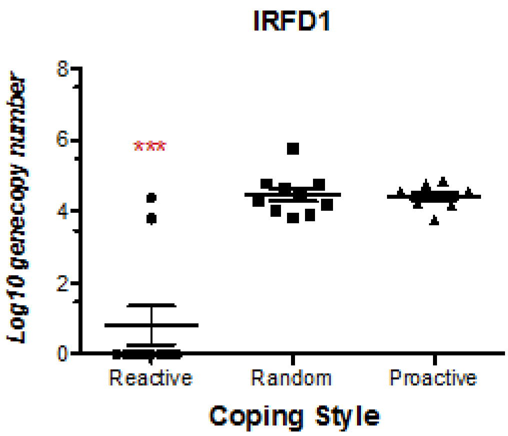


Figure 1.4. Expression of the IRFD1 gene in seabream tested for different stress coping styles. *** means significant difference **p<0.01**

From the mRNAs tested across all three species two target mRNAs (GAPDH and IRFD1) appear to be robust indicators of stress coping styles for seabream, seabass and salmon. We were able to identify 3 different cluster types for seabass and salmon and two for seabream that related to the reactive, proactive and intermediate stress coping styles. Thus stress coping styles have an underpinning genetic framework.

A genetic basis for risk-taking in fish: From the above-mentioned studies we then extended our research aiming to uncover the underpinning genetic information that relates to risk-taking behaviour in fish. These studies were carried out in both seabass and salmon. Here we applied the RNA-Seq technique in an attempt to completely characterize the brain transcriptome. Clustering highlighted the efficacy of this approach and proactive individuals were easily separated from the control population as evidenced by the hierarchical clustering of TMM-normalized FPKM values. We were able to identify a significant set of DEGs related to the proactive phenotype even when applying highly stringent cut-off values (>4 fold change, p<0.001). This allowed for the identification of a 'core' set of DEGs that are contributing to the observed effect in both species. Two genes with a highly similar behaviour were identified to be highly specific to proactive behaviour for both species and an extended cohort of transcript relating to both alternatively spliced isoforms and members of similar gene families were also identified. Interestingly the sea bass had more evidence of using alternatively spliced transcripts in contrast to the salmon.

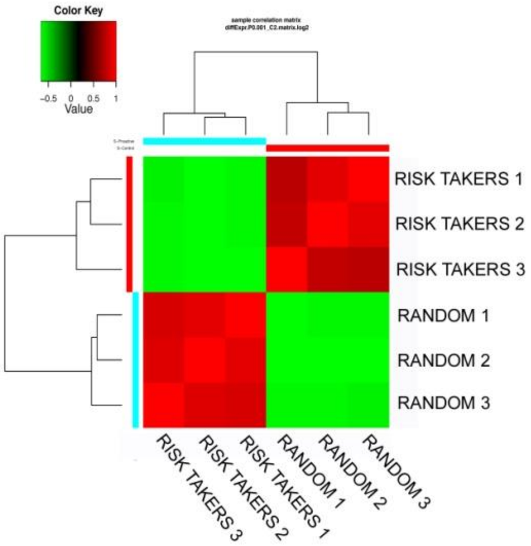


Fig 1.5. Global difference in gene expression measured by cluster analysis in the brains of risk-taking salmon versus a random set of individual salmon. Samples 1-3 represent pooled samples each containing n=6 brain

Further analyses looking into biological function highlighted a set of processes common to both species however often being transposed (activated versus inhibited) for the proactive phenotype. As highlighted by Figure 1.5 we were able to identify a underpinning constellation of genes that contribute to the risk-taking phenotype. Furthermore the abundance levels of measured mRNAs were conserved in both species in several cases. This suggests that risk-taking behaviour has a genetic signature and this has been conserved through evolutionary time.

TASK 1.4 TELCOPE - Telencephalic basis for coping through function, plasticity and memory

Several brain processes and mechanisms are responsible for regulating individual coping responses. In fish, which are increasingly used as alternative to mammals in behavioural and neurobiological research, there is now a growing body of evidence linking consistent individual behavioural differences with brain regulation. In this context, in order to understand how coping strategies may affect farmed fish, it is important to understand how brain responses in different species may be regulating behaviour.

The main objective for the current task was to characterize brain response patterns in individuals of the three target species. Individuals from all three species were in previous tasks (1.1 and 1.2) behaviourally identified and designated into either proactive (active coping response) or reactive (passive coping response) individuals.

In order to have a general understanding on brain activity patterns in the differing coping styles, we first analysed **a gene commonly used as a general marker for initiation of brain activity** (*cFos*). Our results showed that all species showed activity in the same areas of the forebrain (the telencephalon). However, we found that sea bass, which are more stress sensitive, showed higher activation in the brain than both seabream and salmon (See Figure 1.6).

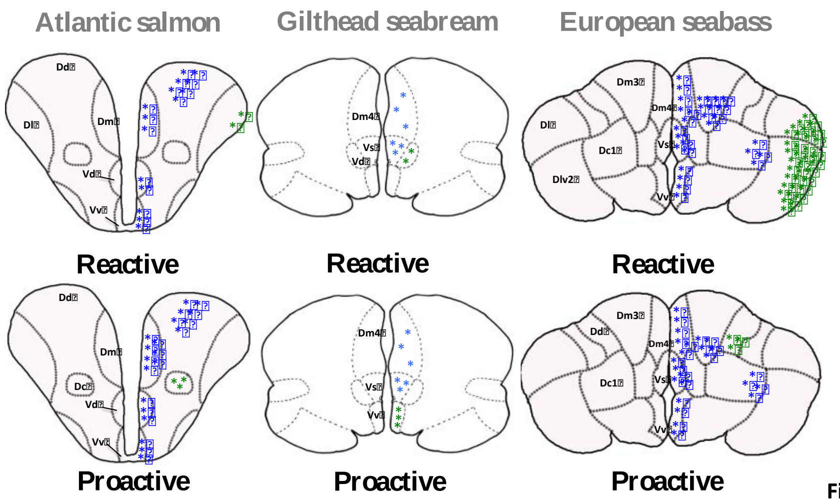


Figure 1.6. Schematic representation of an Atlantic salmon´s, Gilthead seabream´s and European sebass´s telencephalic transverse sections illustrating the mRNA expression of the immediate early gene cFos in reactive (A, B) and proactive (C, D) fish at acute stress conditions. Blue * indicate cFOS expression in both coping styles, while differences in cFOS expression between coping styles are indicated by green *. Central part of the dorsal area, Dc; dorsal part of the dorsal area, Dd; doroslateral pallium, Dl; dorsomedial pallium, Dm; ventrodorsal part of the dorsal area, Dlv; dorsal part of the ventral area, Vd; supracommisural nucleus of the ventral area, Vs; ventral part of the ventral area, Vv.

We then analysed **whole brain telencephalon samples** in all species to try to identify important molecules associated with the differing behavioural coping responses. In seabream, we found no differences in whole telencephalon molecular regulation between coping styles. In proactive sea bass there was an upregulation of molecular markers associated with active responses, which is in agreement with their active coping style. In salmon, our results suggest that proactive individuals had a general tendency to downregulate receptors that are associated with anxiety-like personalities. However, it is important to acknowledge that even though these results give us an idea of general tendencies for the telencephalic regulation of the studied molecular markers, it is hardly specific. That is, the telencephalon is made up of very distinct neuronal populations that may be regulated differently under the same environmental situations. Thus, **region-specific analysis of subareas within the telencephalon**, were required in order to fully understand the underlying brain mechanisms. We therefore also conducted analysis of molecular markers on specific microdissected telencephalic areas. In seabream, we only found one area in the telencephalon showing differences between coping styles in molecular activity markers. This area (the lateral septum-equivalent) is believed to be important in regulating goal-oriented behaviour (for example the goal of escaping a situation) and it was more active in proactive seabream. Sea bass showed no differences in the telencephalon between coping styles, although, both coping styles showed increased regulation of the same telencephalic areas during stress. This is in agreement with previous observations that sea bass is highly stress sensitive. Reactive salmon showed a higher capacity for flexibility in their ability to learn and respond during non-stressful situations in hippocampus-equivalent structures. By contrast, proactive salmon showed upregulation of molecular markers in the amygdala-like, hippocampus-like and lateral septum-like structures associated with an active coping style.

In conclusion, we show for the first time that fish with differing coping styles show clear differences in forebrain area activation. Our results provide new and important evidence of individual perception through complex forebrain regulatory systems. We hope that these results encourage more research into individual differences and their link to disease vulnerability. This, in turn, will help develop aquaculture husbandry practices that will not only improve welfare, but also product quality and effectivity in aquaculture operations.

WP2 APPRAISAL

The second concept focus area was on **how fish do appraise their world** and we claim that the introduction of the **concept of appraisal** in fish will be an important breakthrough in the area of fish welfare. The large individual variation in stress responses in fish does not only depend on the situation to which the individual is exposed, but also on the cognitive evaluation that the individual fish makes of the situation, i.e. on the way the stressor is appraised. Recent studies demonstrate similarity in cognitive complexity between fish and higher vertebrates, suggesting that fish also are capable of appraisal. However, very little is known about the appraisal criteria that fish use, and how appraisal can be measured and the underlying neurophysiological mechanisms. The main goals of WP2 were to demonstrate the occurrence of cognitive appraisal in fish and to understand how fish experience positive and negative stimuli. This has been achieved by assessment of the behavioural, neurophysiological and genomic profiles of fish exposed to stimuli with positive or negative valence, and by providing novel tools and methods to measure appraisal. We have also investigated how appraisal differs between species and individuals with different coping strategies, and explore how predictability and controllability modulate appraisal and coping ability in fish.

This WP consisted in three tasks:

i) Proof of principle of the concept of cognitive appraisal in fish using the model organism zebrafish (*Danio rerio*), for the methodological development of the behavioural assays, as well as physiological and genetic measures.
ii) Apply the optimized methodology to study the appraisal mechanisms in salmon, seabream and seabass.
iii) To study appraisal modulators in salmon, seabream and seabass

Task 2.1: APPRAISEMETHODS Proof of Concept 1: Appraisal mechanisms in fish

This task aimed to demonstrate the occurrence of cognitive appraisal in fish underlying the hypothesis that the way fish perceive a stimulus in their environment, does not depend exclusively on the intrinsic characteristics of the stimuli, but rather on the cognitive interpretation that the fish does of the stimuli. For this purpose different behavioural paradigms described in detail below were tested.

2.1.1- CONDITIONED PLACE PREFERENCE (CPP) TEST

Methods: The goal of this task was to establish a Conditioned Place Preference (CPP) paradigm in zebrafish to assess how individuals evaluate positive/negative stimuli as rewards/punishers. In this task the visual sight of a shoal of conspecifics was used as positive stimulus (social stimulus), and an empty tank as negative stimulus (asocial stimuli). The walls on each side of the tank had distinct visual cues (white side *versus* black circles) that worked as conditioned stimulus (CS) as depicted in figure 2.1. This experimental protocol was performed in two different ways:

1) The fish were individually isolated and returned to the isolation tank after each trial in the testing arena. This protocol was performed over a four-day period, with a total of five training sessions.
2) The test was performed in the home-tank to avoid repeated manipulations of the animal, and conducted over a one-day period, with four conditioning sessions.

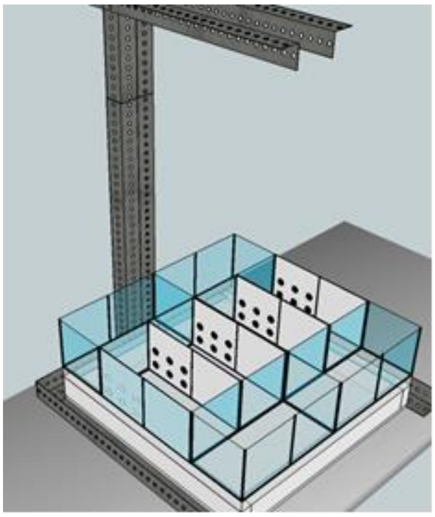


Fig. 2.1. Design model of the four-arena CPP setup

Results and Discussion: The first protocol led to significant losses of subject fish related to stress behaviour (around 40% of total sample size). By reducing the time of the experiment to one day and maintaining the subject fish in the experimental tanks for the entire duration of the experiment, the second protocol achieved a reduction of stress behaviour related losses. However, protocol 2 also failed to accomplish conditioned place preference and changes in place preference elicited by contextual cues (Fig 2.2). Therefore, although it is appealing in terms of stress reduction the home-tank protocol was not recommended for CPP testing.

