Fish welfare and genomics

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Abstract There is a considerable public and scientific debate concerning welfare of fish in aquaculture. In this review, we will consider fish welfare as an integration of physiological, behavioral, and cognitive/emotional responses, all of which are essentially adaptative responses to stressful situations. An overview of fish welfare in this context suggests that understanding will rely on knowledge of all components of allostatic responses to stress and environmental perturbations. The development of genomic technologies provides new approaches to this task, exemplified by how genome-wide analysis of genetic structures and corresponding expression patterns can lead to the discovery of new aspects of adaptative responses. We will illustrate how the genomic approach may give rise to new biomarkers for fish welfare and also increase our understanding of the interaction between physiological, behavioral, and emotional responses. In a first part, we present data on expression of candidate genes selected a priori. This is a common avenue to develop molecular biomarkers capable of diagnosing a stress condition at its earliest onset, in order to allow quick corrective intervention in an aquaculture setting. However, most of these studies address isolated physiological functions and stress responses that may not be truly indicative of animal welfare, and there is only rudimentary understanding of genes related to possible cognitive and emotional responses in fish. We also present an overview on transcriptomic analysis related to the effect of aquaculture stressors, environmental changes (temperature, salinity, hypoxia), or concerning specific behavioral patterns. These studies illustrate the potential of genomic approaches to characterize the complexity of the molecular mechanisms which underlies not only physiological but also behavioral responses in relation to fish welfare. Thirdly, we address proteomic studies on biological responses to stressors such as salinity change and hypoxia. We will also consider proteomic studies developed in mammals in relation to anxiety and depressive status which may lead to new potential candidates in fish. Finally, in the conclusion,

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we will suggest new developments to facilitate an integrated view of fish welfare. This includes use of laser microdissection in the transcriptomic/proteomic studies, development of meta-analysis methods for extracting information from genomic data sets, and implementation of technological advances for high-throughput proteomic studies. Development of these new approaches should be as productive for our understanding of the biological processes underlying fish welfare as it has been for the progress of pathophysiological research.

Introduction: the relationship between stress and animal welfare in aquaculture

Encompassing both an ethical and a factual aspect, animal welfare is a complex subject with prevailing uncertainty in many fundamental areas, including definition and which organisms the concept should apply to (Sandøe and Simonsen 1992; FSBI 2002; Dawkins 2006; Duncan 2006; Fraser 2009). Regarding teleost fishes in particular, there is considerable public and scientific debate whether fishes possess cognitive and emotional capacities that merit welfare concern (Rose 2002, 2007; Braithwaite and Hunting-ford 2004; Chandroo et al. 2004; Huntingford et al. 2006; Ashley 2007; Arlinghaus et al. 2007; Hunting-ford and Kadri 2009). Among the few other terms in biology and biomedicine that have an equally con-flicting history of debate and definition is "stress" (see, e.g., Selye 1950; Lazarus and Launier 1978; Engel 1985; Levine and Ursin 1991; Moberg 2000), a concept is very much related to animal welfare. We will therefore begin this section with a brief clarifi-cation of what meaning could be ascribed to the terms "stress" and "animal welfare."

Fish welfare definition

As outlined in the review from The Fisheries Society of the British Isles (FSBI 2002), one can generally group definitions of animal welfare in three catego-ries: *Feelings-based definitions* refer to subjective

mental states, with good welfare corresponding to states where the animal is both free from negative experiences such as pain or fear, and able to experience positive affective states such as reward (e.g., Duncan 2006). This kind of definition elicits debate, due to the supposition that conscious and subjective components of emotional state are present in animals. *Function-based definitions* involve the animal's ability to adapt to its environment and function well biologically, for instance by showing good health and low stress levels. A much-cited definition put forward by (Broom 1986; Broom and Johnson 1993) falls into this category; here, the welfare of an animal is defined as "*its state as regards its attempts to cope with its environment.*" This definition rests on the assumption that an animal whose physiological systems are functioning poorly is suffering. Finally, *nature-based definitions* rest on the idea that good welfare depends on the animals ability to express its natural behavior, and suffering arises from being prevented from expressing the full repertoire of behavior that is shown in the wild (see, e.g., Kiley-Worthington 1989; Rollin 1993).