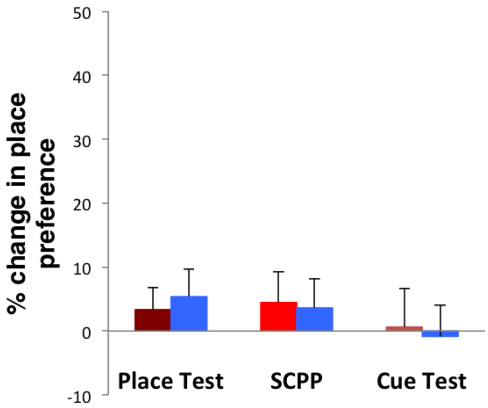


Fig. 2.2. Protocol 2 results. Change in place preference for the shoal side of social-treated subjects (red) compared to controls (blue). Place test (no visual cues); SCPP (circles versus white side); Cue test (circles in opposite side of tank). Mean percentage time +- SEM values are shown (n=13).

2.1.2- CONTRAST EFFECT TEST (CET)

In a second approach a behavioural contrast effect test (CET) was used to assess appraisal. The rationale of this type of test is to train individuals with different reward values, for instance if food is used one individual would be trained with 4 food pellets and another with 40 food pellets, and after a number of trials the reward value would be shifted. If the behavioural response solely relies upon the intrinsic characteristics of the stimulus animals should adjust behavioural responses to a level similar to those when the reward value was constant (no shift).

Methods: To implement this paradigm we took advantage of an existent and robust stress protocol available at a partner Institution (RUN). This protocol has already been validated for zebrafish (Fuzzen et al. 2010), and consisted in using a vortex as a stressor (i.e. forced swimming) for which the intensity and duration of exposure can be easily controlled and quantified. We used different pre-treatments with different stress levels (e.g. 200 rpm, 300 rpm and 400 rpm) and all animals were tested under the same regime (i.e. 300 rpm), so that although the objective stress level in the test phase would be the same for all treatments, fish in one treatment will perceive an increase in the stress level (200 rpm → 300 rpm = negative shift), in the other a decrease (400rpm → 300 rpm = positive shift) and in the third group the stress level will be perceived as similar (300rpm → 300 rpm = no shift control). To validate this protocol in our lab we measured holding water cortisol levels as a proxy of the HPI stress response in zebrafish, which allowed for multiple sampling of the same individuals in a non-invasive manner (Félix et al. 2013).

Results and Discussion: The dose-response curve for the cortisol response to the increasing rpm's did replicate the results reported by Fuzzen et al. (2010). This indicates that changes in rpm do not elicit monotonic changes in cortisol values, and in some cases cortisol even decreases with higher rpm values. Therefore, one cannot rely on cortisol measures as a reliable proxy of stress levels in fish exposed to the vortex stress protocol.

Faced with these results we proposed a contingency plan that instead of a direct measure of appraisal provides indirect evidence, and consists in having fish fighting their own image in a mirror. As fish do not recognize themselves in a mirror they attack their own image and express aggressive behaviour at similar levels to those observed in real opponent fights. However, in mirror fights there is a decoupling between the behaviour being expressed and the perception of victory/defeat. Thus, if a biological response depends on the perception of the fight outcome it should not be present in mirror fighters despite the behavioural output observed (aggressive behaviour). This is indirect evidence that cognitive appraisal is playing a role in the activation of that biological response.

2.1.3 MIRROR ELICITED AGGRESSION TEST

The rational behind this test is that if cognitive appraisal is present in zebrafish, the activation of a biological response to a social interaction must depend upon the perception that the individual has of the interaction outcome (winning vs. losing vs. ambiguous outcome = mirror), rather than on its structure (i.e.

behaviour expressed during the interaction). This way we hypothesized that it would be adaptive for an animal to switch its social status specific internal state only when faced with reliable information on relative competitive ability in comparison to other group members (i.e. Winning or Losing the interaction). Therefore, if cognitive appraisal is needed for the activation of a biological response to changes in the social environment then it is predicted that mirror fights (where individuals cannot appraise its outcome) should not elicit the biological responses (genomic/ endocrine) that are expected in real opponent fights.

Methods: Size matched males zebrafish were isolated overnight before being exposed to a short term (30 min) social interaction, which consisted either in a mirror elicited fight or in a real opponent fight. Aggressive behaviour was quantified and the identity of the Winner and the Loser of the real opponent fights were noted. A reference group remained in social isolation and did not experience any social interaction. Therefore, the experimental manipulations generated 4 phenotypes regarding social experience: mirror-fighters (M), winners of a real opponent fight (W), losers of a real opponent fight (L); and socially isolated fish (I, Control group) (Fig. 2.3).

1) Measures of the expression of immediate early genes (*c-fos* and *egr-1*) in regions of interest in the brain were used as markers of neural activation, allowing us to compare the patterns of brain activation elicited by fight outcomes that are appraised differently.

2) Measures of expression of late effector genes involved in different neural plasticity mechanisms [i.e. activity-dependent plasticity: brain-derived neurotrophic factor (bdnf) and neuronal PAS domain protein 4 (npas4); synaptic plasticity: neuroligin1 (nlg1) and neuroligin2 (nlg2); neurogenesis: wnt3 and neurod] were also used as a way to assess how differences in fight appraisal would differentially trigger these different neural plasticity mechanisms. At the endocrine level cortisol levels were measured as read-outs for appraisal driven changes in internal state. Therefore, in this behavioural paradigm both genomic and endocrine measures were used to read out the biological response to appraisal.

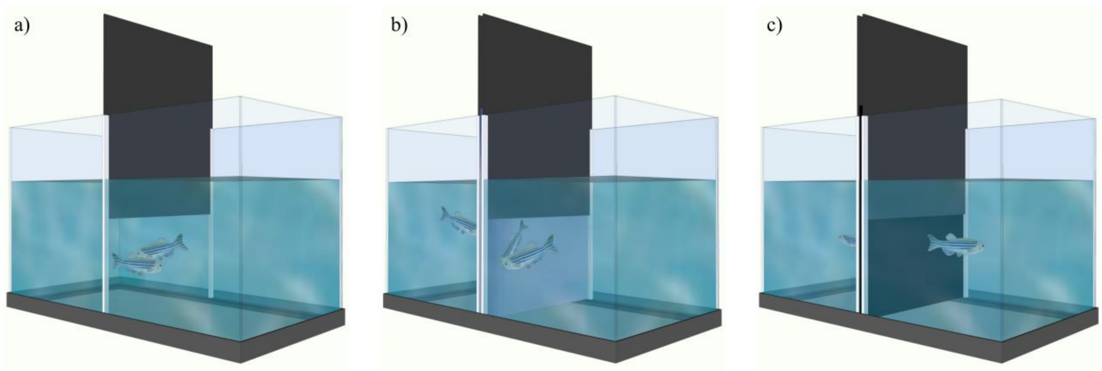


Fig. 2.3. Experimental apparatus: a) Real opponent interaction, fish fought with a conspecific, and a Winner and a Loser emerge; b) Mirror interaction, fish fought with their own image on the mirror but did not experience a change in social status; c) Control group, no agonistic interaction or mirror stimulation. This protocol was performed two times generating two separate data sets.

Results and Discussion: Our results indicate that different social experiences differentially activate gene expression in the nodes of the social decision making network (SDMN) for the IEG's as well as for the plasticity related genes. For all the experimentally induced phenotypes (i.e. Winners, Losers and Mirror-fighters) the pattern of neuronal activation in the SDMN was distinct as depicted in Fig 2.4 for *c-fos* levels.

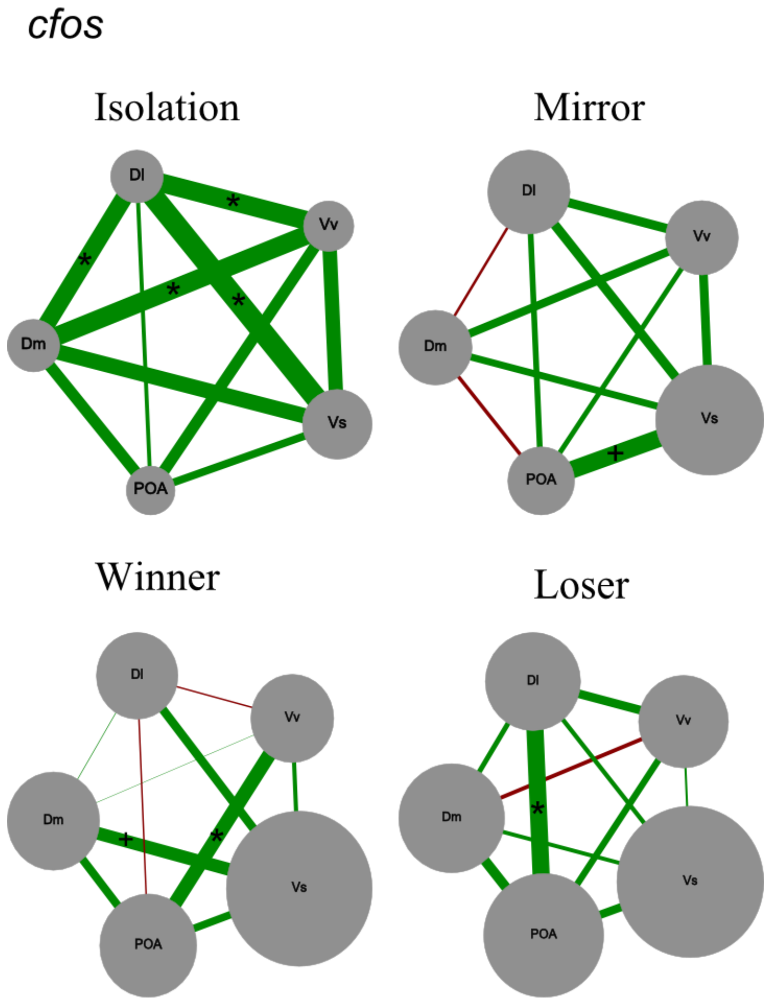


Fig. 2.4. Relative mRNA levels in the SDMN, in response to different social experiences. Circle diameter represents the activity level at each network node: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA). Lines linking pairs of nodes represent the functional connectivity between them as measured by Pearson correlation coefficients of IEG expression, such that: the thickness of the line is proportional to the R value and the colour scheme represents positive (green) or negative (red) correlations. Asterisks indicate significant correlations after p-value adjustment: plus (+) p < 0.1; * p < 0.05; ** p<0.01; *** p< 0.001(Teles *et al*, 2015).

For the plasticity related genes our results show that each social phenotype presented specific patterns of plasticity related gene expression across the SDMN, and that different neuroplasticity genes are differentially expressed in different nodes of the network. Winners expressed unique patterns of gene co-expression, whereas in Losers and Mirror-fighters the co-expression patterns were similar in the dorsal regions of the telencephalon and in the supracommissural nucleus of the ventral telencephalic area, but different in the remaining regions of the ventral telencephalon. These results indicate that appraisal mechanisms rely on multiple neuroplasticity mechanisms across the SDMN, and that there is not a single neuromolecular module underlying this type of behavioural flexibility. Finally individual differences of cortisol levels were also assessed 2h after the social interactions. Our results show that Winners and Mirror-fighters present elevated cortisol levels and Losers decreased cortisol to basal levels. These data are somehow puzzling, and can be a consequence of our behavioural paradigm. At the end of the interaction the respective partitions were placed back (i.e. in real opponent fights the central partition, preventing the interaction between the 2 conspecifics; and in the mirror elicited fights the partitions covering the mirrors), and although animals could not physically interact, odour cues were still available to each pair. Thus, two alternative explanations, which are not mutually exclusive, can be proposed. First, for the Winner, the chemical cues circulating in the tank signal the presence of the Loser, but it is no longer accessible, hence maintaining the anxiety levels; for the Loser, the Winner is still present but it can no longer attack, leading to a decrease in the anxiety state; for Mirror-fighters, the interaction was not solved and anxiety states may reflect frustration. Secondly, it can be related to the degree of activity (swimming) and glucose metabolism that is higher in Winners and Mirror-fighters.

In summary our results indicate that animals need to integrate information at different levels (i.e. not only express aggressive behaviour but also perceive submissive behaviour) in order to respond properly to changes in a social context. One can conclude that cognitive appraisal of fight outcome is necessary for the integration of social inputs and to trigger the transition between different neuronal states.

Task 2.2. APPRAISEFISH – Appraisal mechanisms in salmon, seabream and seabass

The goal of this study was to validate a conditioned place preference/avoidance (CPP/CPA) tests as a method to assess the affective valence of environmental stimuli in gilthead seabream.

Task 2.2.1. Development of a conditioned place preference test for seabream (Sparus aurata)

Animal welfare has been defined as the balance between positive and negative experiences or affective states. Despite the growing evidence of complex cognitive abilities and the expression of affective states such as pain and fear, very little is known about ability to experience memory based affective states in non-mammalian animal models. The goal of this task was to validate conditioned place preference/avoidance (CPP/CPA) tests as a method to assess the affective valence of environmental stimuli in fish. Physiological and behavioural indicators of affective states were used to characterise the response to apriori appetitive and aversive stimuli in CPP/CPA tests in gilthead seabream (Sparus aurata).

Methods: Fish were tested individually in a CPP/CPA tank divided into two halves, with one half uniformly white and one half marked by dotted wall patterns. During an initial habituation phase fish were placed in a central alley for 10 min and afterwards allowed to swim freely throughout the whole tank during 20 min in order to determine their initial preferred and non-preferred side (IPS/INPS). During the training phase, fish were presented either with a single aversive stimulus in the IPS (chasing with a dip net) or with a repeated appetitive stimulus (release of pellets) in the INPS. The test phase consisted of the same procedure as the habituation phase. The behaviour of each individual was video-recorded and analysed with video-tracking software. After this last phase, fish were immediately caught and euthanized with an overdose of 2-phenoxyethanol (1 ‰, Sigma-Aldrich). Blood was withdrawn for cortisol, glucose and lactate analysis and brains sampled dissected for monoamines and respective metabolites determination.

Results and Discussion: Fish submitted to appetitive stimulus significantly increased the time spent and the distance moved in the stimulation side, while fish exposed to aversive stimulus decreased significantly the time spent in the stimulation side, increased the distance moved in the non-stimulation side and showed an increase in cortisol level. Therefore, the use of behavioural (individual swimming activity) and physiological (plasma cortisol concentration) indicators of affective state during the CPP/CPA test allowed us to validate the use of this test as a method to assess the affective valence attributed by fish to different environmental stimuli. For monoamine determination, it was not possible to detect any differences between controls and conditioned fish in NE release and turnover after the test phase, suggesting a lower level of stress/arousal in conditioned Seabream in the present study. Still, in fish where the CPP tests indicated a positive valence of the stimulus the 5-HT levels were lower in DL compared to controls. Finally, this study also shows that fish are able to retain memories of events with positive/negative valence that are retrieved by environmental cues.

Task 2.2.2 Development of a conditioned place preference test for seabass (Dicentrarchus labrax)

Individual variation in the response to environmental challenges partly depends on innate reaction norms and partly on experience-based cognitive/emotional evaluations that individuals make of the situation. In this task, we aimed to ascertain if European seabass with different coping styles could subjectively interpret stimulus with different valences, using once more a conditioned place preference/avoidance (CPP/CPA) paradigm. Expression of the immediate early genes c-fos, egr-1, Bdnf and Npas4 were used as proxies of neuronal activation in specific brain areas with important roles in the mesolimbic reward system, stress response and appraisal.

Methods: ninety juvenile individuals were initially screened for behavioural reactivity using a net restraining test. Thereafter each individual was tested in a choice tank using net chasing as aversive stimulus or exposure to familiar conspecifics as appetitive stimulus in the preferred or non-preferred side respectively (called hereafter stimulation side). Locomotor behaviour (i.e. time spent, distance travelled and swimming speed in each tank side) of each individual was recorded and analysed with video software.

Results and discussion: The results showed that fish that were previously exposed to appetitive stimulus increased significantly the time spent on the stimulation side, while aversive stimulus led to a strong decrease in time spent on the stimulation side. Moreover, this study showed clearly that proactive fish were characterised by a stronger preference for the social stimulus and when placed in a putative aversive environment showed a lower physiological stress responses than reactive fish. In terms of monoamines, the changes in NE signalling observed in DL and in DM might be related to increased arousal, which is independent of the valence of the stimuli. IEG's expression analysis demonstrates us that fish under either aversive or appetitive stimulus have differential gene expression levels when compared with control and non-stressed individuals, namely a decrease of Bdnf in Dm. It was also evident with our results that the stress caused by the different treatments, when compared to control fish, promotes a decrease of neural activity in fish brain. In conclusion, this study showed for the first time in seabass, that the CPP/CPA paradigm can be used to assess the valence (positive vs. negative) that fish attribute to different stimuli and that individual behavioural traits is predictive of how stimuli are perceived and thus of the magnitude of preference or avoidance behaviour.

Task 2.2.3 Conditioned place avoidance test in Atlantic salmon (Salmo salar)

In the present experiment we employed conditioned place avoidance (CPA) tests on Atlantic salmon parr and studied possible effects of individual differences in coping style.

Methods: Conditioned place avoidance test (CPA) in salmon parr was tested with two levels of negative salience. A setup was developed that allowed exposing patterns during sessions and hiding patterns outside sessions in the home tank of each fish. Each fish (previously screened for coping style) was presented two patterns (stripes and spots), one pattern on each of two opposite walls, and allowed to swim freely for 20 min to assess baseline pattern preference. In three subsequent sessions of 20 min fish were presented the same pattern on both walls (stimulation pattern) while they were being stressed every 3rd min. Controls were presented the "stimulation pattern" after the 20-min stress period. The following day they were again presented both patterns for 20 min to assess if pattern preference had changed after association with the respective stimulation pattern, and thereafter the stimulation pattern on both walls for another 40 min before sampling of plasma (for cortisol analysis) and brain (for characterization of gene expression in the midbrain).

Results and discussion: No evidence for CPA was found. There was no effect of procedure (CPA or control), salience or any procedure × salience effect on the change in distance to the stimulation pattern or on change in swimming activity. There was an effect of salience on cortisol levels at the time of sampling, while no effect of procedure or interaction was found, suggesting that higher cortisol levels at sampling resulted from higher stress during training the previous day, irrespective of CPA or control procedure. That fish remained stressed on the test day was supported by elevated swimming activity in all groups in the test compared to base line.

Even though no behavioural differences between control and CPA fish were evident, there were clear molecular differences between groups, suggesting an expectancy preparation towards the impending aversive stimuli. That is, we found that the glucocorticoid receptor 2 (GR2) was down-regulated in the telencephalon and up-regulated in the hypothalamus of CPA fish, compared to controls. In addition, the serotonin receptor 1Aα (5-HT1Aα) was also down-regulated in CPA groups in the hypothalamus, showing a similar tendency in the telencephalon. Interestingly, we found that there was a dramatic up-regulation of the serotonin receptor 1Aβ (5-HT1Aβ) in groups subjected to high intensity aversive stimuli. These results suggested that several components of the hypothalamic-pituitary interrenal (HPI) axis are being regulated in a context-specific manner.

TASK 2.3.1. APPRAISEMODUL - Explore how predictability modulate appraisal and coping ability in fish

Task 2.3.1.1 and 2.3.1.2 **-** The goal of this research was to investigate how Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) use predictability as an appraisal modulator and whether different coping styles differ in behaviour and neuro-endocrine responses to predictable, unpredictable and loss of predictable conditions.

Methods: The paradigms used were based on conditioning learning: groups of fish were trained to learn an association between a visual cue (CS) and either a predictable or unpredictable appetitive (US+) or aversive (US-) stimulus in glass tanks of 80 L. After such association being established, the visual cue acquire a positive or a negative value eliciting afterwards approaching behaviour or avoidance behaviour as freezing or withdrawal behaviour, respectively. Seabream were tested in homogeneous groups of coping styles under both stimuli valence while either a heterogeneous group or fish in isolation was used in seabass and tested only under aversive stimulus. Behaviours were video recorded and measured during CS signalling throughout the conditioning sessions. After the last session, circulating cortisol and immediate early gene (IEGs) expression were determined to assess how the proximate states underlying predictability affects the stress response.

Results and discussion in seabream: Different behavioural patterns between the experimental groups throughout the conditioning sessions were found. Coping styles do not affect the way either valence or predictability of the stimuli is perceived. Both stimulus valence and predictability affected the observed behavioural patterns. Fish under appetitive stimulus had shown a lower number of escape attempts throughout the conditioning sessions as compared to fish under aversive conditions. The opposite pattern was seen for individuals under aversive stimulus, where they spent the time trying to escape and substantially neglected the interactions between counterparts. During the feeding event fish interactions tended to be significantly more frequent under predictable conditions rather than in unpredictable conditions. The same pattern was found for fish escape events under aversive conditions. Whether under aversive or appetitive stimulus, the experimental groups under unpredictable conditions paid more attention to the visual sign, showing higher freezing time. Plasma cortisol concentration was affected by both valence and predictability of the stimuli. Fish under unpredictable conditions expressed higher cortisol levels whether under appetitive or under aversive conditions. Fish under aversive conditions expressed higher cortisol levels. Different behavioural and physiological (plasma cortisol concentration) results evidence that fish are able to retain memories of environmental events retrieved by environmental cues, which could have profound positive ecological repercussions. IEGs expression assessed from specific telencephalic areas, that are related with learning, memory and emotional processing, were differentially activated under the different experimental conditions. In conclusion, this study highlighted the ability of fish to appraise stimuli with different valences as well to interpret different stimuli presented either in a predictable or unpredictable way. Different neuronal expression patterns underlying the different experimental conditions suggest that appraisal of stimulus valence and predictability induces emotion-like states in fish and that the proximate states underlying such expression appear to be conserved across divergent species during evolution.

Results and discussion in seabass: Coping styles did not affect how predictability or social context was interpreted. Throughout conditioning, distinct patterns of time freezing, escape attempts and exploratory behaviour were found between experimental conditions. Sociality was identified to contribute most for the observed differences. Non-social conditions induced higher times spent freezing as compared to homologous conditions for social groups. Fish expecting to receive the stimulus showed lower escape time freezing than fish under unpredictable conditions. Similar patterns were found for escape attempts under social conditions while under non-social conditions the number of escape events was lower under unpredictable conditions. Fish tested under predictable conditions explored more their environment regardless of the social context. Under social conditions, fish tested under unpredictable conditions showed higher shoal cohesion throughout the conditioning sessions, whereas fish expecting the incoming stimulus were spread evenly throughout the experimental tank.

Plasma cortisol concentrations were significantly different between predictability and unpredictability for each of the social conditions. Fish tested under unpredictable conditions showed higher cortisol levels for both social conditions, when compared to fish tested under predictable conditions. Interestingly, no differences were found between fish tested under predictable conditions for both social conditions, while unpredictable social groups showed higher levels than unpredictable non-social individuals. IEGs mRNA expression were measured from telencephalic regions known to modulate emotional processing, learning and memory (i.e. Dm, Dld and Dlv).

Our results demonstrated that unpredictability promoted an up-regulation of neural activity in the different brain regions studied, regardless the social condition. The effect of predictability likley modulated by proximate neuronal states was clearer for fish tested under non-social conditions. IEGs expression in the Dlv suggests that this telencephalic region modulates the effect of predictability in this fish species. Our study put forward that the collective response to stimulus is governed by physiological constraints and masked the cognitive bias regarding the effect of predictability. Lastly, we have shown the presence of affective states by the existence of divergent social interactions between experimental conditions and how they can be implicated in the group behavioural paradigm.