The "five freedoms" (Brambell Committee 1965), i.e., freedom from hunger and thirst, discomfort, pain, injury, disease, fear and distress, as well as the freedom to express normal behavior, is a commonly cited framework to assess welfare issues which apply to both state- and nature-based concepts of animal welfare. It is generally considered that this frame-work is also relevant to teleost fishes, but there are some exceptions to consider (Huntingford 2008). For instance, animals living under seasonal conditions may have evolved to exist without food for extended periods. In fishes, reports do not unequivocally document any neurobiological effects associated with starvation alone in northern species (Winberg et al. 1992; Jobling et al. 1999).

Regardless of definition, the concept of animal welfare is based on the belief that animals have subjective emotions and have the ability to feel pleasure and suffer (Dawkins 2006). This view is in contrast to a positivistic and behavioristic scientific view, where science deals only with the material world, and not with emotion. Undoubtedly, our ability to "measure" welfare, beyond objective states of disease and physical injury, is limited. However, even emergent phenomena such as emotion and cognitive processes are rooted in the material world,

specifically in measurable physiological processes creating the communication between networked nerve cells. Current animal welfare research, thus, suffers from a schism between reductionistic studies and anthropogenic and ethical views on animal suffering (Fraser 2009). We will suggest that physiological, behavioral, and cognitive/emotional responses should be seen as adaptive responses to changing situations and environments and develop this argument as a framework for how to include the concept of "animal welfare" as a functional element in biological science.

Fish stress and allostasis

Similarly elusive, but yet very much related to animal welfare, is the concept of stress. Moberg (2000) related a definition that has become somewhat of a consensus, when stating that "stress" is "*the biological response elicited when an individual perceives a threat to its homeostasis.*" This definition encompasses the recent notion that psychological processes associated with how an individual assesses a given situation might be equally important factors as the actual physical challenge in determining the severity of the stress response (reviewed by Von Holst 1998). Korte et al. (2007), however, recently advised on a potential pitfall in the relationship between homeostasis and animal welfare: The concept of homeostasis implies that the purpose of physiological regulation is to maintain physiological parameters at pre-defined settings. However, successful acclimation to changing environments involves significant resetting of neural, endocrine and immune mechanisms. This process is referred to as allostasis, or "*stability through change*" (Sterling and Eyer 1988; McEwen and Wingfield 2003, 2010). Allostasis thus describes regulatory mechanisms mediating change through prediction of activity required to meet new demand. In this concept, impaired welfare arise only when "allostatic overload" arises from chronic, unpredictable, or uncontrollable conditions which do not merit successful allostatic adjustment (McEwen and Stellar 1993; Korte et al. 2007). This realization might clarify some of the confounding and unproductive semantic debate on whether and when stress is relevant for animal welfare.

The concept of allostasis is developed in parallel with stress research taking on an integrative and evolutionary approach, i.e., physiological and behavioral stress reactions are increasingly viewed as adaptive responses that are crucial for survival in a continuously changing environment (e.g., Korte et al. 2005; Romero et al. 2009). Typical proactive responses such as aggression and active avoidance, for instance, are likely to increase fitness when stressors are mild, predictable and of short duration (Wingfield 2003). Under chronic, severe, or unpredictable stress, the organism is better off by reducing risk taking and conserving energy by passive behavioral responses. In line with this evolutionary line of thinking, the functionalist approach to emotions (Frijda 1986) holds that emotions have evolved for a particular function, such as inducing appropriate behavioral responses to potentially dangerous stimuli. It follows that cognitive changes and emotional distress are likely an essential component of passive coping. Therefore, individual variation in the threshold for when a challenge becomes inhibiting rather than stimulatory, i.e., coping style (Koolhaas et al. 1999), is likely correlated to the individual's subjective experience of welfare in a given situation. In this view, welfare is the animal overall subjective experience, a real phenomenon emerging from the brain's motivational and cognitive systems, developed through evolution as an integrated part of the survival mechanisms in animals with advanced central neural systems. This stance circumvents any notion that emotions or affective states are inherently outside the realm than can be subject to the scientific scrutiny; on the contrary, such phenomena are evolved adaptations that play important roles in causing behavior and therefore warrant investigation as biological phenomena.