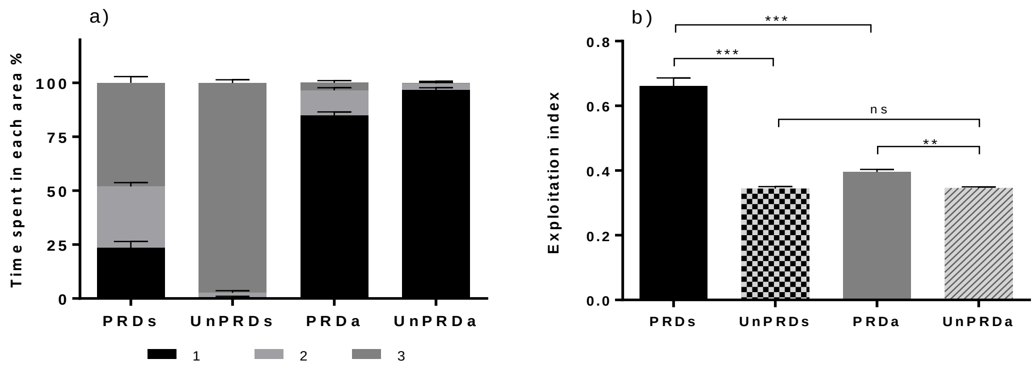


Fig.2.5 a) Raw data with the time fish spent in each area of the tank (1 – net confinement area; 2 – centre of the tank; 3 – visual cue area) (Mean ± SEM) during CS of the last conditioning session for each of the experimental conditions tested (PRDs – predictable social; UnPRDs – unpredictable social; PRDa – predictable nonsocial; UnPRDa – unpredictable nonsocial). **b)** Fish exploitation index (Mean ± SEM) measured from the ratio between the time fish spent in each one of the areas of the tank and the maximum time found for any of the areas tested. One-way ANOVA with planned comparisons significant levels are indicated (PRDs vs. UnPRDs; PRDs vs. PRDa; UnPRDs vs. UnPRDa; PRDa vs. UnPRDa): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns – nonsignificant.

Task 2.3.1.3 – This research was carried out to investigate how predictability affects the ability of Atlantic salmon to cope with an environment containing positive and negative challenges. All fish in the experiment received the same number of positive (feeding restricted in space and time) and negative (shelter being lifted above the water surface) challenges, but with different levels of predictability. The role of predictability was evaluated by assessing both behavioural outputs, as well as plasma cortisol and gene expression levels for target genes in the hypothalamus.

Methods: Individuals pre-screened for coping style by a net restraint test, were allowed to acclimate for 7 days before conditioning learning. During 14 days (learning phase) each fish was subjected to either a paired or unpaired CS-US paradigm (i.e. predictable or unpredictable regime, respectively, Fig 2.6). A diode light used as a CS negative (CS_{neg}) and a hose connected to an air pump used CS positive (CS_{pos}). In the predictable treatment the lifting of the shelter was announced by light flashes for 10 s (CS_{neg}) while feeding was announced with 10 s of air bubbles (CS_{pos}). In the unpredictable treatment light flashes and bubbles were given 30 min after the US (shelter lift and feeding) so that there was no CS/US pairing. After the learning phase, half of the fish experienced a loss of predictability (illustrated as unsafe). This was achieved by randomly switching the CS_{neg} (light flashes) and CS_{pos} (air bubbles) in 50% of the trials for 7-days, so that each CS did not always predict their respective US. Video recordings were conducted throughout all trials in the learning and unsafe phases (a total of 21 days) for later analysis. Blood and brains were collected in the end of the experiment to assess how the internal states processing the effect of predictability of stimuli with different valences.

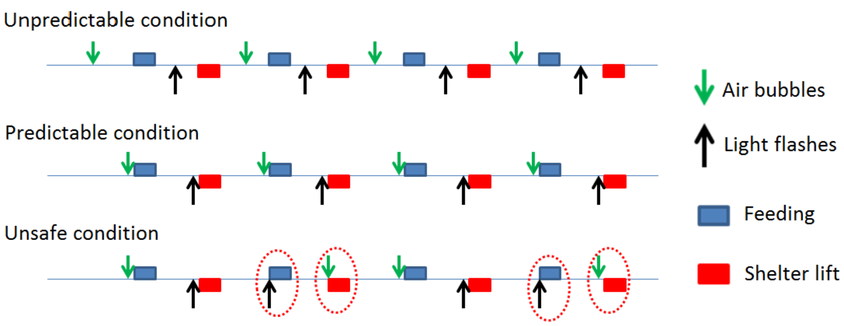


Fig. 2.6 Illustration of the CS-US relationships tested in the three experimental treatments.

Results and discussion in salmon: Behavioural results to CSneg showed that there was a tendency for fish under unpredictable conditions to use the shelter less than fish in the predictable and unsafe treatments. Generally fish infrequently consumed food (in total 17% of the feeding trials), with a higher tendency displayed by fish subjected to the unpredictable treatment. Notably, except for single case, fish occupying the shelter at the onset of the feeding trial did not leave (one fish in the unsafe treatment left once), and the probability of a fish to consume food was negatively correlated to the probability that the fish used the shelter. Hence, explaining why fish in the unpredictable treatment, that used the shelter less frequently, consumed food more often. No differences for cortisol concentration were found between the different treatments. From eleven genes normally associated with stress, only gene CRF1 mRNA expression was elevated in fish tested under unpredictable conditions compared to unsafe conditions but not to fish tested under predictable conditions. However, the highest mRNA expression displayed by unpredictable groups, suggested that these individuals experienced a more stressful environment than the other groups. However as the lack of physiological and gene expression response coupled to the behaviour of fish from the predictable treatment differed little from that of the unpredictable treatment we could not demonstrate any clear benefits of predictability for salmon, and similarly no negative effects of losing control of the information. In the future, it could be useful to include a higher number of individuals, but also a pre-selection of coping styles in order to group individuals into a more consistent behavioural coping strategy, which could help elucidate general behavioural and physiological patterns to predictability.

TASK 2.3.2. APPRAISEMODUL - Explore how controllability modulate appraisal and coping ability in fish

This task aimed to use controllability as appraisal modulator of environmental cues in seabream and seabass. Offering to fish alternative strategies for dealing with stressful conditions it is hypothesized that it should improve their coping ability, as well as to demonstrate their appraisal capability on using surrounding cues to monitor their environment.

Methods: Avoidance learning conditioning was used as a paradigm. In a first stage, individuals learnt to associate a warning stimulus (CS) to a negative reinforcer (US^-) after a training period. In stage two, the learner experiences operant conditioning, whereby the individual realizes that an action by its own part can ameliorate the stressful outcome. Different experimental conditions were tested: controllable event (CTR: possibility to escape from a signalled (light) stimulus); uncontrollable event (UnCTR: impossibility to escape from a signalling event) and loss of control over the event (CTRUn: possibility followed by impossibility to escape). Assuming that CS itself elicited the same response as the US after the association being established, in the last conditioning session only the CS was presented to the fish. Behaviours were video recorded throughout the conditioning period during CS. After the last session, both circulating cortisol, and immediate early genes (IEGs) expression from specific brain areas related to learning, memory and emotional processing were determined to assess how the proximate states underlying controllability affects the stress response to environmental challenges.

Results and discussion: Our study showed a positive effect of CTR conditions for both species when compared to UnCTR and CTRUn. As in the previous task, coping styles had no effect on the experimental conditions tested and hence, removed from the analysis. Control over the income predictable aversive event was shown to significantly decrease the number of escape events as well as to promote higher dispersion of the fish (shoal rank) throughout the tank. Within experimental conditions, under CTR seabream showed significantly higher time freezing than under UnCTR and CTRUn conditions, while no differences were found between these last two groups. In seabass the time freezing was lower in fish under CTR conditions, whereas fish under CTRUn conditions presented significant higher levels when compared to UnCTR and CTR conditions. Exploratory behaviour analysis showed that seabream under CTR conditions, was more exploratory with no differences between UnCTR and CTRUn. In seabass no differences were found between CTR and UnCTR; however a negative effect of CTRUn conditions depicted by the lowest exploitation index suggests that having control over an aversive event and losing it afterwards can be more detrimental for fish. Controllability affected circulating cortisol in similar way in both species (Fig 2.7). UnCTR and CTRUn conditions showed to significantly increase the plasma cortisol levels whereas CTRUn conditions seemed to be more detrimental for the fish with the highest levels observed. Seabass showed higher levels of cortisol as compared to seabream between parallel experimental conditions.

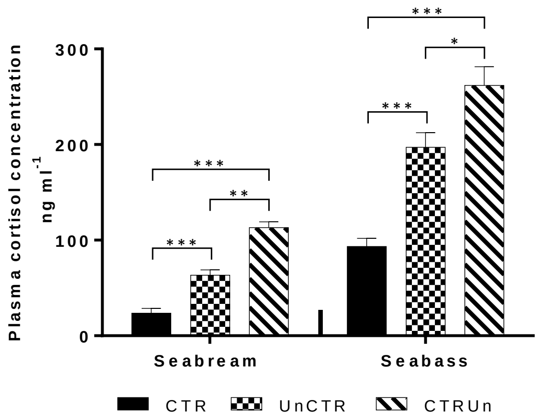


Fig.2.7. Seabream and seabass plasma cortisol concentrations (Mean ± SEM) measured from fish tested under each experimental condition (i.e. CTR, UnCTR and CTRUn). One-way ANOVA with planned comparisons significance levels are indicated; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

IEG gene expression analyses depicted different patterns of neuronal activity between experimental conditions. The effect of controllability resulted in a down-regulation of IEG gene expression. In seabream, Vv seems to modulate the way the individuals interpreted the controllable event. In seabass, CTRUn conditions promoted a significant up-regulation of all the genes tested in the Dlv brain region suggesting a major role of this area in the mediation of how fish will respond to a loss of control over an aversive event. In conclusion, this study suggests that providing fish with appropriate environmental features can change the way they perceive the environment with positive outcomes for successful coping ability. Insights regarding neuromolecular basis underlying controllability cognitive appraisal suggests to be ecologically conserved either throughout divergent species or between different appraisal modulators.

WP3 ALLOSTASIS

The third concept focus area was aimed at verifying the concept of **allostasis** as applied to fish in aquaculture. In WP3 the main objective was to demonstrate allostasis as fundamental regulatory process to explain variation in stress responsiveness of salmon, seabass and seabream. Allostasis, “stability through change” (Sterling and Eyer, 1988), is defined as an efficient regulatory system in which anticipation of needs is key to satisfy them before they arise (Sterling 2012). This process is coordinated by the brain that, while constantly monitoring internal and external parameters to anticipate the required changes, evaluates priorities and prepares the body for adjustment before they lead to errors. The capacity to prevent errors from occurring is more efficient than correcting them once occurred (Koolhaas et al., 2011). Changes involve stress hormones (cortisol, adrenalin), cytokines and parasympathetic activity to adapt to a new situation or environmental challenge. There is a large variation in individual perception (*i.e.* appraisal; see WP2) and allostasis allows customization of the individual response to a changing environment (McEwen & Wingfield 2010). Deviations from ‘normal’ environmental states generate a situation called allostatic state, in which the regulatory capacity of an animal changes (Koob & Le Moal 2001; Korte *et al.* 2007; McEwen & Wingfield 2010). This condition is characterised by increases in primary mediators of allostasis as defined above and can only be sustained for a relatively short period. The cumulative result of allostatic states is referred to as allostatic load. This can be regarded as the cost an animal has to pay to realise homeostasis *via* allostasis (McEwen & Wingfield 2003). Although being an adaptive response at first, prolonged and/or additional exposure to novel stressors leads to a situation named allostatic overload, in which pathologies and diseases occur, as a result of ‘wear and tear’ of the body (McEwen & Wingfield 2003; Korte *et al.* 2007; McEwen & Wingfield 2010) which results amongst others from lasting (unsuccessful) actions of adrenalin and cortisol.

It will be clear that the concept of allostasis plays a key role in our understanding of individual variation in stress responsiveness, coping styles and animal personalities: indeed, perception is central in this concept and perception varies individually (as are underlying physiologies). In WP3, we achieved that goal by testing how chronic exposure to different levels of allostatic load (degrees of stress), ranging from hypo-stimulation (as little as possible environmental stimuli) to a high stress load, activate the various components of the stress axis, and how this affects the ability to deal with an additional acute stress test (task 3.1 –

ALLOCOPE), how learning (through fear-conditioning, a test based on anxiety as strong learning stimulus) affects the response to stressors and affects allostatic load (task 3.2 – *ALLOPREDICT*), how hypo-stimulation affects allostatic prediction and learning ability (task 3.3 – *ALLOHYPO*) and how allostatic principles are applied in the case of exposure to deviating light regimes, *i.e.* constant light (task 3.4 – *ALLOLIGHT*).

Task 3.1 - Allostatic regulation in salmon, seabass and seabream

In the first series of experiments (task 3.1) we exposed Atlantic salmon, seabass and seabream to increasing chronic (*i.e.* three weeks) stress. Salmon were not disturbed other than for feeding (which served as control), repeated chasing with a net (RCS; chasing for 5 minutes in their home tank) or unpredictable chronic stress (UCS; a plethora of different stressors *i.e.* chasing, handling, confinement, and air exposure). In these experiments we assessed a physiological mediator of allostasis: cortisol. Catecholamine's (e.g. noradrenalin and adrenalin) are difficult to measure in animals, and particularly in fish. The adrenalin response is activated within seconds of the application of a stressor, and therefore it is near impossible to measure a true 'baseline' (and control) level of (nor-) adrenalin. Besides plasma cortisol, we characterised gene expression in tissues that make up the hypothalamus – pituitary – interrenal (endocrine stress) axis.

Fish exposed to UCS showed a reduced cortisol response, which correlated to a reduced pituitary capacity to mount a powerful enough stress response. Interestingly, the hypothalamic and interrenal capacities within the stress axis to respond to a novel stressor were not markedly affected by UCS (Madaro *et al.* 2015). Exposure to RCS resulted in rapid habituation of salmon to the stressor. In our study where we assayed plasma cortisol levels throughout the experiment, stressor-induced levels of the hormone rapidly restored to baseline. Despite this seemingly clear habituation, the phenomenon of allostatic state was reflected when we considered cortisol levels following exposure to a novel stressor. Here, a change in set point and regulatory capacity was observed, as the mounted cortisol response was lower compared to controls (Madaro *et al.* 2015; 2016b).

Seabass and seabream experiments were conducted in the same laboratory (Crete), using for these fish an identical experimental protocol, set-up and biochemical analyses, and the results can therefore be compared directly. Both species were exposed to increasing levels of stress for three weeks, resulting in increasing levels of allostatic state and load. Feed intake and growth were reduced in chronically stressed seabass, whereas no effects were observed in seabream. Notable differences between these two Mediterranean species occur also at the level of plasma cortisol: whereas seabream responded to the novel stressor with a potentiated cortisol increase in all groups, seabass only responded to the novel stressor in control and low-intensity chronic stress groups. The group exposed to the highest intensity of stress responded with an attenuated cortisol response, much like Atlantic salmon, and in accordance with effects of habituation as predicted by the allostasis concept. This group of seabass was further characterised by a markedly increased hypothalamic *gr/mr* ratio, which feeds the idea of a profound difference in cortisol feed back to the hypothalamus and the processing of plasma cortisol in the brain, and indicates the site of neuronal networks that execute the allostatic control

TASK 3.2 ALLOPREDICT - Conditioning to stress

The ability to predict the outcome of a current situation, based on prior experience is fundamental to the allostasis principle (McEwen & Wingfield 2003). Based on earlier experiences, behavioural and physiological set points are reset, in order to (better) cope with the challenge or changed environmental conditions by a fine-tuned and 'tailored to the situation' physiological stress response. To demonstrate this process in fish, Atlantic salmon were conditioned (*i.e.* they received a conditioning stimulus before the onset of a stressor) using a light signal. Although no difference in cortisol was found between conditioned and non-conditioned groups, a profound difference in behaviour was observed (Madaro *et al.* 2016a). Conditioned fish changed their swimming pattern when the conditioning stimulus was applied, demonstrating that the fish were anticipating an event, yet without an apparent (predicted) endocrine response. Several hypotheses may be advanced as to why predictability of the stressor did not reduce the stress response in our experiment: first, the biological relevance of light flashes and chasing and a natural link between these stimuli could be questioned as they do not occur in the wild. On the other hand, sudden changes in light or shadow are naturally connected with danger, *i.e* predators. However, the behavioural response to the conditioning stimulus shows that the stimulus was associated with the stressor. Second, as chasing occurred in the same manner every time with no severe consequences, the fish could habituate to the procedure, and habituation to the stressor resulted over time in a low beneficial physiological stress response (conditions of eustress) in all treatment groups. Habituation may in this way have masked possible effects of the announcement of chasing. Perhaps the stressor was not 'strong' enough to disentangle the effect of habituation and conditioning. Finally, unconditioned fish might have been able to predict the stress event because of experimental handlings such as removing the window of the tank, or the regular times of chasing could permit unconditioned fish to anticipate the stress by temporal predictability (Madaro *et al.* 2016a).

Prediction experiments were also conducted with seabass and seabream. Unfortunately, the chosen conditioning stimulus (light flashes 1 min before the onset of the stressor) proved to elicit a frantic response in seabass. Fish swam chaotically in the tank and bumped into the tank walls. Clearly, the chosen stimulus was not useful to study predictability in seabass. In contrast to seabass, seabream showed a clear and significant decrease in plasma cortisol levels as a result of the conditioning protocol (mean 15.0 ng·ml^{-1} *vs.* 50.2 ng·ml^{-1}). Moreover, the light stimulus *per se* had no obvious effect on the seabream as videotaped fish continued to swim calmly through their tanks. These results demonstrate that seabream are able to predict a stressor, and adjust their stress response accordingly: it was significantly lower in intensity compared to when the fish cannot predict the stressor. This series of experiments demonstrates prediction and the effects thereof on allostasis in Atlantic salmon and seabream. These findings may be applied in an aquaculture setting; by providing fish with stimuli before the onset of a handling or event (which are likely perceived by the fish as stressors, *e.g*. sorting, transport), the stress response may be mitigated – thereby decreasing possible losses and negative influences on welfare and product quality.

TASK 3.3 ALLOHYPO - Effects of hypostimulation on allostatic regulation

The above-mentioned studies deal with an increase in allostatic state and allostatic load. Experiments on salmon were undertaken in order to evaluate the possible negative effects of hypostimulation, *i.e*. effects of an as low as possible level of stimuli. Our hypothesis was that a stimulus-poor environment (with the lowest possible allostatic load) should lead to lower the ability of allostatic prediction and regulation, a poorer learning ability and behavioural flexibility, but this may be overcome by regular activation of stress and reward systems.

Salmon fry from the same egg group were raised individually from hatching, with as little stimuli as possible, or individually with daily stress stimulation, and as control, communally in tanks. Raising salmon individually without disturbing them presented a huge logistic and experimental challenge. Indeed, several issues arose during the experiment, such as fungal growth in the tanks (thus necessitating cleaning of the tank and therefore confounding the experimental 'design of no stimuli'). Unfortunately, these issues posed such significant problems that the experiment was terminated prematurely when the fish were around 4 cm. At this stage we found no difference between treatment groups in acute stress (cortisol) response or in learning ability in an escape-learning task, but we could not be sure our experimental set up gave hypostimulated animals. The effects of hypostimulation on the principle of allostasis in fish still provide a challenge and relatively unexplored venue for further research, but although theoretically interesting as a proof of principle for the allostasis model, hypostimulation may not be a relevant problem in aquaculture.

TASK 3.4 ALLOLIGHT - Effects of constant light on allostatic load

As a practical application of the allostasis principle, the model of continuous light was chosen. Seabream and seabass were exposed to either control (12:12 light:dark; LD) or continuous light (24:0 LD). These fish were equipped with electroencephalogram (EEG-) and electrocardiogram (ECG-) electrodes and (because a free-floating logging device was used) were able to swim freely in their home tank. Brain activity was assessed by evaluation of brain wave frequencies and brain activity by 'correlation dimension ' analysis. Both seabream and seabass displayed alternating high and low brain activities during the day (independent of light or dark). However, only in seabass brains wave frequency changed as a result of changed light regimes. For seabream, no differences in brain wave distribution were found. The results in seabass corroborate similar results in common carp (the studies carried out to develop the methods applied). They for the first time prove by physiology (EEG) that fish show (non-REM) sleep. Sleep occurs in carp in bouts throughout the day (compare to dogs that so similar sleep patterns). Importantly, sleep deprivation such as could result from altered light regimes as often applied in aquaculture settings may contribute to allostatic load or even overload.

WP 4 - ONTOGENY

The fourth WP concept focus related to the ontogeny of neuronal function and neuroendocrine stress responses in salmon, seabass and seabream. To this end, the ontogenesis and regulation of the physiological stress responses and the impact of early chronic stress experiences on brain cytoarchitecture, neurogenesis, apoptosis and neurotransmitter systems at later stages of development have been investigated. Studies also included the effect of larval rearing system (intensive vs. mesocosm) and common husbandry practises on operant conditioning, brain function, allostatic states and performance of seabass and seabream in subsequent stages of development. The application of chronic stress models in all species at early ontogeny and the choice of advanced learning challenges, allowed to separate developmental and population factors and to identify how the rearing environment impacts brain function plasticity.

At hatching the brain takes up a large proportion of the cells in the fish body, showing the importance of the brain as the central regulatory organ (Fig 4.1). Early life stage history is important for the ontogeny of the brain and related neuroendocrine organs that will affect fish performance at subsequent developmental phases. Fish reared under intensive culture conditions are subjected to several environmental, social, and husbandry related stimuli that may have potentially noxious or stressful effects. In fish, development of the endocrine organs and factors involved in regulation of the HPI-axis (hypothalamus-pituitary-interrenal axis) take place during embryogenesis and post hatch stages. The overall aim of WP4 was to investigate the impact of early-life stress experiences on ontogenesis, the performance of allostatic mechanisms and on coping ability later in life of all three target species.

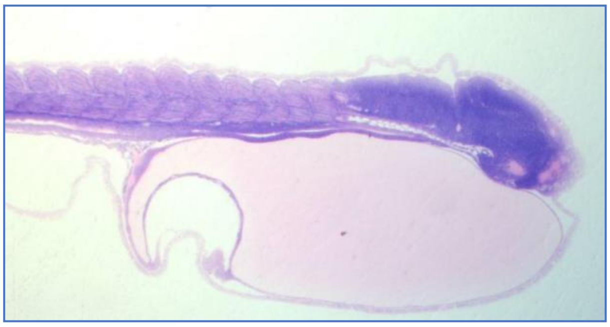


Figure 4.1. Seabass larvae at day 1 post hatching (dph). The brain covers the majority of the head area.

Task 4.1 STRESSONTO - Ontogeny of neuronal function and of neuroendo-crine stress response in Atlanitic salmon, gilthead seabream and European seabass.

The specific aim was to investigate the ontogeny of neuronal function and of neuroendocrine stress response. Acute stress protocols were developed and applied at specific developmental stages during early development of **Atlantic salmon** (fertilization, eye stage, hatch, first feeding), **gilthead seabream** (embryos, hatch, first feeding, flexion, formation of all fins), and **European seabass** (embryos, hatch, mouth opening, first feeding, flexion, formation of all fins). Samples were taken to study ***molecular*** (key genes encoding (i) proteins and receptors critical for the functioning of the HPI axis, (ii) enzymes involved in the biosynthesis and degradation of cortisol, and (iii) certain neurogenesis and neuroplasticity markers), ***endocrine*** (whole body concentrations of cortisol, T_3, T_4, aMSH), and ***neural*** (ontogenesis of adrenergic receptors, brain cell proliferation and apoptotic activity) modulators of allostasis, in relation to the ontogenesis of the HPI axis.

The ontogeny of the stress response (cortisol, a-MSH and of the adrenergic system), and the molecular programming of the corticoid axis during early life were characterised for the first time for these species. For ***Atlantic salmon***, most HPI – axis related genes were turned on around 300 day degrees, the exception being gr1 and gr2 mRNAs, which were maternally provided. Maternal gr mRNAs together with maternal cortisol may indicate a glucocorticoid signalling pathway during initial stages of embryogenesis which is of particular interest (confer findings in other species). Unfertilized A. salmon egg contained relatively high amounts of cortisol, which is most likely of maternal origin. Cortisol content decreased during early development to a nadir around the "eyed stage" (c.250 DD). Thereafter cortisol content was low until the time of hatch (c.520 DD). From hatch onwards cortisol content increased gradually towards start feeding (880DD). The increase in cortisol after hatch is likely to be a result of endogenous steroid synthesis.

European seabass and ***gilthead seabream*** are able to respond to external noxious stimuli as early as around first feeding. The magnitude and duration of the cortisol stress response is higher and a pattern similar to that observed in juvenile and adult fish is established from flexion until the formation of all fins. This was further supported by histological data showing that while the kidney tubules with a distinct morphology were present at hatching, a clear morphological differentiation of the interrenal and chromaffin tissue was possible to identify only around flexion. Expression data of genes involved in corticoid biosynthesis (*11β-hydroxylase*), degradation (*11β-hsd2*) and signalling (*gr1*, *gr2*, *mr*, *crf*) also verified that the HPI axis seems to be fully mature only at the stage of the development of all fins. Moreover, the mRNA transcripts of *11β-hydroxylase* and *11β-hsd2* showed a strong correlation with the whole-body cortisol concentrations throughout early development.

Task 4.2 SALPROG - Early stress and life history programming in Atlantic salmon pre-smolts

This task investigated whether chronic stressors applied during early life of Atlantic salmon, may reduce the offspring's performance and potential to cope with environmental perturbations during later developmental stages.

Fertilized Atlantic salmon eggs (Aquagen strain) were incubated at 7 °C. Four treatment groups: 1) unstressed control, 2) eggs stressed during embryogenesis, 3) fry stressed during post-hatch stages, 4) embryos and post-hatch fry stressed during both stages. Groups 2-4 were exposed to bouts of stress (cold-shock and air exposure - drop from 7 °C to 0.2 °C for 1 min, followed by air exposure (15 °C) for 1 min and then transfer back to 7 °C) either during embryogenesis (5 times from 250 to 450 day degrees (DG) or during yolk-sac stages (4 times, from 540 to 880 DG), or both. To investigate the influence of stress treatment on developmental timing and HPI-axis functionality of the stress response, a stress test was performed just prior to start feeding (880DG), when the HPI-axis is assumed to become functional. Stress was applied as indicated above, and fry was sampled for molecular and endocrine analysis at 0, 1, 3 and 24 hours post stress. Whole body cortisol was determined by EIA. Quantitative PCR (qPCR) was conducted using sequence specific primers for *crf1*, *crf2*, *pomcA1*, *pomcA2*, *pomcB*, *mc2r*, *mr*, *gr1*and *gr2*.

Stress treatment did not result in embryo or fry mortality and had not significant effect on growth. At 880DG baseline expression of most HPI-axis related genes were reduced in the stress treated groups compared to that of the control (Fig. 4.2). This implies that the effect of stress during embryogenesis on gene expression was maintained for at least 2 months after the last stress exposure.

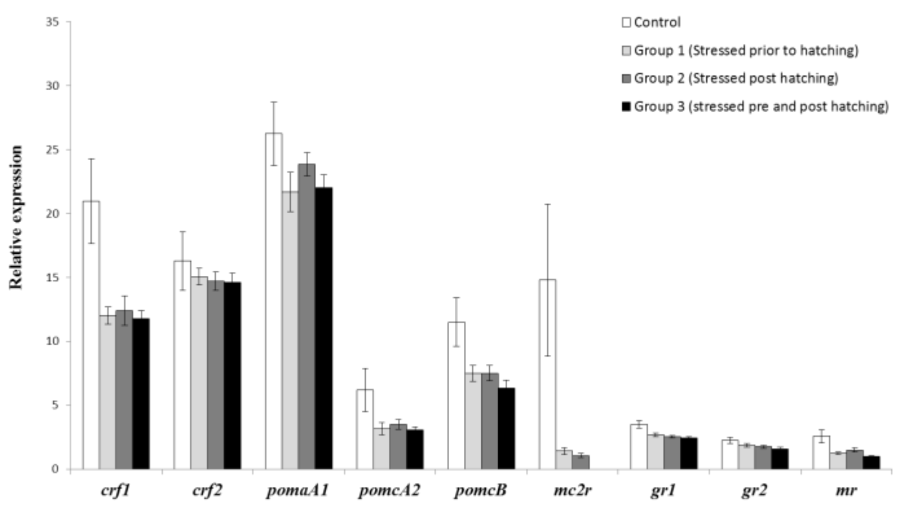


Fig. 4.2: Relative expression of stress related genes prior to start feeding at 880 day degrees in the four treatment

Previous stress treatment did also influence HPI-axis related gene expression after exposure to acute stress. For example, corticotrophin releasing factor (*crf1*) gene expression was clearly responsive to stress and was also influenced by previous stress exposure, resulting in a more rapid down regulation in group 2 and 3 (Fig. 3).

Compared to the control group, stress treatment resulted in a delayed elevation in whole body cortisol in the pre hatch and pre-and post- hatch stressed groups. Since temporal change in cortisol was less influenced in the post hatch stressed group, it appears that development of the HPI-axis is more susceptible to stress in the period before rather than after hatch.

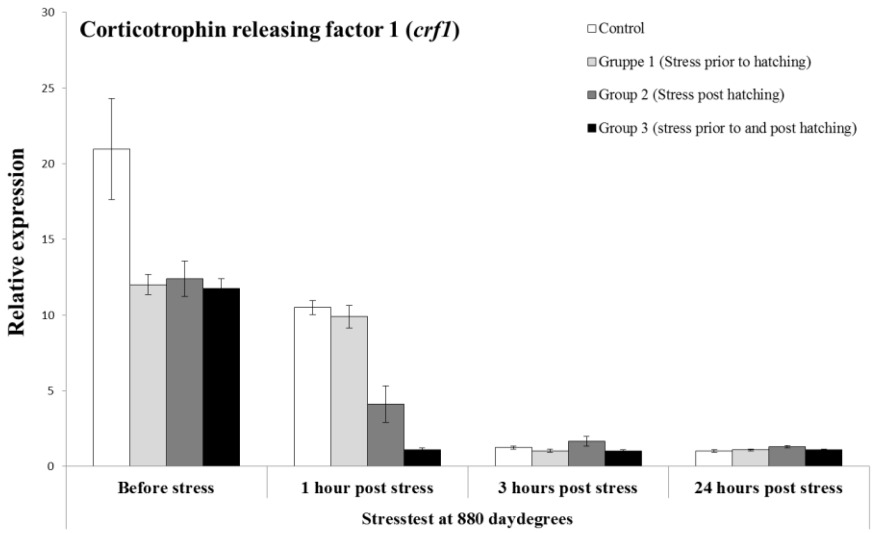


Figure 4.3: Influence of stress on mRNA levels of Corticotrophin Releasing Factor 1 (crf1) prior to start feeding at 880 DG of the four treatment groups.

Stress applied at the time of start feeding resulted in a modest increase in cortisol (c. 1.5-3.0 fold increase) one hour after exposure (Figure 5). Three hours after stress, cortisol content was generally back to pre-stress levels. The pre- and post-hatch stressed group appeared, however, to mount a slightly lower response and/or a more rapid decline in cortisol in response to stress. This is in agreement with the delayed elevation in endogenous cortisol in the period prior to start feeding (see above). The cortisol response to stress also varied considerably within treatments, supporting the notion that the capacity to produce cortisol and/or respond to an environmental stressor was still under development. Based on the present cortisol data, we suggest that the HPI axis of A. salmon is not yet fully functional at this time.

It appears that stress exposure during early life stages of Atlantic salmon may have the potential to induce long term effects on gene expression which is central to the functionality of HPI-axis. Further, embryonic stress may also alter responses in gene expression and endocrine changes to acute stress during later stages of development. If such altered stress coping abilities also manifest into juvenile and adult stages, it may hold great potential for the production of a more robust salmon in the future.

Task 4.3 MEDPROG - Early stress and life history programming in seabass and seabream juveniles

Here the aim was to determine the impact of long term unpredictable chronic mild stress applied early in life on brain structure, coping abilities and performance of juvenile European seabass and gilthead seabream individuals. An ***unpredictable chronic low intensity stress protocol*** *(UCLIS)* was developed and applied, for the first time, at three different developmental phases (first feeding to flexion; flexion to post-flexion; and formation of all fins to melanophores) in early life of European seabass and Gilthead seabream. To evaluate the effect of stress, survival and performance data were recorded and water samples were collected from the larvae rearing tanks to determine water-born cortisol concentrations. At the end of each respective experimental period, whole-body pooled samples were collected for massive sequencing and microRNA analysis. Subsequently, all groups were reared under similar weaning conditions and juveniles were subjected to an acute stressor to determine if early stress experience has long lasting influences on brain structure and function of juvenile fish. Samples were collected for ***hormonal*** (whole-body concentration of cortisol and brain neurotransmitters), ***molecular*** (massive sequencing, microRNA and mRNA studies), and ***neural*** (brain cytoarchitecture and neurogenesis) determinations.

Exposure of fish to an unpredictable chronic low intensity stress protocol (*UCLIS*) based on optical, mechanical and social mild stimuli, for a period of two-weeks at three different periods during early life, had a species specific effect on fish performance, cortisol stress response, neurogenesis and transcriptomic changes in European seabass and gilthead seabream. ***Overall, the data verified that European seabass is more sensitive than gilthead seabream in ordinary stimuli and practices that may occur during intensive rearing***. In particular, ***seabass*** larvae were sensitive to mild husbandry stimuli with consequences even at subsequent stages of development (*i.e.* juvenile fish of approximately 15 cm in length), with the ***stages of first feeding and the development of all fins being the most critical***, providing the necessity to reconsider common rearing practices. UCLIS application resulted in higher ***water cortisol release rates*** in all groups compared to the controls provided ***a reliable non-invasive indicator of stress event during early ontogeny***. On the contrary, application of the same protocol did not affect significantly water cortisol release rates during early life of ***seabream***. In addition, the application of the UCLIS, had no significant effect on the growth rate of larvae at the end of the period that it was applied (*i.e.* until 60 dph). However, as in the case of seabass, two months after the end of the UCLIS trial period, juveniles (approximately 8.5 cm) experienced early life stress during the development of all fins stage showed significantly lower body weight and total length than controls.

UCLIS application during flexion and all fins developmental stages had no impact in the juvenile brain proliferation pattern. In contrast, the acute stress exposure of naive juveniles, not previously exposed to UCLIS, significantly reduced brain proliferation, in agreement to studies in mammals, ***suggesting that early life chronic adverse experience reduced acute stress responses of juvenile brain cell proliferation, possibly underlying an allostatic mechanism leading to more effective coping styles***.

For the first time distinct expression patterns between individuals exposed to UCLIS during different development periods, as well as between juveniles exposed to acute stress according to their larval stress exposure, were revealed. These data will be further analyzed towards identifying the appropriate paralogues of the genes involved in the stress response at early ontogeny.

Task 4.4 SALCHRON - Impact of long term chronic stress in pre-smolts on allostatic states, coping style and performance of salmon in subsequent stages of development

Ongoing rapid domestication of Atlantic salmon to aquaculture conditions implies that natural selection has not “prepared” fish for the many stressors encountered under conditions of artificial rearing. Animals show a variable capacity to modify phenotypic responses in response to environmental change. Windows of opportunity have been identified at early-life stages that have been particularly important in shaping such phenotypic plasticity. In this task we investigated whether “programming” salmon to cope with chronic stress during the early freshwater life period could potentially improve performance at later life stages. 10 month old salmon parr were assigned to one of 3 treatments: chronic unpredictable stressors (CUS), chronic predictable stressor (CPS) or no stress (control) for 23 days and this was followed by routine rearing for 6 weeks under continuous light (which induces smoltification) and then by 4 weeks in seawater (See task 3.1 or Madaro 2015).

We found that previously stressed groups that had highly reduced growth rates during the chronic stress phase, compensated during recovery with higher growth rates compared to controls particularly during the continuous light (smoltification) phase before seawater transfer. Four weeks after seawater transfer there were no difference in weight between groups. When subjecting fish to an acute stress at the end of the experiment, we found that CUS groups had an overall lower brain monoaminergic response to stress compared to CPS as well as control groups. Notably serotonergic activity at both basal and acute stress conditions was negatively correlated with specific growth rates, which implies that serotonin responsive individuals suffered growth disadvantages

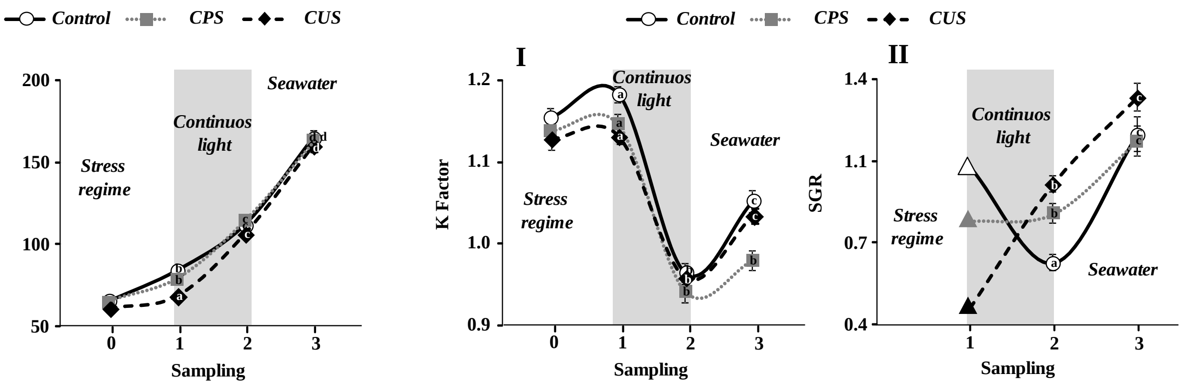


Figure 4.4. Mean weight (g, to the left), mean (±SEM) K factor (**I**) and specific growth rate (SGR; **II**) for Atlantic salmon at start of the experiment (sample 0), after a stress regime (*i.e.* unpredictable chronic stressor (UCS), predictable chronic stressor (PCS) or control; sample 1), after smoltification triggered by continuous light (sample 2), and after seawater transfer (sample 3).

Sampling on the final day (10 weeks after chronic stress) was conducted in order to analyze basal monoaminergic activity as well gene expression of target genes in the brain stem, hypothalamus and telencephalon. We found that post-hoc selection of serotonergic activity high (HR) and low (LR) responders resulted in the elucidation of several individual differences associated with developmental plasticity. While the growth rate in LR fish at end of the experiment was significantly increased in CUS groups only, in HR fish the control groups showed the highest variation (the lowest and highest increase after smoltification and seawater periods, respectively). Furthermore, CUS groups had higher serotonergic and dopaminergic activity in the hypothalamus. In addition, in CUS groups, LR fish had a significant lower telencephalic expression of the serotonin receptor 5-HT1A, while HR individuals had higher CRH and CRHBP expression. These results suggest an anxiety-like profile for individuals previously exposed to a CUS regime.

Finally, we show that while there is a positive relationship between brain stem 5-HT activity and brain derived neurotrophic factor (BDNF), this relationship is opposite in stressed treated groups, suggesting a long-term disruption of this relationship by an early-stress environment. In conclusion, we here have shown that selecting individuals for their basal serotonergic activity may be a useful tool to study developmental plasticity.

Taken together our results suggest that experiencing a higher stress regime during early life may prepare individuals to perform better in aquaculture environments. These results may be directly applicable to improving husbandry practices in aquaculture by training fish to cope better with stressors relevant for aquaculture.

Task 4.5 OPERCONDIT - Effect of larviculture technique, fish weight and density on operant conditioning, by the use of self-feeder

The aim was to test the hypothesis that the probability of rearing error is higher in intensive as opposed to mesocosm larval rearing techniques, and therefore, seabream and seabass larvae originating from intensive hatcheries should present a higher risk of a compromised nervous system in contrast to those reared in a mesocosm and thus should not learn so easily an operant conditioning task.

A factorial experiment was conducted, consisting of juvenile European seabass and gilthead seabream, (i) originated from two different larvae rearing systems (intensive or mesocosm), (ii) reared at three different density groups (50, 250 and 500 individuals) and (iii) starting at four different body weight classes (25, 50, 100 and 250 mg). All groups were weaned overnight on adequate size commercial diet, with the use of ***a novel in-house built self-feeder***, to which they had not been previously exposed. Performance parameters (survival, biomass production, conversion ratio, growth heterogeneity) were estimated and fish behaviour was monitored by the use of video cameras and image analysis.

Ordinary husbandry practices also affect significantly fish performance and coping ability. ***Larvae rearing method*** (mesocosm vs. intensive), ***stocking density*** and ***sorting*** affected the behaviour, stress response and performance of fish at subsequent phases of development in a size and species dependent manner. Thus, fish originating from *mesocosm* (*i.e.* low stocking density) that were then transferred to high weaning densities, exhibited ***higher mortality due to cannibalism***, compared to intensively (*i.e.* at higher stocking densities) reared larvae. At ***higher stocking densities*** (250 and 500 individuals per 50 L), ***the probability of fish learning and using the self-feeder was higher and faster***. In addition, ***larger sized juveniles*** (100 mg and 250 mg), originating from both larval rearing systems, ***learned to manage the self-feeder faster and use it more frequently than smaller individuals*** (25 and 50 mg). Finally, at ***the lower weights the "intensive" fish, of both species, tended to use the self-feeder more than the "mesocosm" fish***.

Task 4.6 MEDCHRON - Impact of larval rearing environment and common husbandry practices on allostatic states, coping and performance of on-growing seabass and seabream

Batches of European seabass and gilthead seabream eggs were reared with two different larvae rearing systems (intensive or mesocosm), transferred to weaning installations and one cohort from each respective rearing system was treated according to the standard aquaculture procedure, i.e. with sorting – size selection, so that finally (120 dph) three experimental groups were created, consisted of Large (L), medium (m) and unsorted (U) sized juveniles (in the U group, only large individuals were expected to survive, due to cannibalism). Furthermore, to characterise fish coping style (proactive vs. reactive individuals), fish in all groups were further subjected to a risk taking test (RT) developed in WP1, modified and validated from small-size laboratory conditions (WP1) to large industrial ones (WP4). The RT test was based on escape behaviour, and possible differences in the coping style among the experimental groups was assessed by counting the number of escapees, over a time course of 4 hours. At the end of the Risk Taking test, an extra acute stress was applied and samples were collected for whole-body cortisol determination and brain expression studies. Fish were then transported for further on-growing in net-pen cages, for a period of four months and then an UCLIS protocol was designed and applied for a period of 14 days. During stress exposure fish were monitored to estimate their spatial distribution and feeding behaviour by the use of a hydro-acoustic echo integrator system. Performance data were estimated, blood stress indicators were determined and brain gene expression studies were performed, to investigate differences among the experimental groups during on-growing and to identify possible differential response to stress.

In terms of growth performance (during on-growing in open sea net-pen cages), results support the hypothesis that individuals reared with the Mesocosm technology, performed better than the ones from intensive rearing. Furthermore, results also support the ordinary husbandry procedure adopted by the farmers (i.e. sorting), as medium size groups performed better and reach a similar size as large individuals. On the contrary unsorted groups performed poorly justifying the selection procedure.

Regarding the RT test, it was the first time that such a test was performed under big volumes and with a large number of fish. The percentage of escapees was similar between replicates. For both species, the original hypothesis that unsorted and large-sized groups may have a higher percentage of bold individuals was partially supported by the results.

Statistically significant differences in bdnf, mc2r, gr1 and gr2 transcripts were observed between the different sizes in European seabass individuals. In addition, bdnf and gr2 expression showed statistically significant higher and lower levels, respectively, in bold fish of the medium size group compared to the shy fish. The extent that such differences may be related to fish performance remains to be elucidated.

During the on growing phase, the application of the chronic stress did not result in decreased growth performance, in both species, verifying the mild nature of the stimuli. However, in the case of European seabass high level of disturbance in swimming behavior, low mortalities following lights on at night, as well as differences in plasma cortisol concentrations depending on fish size and further acute stress treatment were observed, ***indicating that this species is more sensitive than seabream in common husbandry practices***.

4.1.4 IMPACT

Finfish aquaculture is a growing industry in Europe and is central to EU food security concerns; it provides a steady supply of healthy and high-quality (sea) food and secures income in particular to people in coastal areas. The recent development of large- scale intensive fish farming of species such as Atlantic salmon, rainbow trout, European seabass and Gilthead seabream, has highlighted welfare problems and as a direct consequence the realisation that there is a very limited knowledge of fish welfare in aquaculture systems.

In Europe concerns around the welfare of farmed production animals including fish is steadily increasing; in European animal welfare law farmed fish are defined as “sentient” animals with the ability to suffer and experience pleasure. The biological basis for this definition is still debated with supporters on both sides of the argument (e.g. EFSA- Q-2008-708, Key 2015, Rose, 2002; Huntingford et al., 2006;Rey et al, 2015). Many of the researchers in Copewell are directly involved in this on-going philosophical task. As EU society generally accepts that the use of any animal for the purpose of furthering human interests entails the ethical responsibility to optimally ensure their welfare. Ethical considerations, including the very concept of “animal welfare”, are based on the belief that animals have subjective emotions and the ability to experience pleasure and suffer (Dawkins, 1990). This is very much in line with most people’s everyday thinking, but in sharp contrast to the dominating scientific views during the 20th century, which were strongly under influence of positivism and behaviourism, where emotional and affective states were considered outside the scope of science and banished as explanatory concepts (Fraser, 2009). Animal welfare science today in fact suffers from a certain “schizophrenia” with positivism-rooted studies of neurobiology, physiology, and behaviourism on the one side, and anthropogenic and ethical views on animal suffering on the other. Copewell has made a significant contribution to the above through both high quality published science and associated dissemination activities toward providing a platform to discuss and validate results and to provide a biological framework to further understand welfare in fish in the EU.

However in the industrial context of aquaculture current laws and regulations are present and demand that fish farmers have sufficient competence and technology to secure the welfare of their animals. Operational definitions of fish welfare and established methods to assess welfare are lacking, and it is, therefore yet impossible to know how to comply with existing regulations, as it is unfeasible for authorities to control or enforce regulations. During the last decade a range of EU funded projects provided a knowledge basis to secure welfare in European farmed fish such as SEAFOOD+, WEALTH, FASTFISH, BENEFISH and the Cost project Wellfish (Cost Action 867). These projects had centred upon the definition and concept of welfare, focusing their research on specific welfare related concepts such as “stress level”, “stress genes” and “immune defence”, however application of scientific theory that includes the concept of animal welfare as an integrated functional element in biological science was still lacking in such research. Recently, several groups in the European Food Safety Authority (EFSA) have reviewed the knowledge status and conducted risk analyses related to welfare of European farmed fish species. These reviews show an extensive knowledge base for the basic biology of these fishes and the impact of various husbandry procedures on fish health and welfare; but they underline that our knowledge is fragmentary at best and that we need to improve our basic knowledge about how fish experience their environment and the underlying mechanisms, including neural and neuroendocrine functions and responses to stressful situations.

A main objective of the COPEWELL project has been to improve scientific understanding by addressing the present gap between the ethical-philosophical conceptualisation and hypothesis-driven biomedical research. This is in turn will facilitate translational studies that will benefit from the scientific framework developed in COPEWELL. Importantly and perhaps in contrary to current trends in biological research this was achieved by applying the comparative evolutionary approach. COPEWELL has applied a hypothesis-driven multidisciplinary approach to explore links between brain function, behaviour and adaptive stress physiology, and tried to understand how these features change as the animal develops from egg to adult. For example, early development in environments that are both varied and variable, relevant for ontogeny in commercial aquaculture operations, have been studied to understand the extent to which experience alters subsequent performance under production conditions.

The project focused upon Atlantic salmon (*Salmo salar*), European seabass (Dicentrarchus labrax) and Gilthead seabream (*Sparus aurata*), commercially important species with different behaviour, life histories and physiologies in culture conditions; additional research on zebrafish and specific strains of rainbow trout addressed different stress coping styles, and facilitated our approach to appraisal and the comparative genomics of stress across the Teleostei. The project was organized in 4 scientific Work Packages: WP1 Coping Styles, WP2 Appraisal, WP3 Allostasis and WP3 Ontogeny.

WP1: COPING STYLES

Consistent over time and across contexts variation in how individuals react to positive and negative situations is often referred to as coping style (proactive vs. reactive), or animal personalities. These coping styles are the result of how individuals perceive their environment and how this information is processed in order to decide on an appropriate response to cope with changing environments.

In the course of the COPEWELL project, we have developed and validated methods and associated experimental assays to analyse in-depth stress coping styles in Gilthead seabream, European seabass and Atlantic salmon. This research has resulted in a significant advance, as highlighted by the number of high quality scientific manuscripts published, in our understanding of the underlying mechanisms of stress coping styles and importantly a series of methodologies that describe how to measure stress coping styles in each of the fish species in COPEWELL. The three major impact points are listed below:

1) Assays to parameterize common behavioural and physiological correlates for each of the three species addressed. A common approach to analyses and identification of species-specific traits that will be fundamental toward future translational research efforts.
2) The identification of relevant gene products as biomarkers for stress coping styles in each species and across all 3 species. Transcripitomic based analyses of risk-taking across species leading to the identification of an underpinning genetic framework conserved through evolutionary time.
3) The construction of neuroanatomical maps with histological and neurotransmitter markers with the telencephalic description and defined micro-dissection protocols for gene expression and neurotransmitters. For the first time we have shown that fish with differing coping styles show clear differences in forebrain area activation. New evidence highlighting that variation in individual perception is through complex forebrain regulatory systems. Confirmation of the hypothesis that structural mechanisms and neural plasticity are essential to contrasting stress coping styles has underpinned the analysis of cognitive functions.

Coping styles may have implications in diverse fields (from ecology to aquaculture), so the development of appropriate screening methods and the understanding of their genomic and neurologic correlates has been a highly effective way to extend our knowledge and to incorporate behavioural responses into common practices for example selection-based breeding programmes, to improve the welfare of farmed fish. Apart from demonstrating the extent of individual variation in aquaculture fish populations, and thus showing the validity of an evolutionary approach that takes such variability into concern, the knowledge generated can also be used directly by the aquaculture industry to improve rearing conditions for farmed fish. We hope that these results encourage more research into individual differences and their link to stress and disease vulnerability. This, in turn, will help develop aquaculture husbandry practices that will not only improve welfare, but also product quality and efficacy in aquaculture operations.

WP2: APPRAISAL

The way environmental stressors are so ambiguously perceived by animals can explain why some individuals react so differently under similar stressful conditions. The large individual variation in stress responses in fish should not solely depend on the situation to which the individual is exposed, but also on the cognitive evaluation that the individual makes of the situation, i.e. the way the stressor is appraised. Such evaluation is based on a limited number of criteria and can be used to manipulate the way individuals interpret such events, either by decreasing the negative effect of an environmental challenge, or by increasing the positive effect of a rewarding situation. Both predictability and controllability of the environment can be used to modulate the stress response depending first on the degree of conflict of the event with the individual's needs or expectations, and second on the individual's coping possibilities offered by the environment. These criteria can be used as a simple and inexpensive tool to promote physical, physiological and psychological wellbeing of farmed fish. Additionally, the study of the neural mechanisms underlying cognitive appraisal processes will allow a better understanding of the neuronal networks that process environmental information.

In the COPEWELL project we have shown appraisal evidences in fish suggesting a relevant role for the environmental context on the internal state of the animal, and thus on the behavioural output responses. We also revealed for the first time that fish with different social experiences have different functionally connected brain networks. The social plasticity mechanisms underlying these networks occur independently in discrete relevant brain nuclei given rise to different neuronal states in zebrafish. We also developed methodologies and paradigms to examine if salmon, seabream and seabass exhibit behaviour, gene expression and neurophysiological profiles specific for different environmental conditions (positive and negative stimuli). We found behavioural and physiological correlates, and identify species-specific traits in different coping styles (behavioural consistency between events or across situations). Additionally, we explore how the same fish species used predictability as an appraisal criteria, and whether different coping styles differ in behaviour and neuroendocrine responses to predictable, unpredictable and loss of predictable conditions. Different behavioural and physiological outcomes have shown that fish are able to retain memories of environmental events (retrieved by environmental cues), and predictable rather than unpredictable environments have demonstrated a positive effect. These results emphasize the positive effect of control, rather than the negative effect of a total lack of control in an aversive situation. We have also shown for the first time how controllability modulates the way fish perceive their environment and identify the complex forebrain regulatory systems of individual perception. The ability of fish to appraise stimuli with different valences presented similarities to what has been found in other vertebrates, indicating a conserved mechanism throughout evolution.

The positive outcomes from this project regarding fish appraisal may be a clear start point to understand the adaptive physiological and behavioural responses towards ecological threats or opportunities, with repercussions for fish farming welfare. Also, evidences of emotional states in fish driven by the different neural states and expressed by different behaviours can promote new possibilities for future research. Understand how stress is constrained by the emotional state of the individual and how it can influence their counterparts will improve aquaculture practices and management with the main objective, nowadays an evident spin-off of public concern, improve the welfare of fish kept and reared in captivity.

WP3: ALLOSTASIS

In WP3 the relatively new concept of allostasis, constancy through change, as a basis for regulation of dealing with animals stress situations and various demand implies that fish make predictions to adjust regulatory set points as required by the ever changing demands of the variable and varying environment was addressed.

Within WP3 ALLOSTASIS, the general, scientific knowledge on fish stress physiology within a context of allostasis has been tested and proven. Key components of the allostatic concept:

1) A change in physiological output in response to environmental challenges (allostatic state).

2) The effect of predictability on the outcome of the stress response (allowing the fish to reset and fine-tune its internal set points and equilibria).

These results have been published in international, peer-reviewed journals, and presented at international conferences, attended by the scientific community as well as stakeholders, i.a. people from the aquaculture industry. Several publications are currently under development and we expect to submit these manuscripts in 2016. Although fundamental in nature, the knowledge provided by the work package allostasis provides scientists with novel physiological insights to further develop hypotheses and concepts with respect to stress physiology and animal welfare as well as aquaculture industries to develop new protocols that can promote animal welfare. The results strongly indicate that we can improve the stress tolerance and performance of farmed fish by implementing training methods at the hatchery stage. A Ph.D. student (A. Madaro), appointed at the Institute of Marine Research (IMR – Bergen, Norway) and the University of Bergen (Norway) defended his thesis successfully in December 2015.

WP4: ONTOGENY

The overall aim of WP4 was to investigate the impact of early-life stress experiences on ontogenesis, the performance of allostatic mechanisms and on coping ability later in life of all three species. Three major impact points are listed below:

1) A detailed description of the ontogeny of neuronal function and of neuroendocrine stress response in the three study species with emphasis upon the stress axis.
2) Stress exposure during early life stages may have the potential to induce long term effects on performance. This may hold great potential for the production of a more robust fish in the future.
3) Manipulation of the neuroendocrine system during development has significant impact upon later life and related performance. These results may be directly applicable to improving husbandry practices in aquaculture by training fish to cope better with stressors relevant for aquaculture.

WP4 has combined state of the art neuroendocrine analyses and molecular biology with experimental designs that are directly relevant to aquaculture. We have provided a road map for studies addressing ontogenesis (intensive vs. mesocosm) and regulation of the physiological stress responses and the impact of early chronic stress experiences on brain function through later stages of development. The application of chronic stress models in all species at early ontogeny and the choice of advanced learning challenges, allowed to separate developmental and population factors and to identify how the rearing environment impacts brain function plasticity. WP4 therefore has provided important in sight into how the three species respond to husbandry practise and how this can be bettered in subsequent studies by setting out a series of important baselines.

Relevance to the wider social and policy objectives and relevance to the Treaty of the European Union and the European Research Area

In a wider perspective, COPEWELL will contribute to the building and consolidation of the European Knowledge Based Bio-Economy (KBBE), by providing knowledge on fish biology and welfare in aquaculture systems in line with the strategy for sustainable development of European aquaculture in the Common Fisheries Policy (CFP), and the broader context of an EU strategy for marine and maritime research regarding "The ocean of tomorrow".

Fish welfare is a key component of sustainable aquaculture development and therefore for EU food security. There is an increasing global demand for seafood and traditional fisheries appear to have reached their limits on a global scale, whereas aquaculture is rapidly increasing and is regarded as the main source for future growth in sea food production. On one hand, aquaculture, including European finfish production, can provide a steady supply of high quality (sea) food that is also considered to be greatly beneficial to human health. On the other hand, there is increasing controversy regarding the sustainability of intensive European fish farming including; environmental impact on marine ecosystems and wild populations, the sustainability of feed sources that are used in fish farming, and also fish health and fish welfare in such systems. The introduction of novel feed resources, such as land derived plant sources to aquaculture fish also raises new and currently unknown fish health and welfare problems.

The increased use of fish in intensive farming systems has increased the general awareness of fish welfare, and questions are raised whether fish are sentient beings and to what degree they can feel pain and fear. Farmed fish are protected by similar legislation as domestic farmed animals, but the knowledge base for fish welfare is far less developed leading to a lack in the EU regulatory framework. Food produced in the EU is being retailed in markets where concern for the welfare of animals in capture is steadily increasing. Thus, farming industries capable of defining coherent criteria for animal welfare, and which incorporate these factors in the rearing process, will be at a competitive advantage. Furthermore, it is a generally accepted ethical stance that animals used for furthering human economic development must be protected from suffering. Notably, recent laws and regulations encompass not only protection from physical pain, but also suffering from poor environments and lack of fulfilment of behavioural needs. Fulfilling the intention of these regulations requires a more extensive knowledge of specific environmental requirements, behavioural biology and neurophysiology of the animals in concern. COPEWELL has provided new basic knowledge about fish welfare with focus on three of the main farmed fish species in Europe, the Atlantic salmon, European seabass and Gilthead seabream. It contributes to the continued development of European fish farming that can provide attractive jobs in coastal communities and a steady increase in healthy seafood and associated food security for EU citizens.

The COPEWELL project has contributed to strengthen the European Research Community on Fish welfare thereby also supporting the structuring of ERA. This builds on the continued effort of the EU commission to support European projects in the field of fish welfare and fish stress research which includes recent projects like WEALTH, SEAFOOD+, AQUAMAX, FASTFISH, BENEFISH and WELLFISH (Cost Action 867). The partners of the COPEWELL consortium have been central in developing and conducting these projects, including coordination. Moreover, COPEWELL has built upon recent European and national projects to develop genomic resources and tools for amongst others Atlantic salmon, rainbow trout, European seabass and Gilthead seabream as well as zebrafish; it will implement these methods in aquaculture and aquaculture research, including European projects such as STRESSGENES, AQUAFIRST and AQUAGENOME, where several of the COPEWELL partners contributed. Recently, Copewell partners also got an ERANET ANIHWA WINFISH project coordinated by DTU Aqua (also beneficiary in COPEWELL), a Marie-Curie ITN 2016 project, established the Centre for Research-based Innovations in Controlled-environment Aquaculture (CtrlAQUA) where Uni Research leads the Department of Fish Production and Welfare. Built on Copewell results ISPA got research grant from BIAL Foundation, Portugal, on cognitive bias in fish, and IMR the RCN project BEHAVEGENES that look at underpinning genomic and epigenetic mechanisms behind variation in behaviour and coping ability in farmed salmon.

Carrying out this project at the European level has had many advantages. COPEWELL brought together a critical mass of leading European research teams that are at the forefront of fish welfare research, and made them share excellent experimental and unique research facilities. These partners facilitated our unique multidisciplinary approach, from leading experts in fish genomics, neuroendocrinology, stress physiology, fish behaviour, fish experimental biology and aquaculture. The critical mass and diversity of expertise of the consortium would be difficult if not impossible to establish at any national level. The success of the COPEWELL consortium highlights the huge benefits of this approach.

COPEWELL provided an excellent opportunity for exchange of ideas and methods across species and laboratories, both through the cooperative experimental work and publications, several scientific workshops, visits between partners and five annual meeting. This has substantially strengthened the research capacity at both the national and European level beyond the current situation, and many partners have continued the cooperation in new projects. The consortium has involved 13 PhD-students in the project, and facilitated mobility between personnel of the laboratories to exploit specific expertise, and the unique experimental fish holding facilities.

Several of the participating research groups have made unique national resources for fish experimental research available to the broader EU scientific community. This includes highly specialised and world leading fish welfare research facilities at IMR Matre, at NOFIMA Tromsø, at the RUN in Nijmegen, HCMR, Crete, CCMAR, Portugal, DTU, Hirtshals, Denmark, and the IoA Stirling University, Scotland.

To ensure impact on the European aquaculture industry, on aquaculture policy development and future fish welfare research, COPEWELL have disseminated results and conclusions through websites, workshops and continuous dialogue with the aquaculture industry. In 2014 the main results from the project were presented at the European Aquaculture Society conferences in Donostia/San Sebastian, Spain, where a separate COPEWELL session was arranged including 12 oral presentations. In February 2015 COPEWELL was introduced to the Greek fish farming industry in a 2-day course in Fish welfare for aquaculture specialists in Athens.

COPEWELL have provided extensive data on basic questions related to stress coping styles, fish appraisal, allostatic stress regulation and distinction between eustress and distress, and novel knowledge on how stress exposure in early life-stages affects coping and allostatic regulation in later life. This will have major bearings on how to understand and manage fish welfare in aquaculture, both from a governmental perspective and in implementing best practices in the farming industry. The project have also provided new insights on how predictability and controllability of stressors, e.g. by conditioning, can improve how farmed fish handle unavoidable disturbances and handling in aquaculture. The improved basic knowledge on stress physiology and behaviour will be the basis for new operational welfare indicators that can be implemented in aquaculture operations, providing a tool for European fish farmers to protect the fish against harmful situations.

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