Coping style and individual variation

Understanding individual differences in coping ability and coping style has indeed become a paramount task in psychiatry and stress research (see, e.g., Susman et al. 1999; Bartolomucci et al. 2005; Korte et al. 2005; Koolhaas 2008). Apart from the now well-established connection between coping and individual health, individual variation in responsiveness is of particular importance for animal welfare (see, e.g., Huntingford and Adams 2005), since conditions that are well tolerated by some individuals may be detrimental to others. Even in fishes, cognitive abilities seem to differ between divergent coping styles, with proactive individuals retaining learned

responses to external cues longer than reactive individuals (Moreira et al. 2004; Ruiz-Gomez et al. 2011). This observation suggests differences in learning and memory consolidation, an observation that is in line with an increasing amount of evidences from mammalian models showing that differences in stress-coping characteristics are associated with divergence in cognitive function (see, e.g., Tomie et al. 2000; Aguilar et al. 2004).

The proximate and ultimate mechanisms involved in maintaining individual variation in stress-coping style are, however, poorly understood in teleost fishes. Both genetic and environmental factors contribute to extensive inter-individual variation in how stress affects individuals (e.g., Øverli et al. 2007; Prunet et al. 2008). A direct link between coping style and variation in gene expression as a response to adaptive physiological changes was recently described in a teleost fish species, the common carp *Cyprinus carpio* (MacKenzie et al. 2009). This study suggests that combining behavioral screening for coping style with gene expression studies provides a powerful approach to exploring the link between gene expression and adaptive change. Reciprocally, failure to take such variation into account severely limits the interpretative value of gene expression and other physiological data (MacKenzie et al. 2009). It should, however, be kept in mind that some trait correlations are flexible and can be dissociated by experience and modulation of the environment (Ruiz-Gomez et al. 2008).

In view of the above, providing tools to reliably identify individuals with contrasting responses to allostatic load, and providing a causal framework for this variation, may considerably underpin efforts to improve animal welfare in aquaculture. Presently, our understanding of welfare in fish species is limited, and further significant progress on these issues will rely on our ability to (1) complete our knowledge on the various disciplinary aspects of the adaptive responses to each specific stressor (2) to aggregate all this information to provide an integrated biological description of the adjustments made by the animal and (3) to consider individual characteristics and responses and not only group analysis.

Genomic and fish welfare

The recent development of genomic technologies provides new approaches to study gene expression analysis and, more importantly, lead us to significantly modify our approach to biological questions. The possibility to simultaneously study the expression of many genes through RNA analysis (transriptome analysis) (Baron et al. 2007) or through protein analysis (proteome analysis) (Becker and Bern 2010) allows genome-wide surveys. These genomic approaches make no assumption about which genes or other expression products are worthy to study and also favor development of system-wide analysis of biological processes, all issues that are important points when dealing with welfare questions. Association of this wide information with specific phenotypes characterized at the individual level is the basic approach developed in such studies which should lead to discovery of new aspects of the adaptative response. In this context, genomic approaches appear to bring original and important perspectives not only for the discovery of new aspects of the adaptative responses to stressors, but also for providing an integrated view of animal welfare. The present review of the main publications related to gene expression and fish welfare will start with analysis of candidate genes and extend to large-scale gene expression studies of transcriptome and proteome. Along this review, we will illustrate how these approaches may give rise to new biomarkers for fish welfare and also increase our understanding of these complex interactions between physiological, behavioral, and emotional responses.

Expression of candidate genes and fish welfare

A first obvious step in the analysis of fish welfare in the genomic context is the study of candidate gene expression. Gene expression regulation (i.e., the modulation of any stage of the process by which cells transform the information coded in their DNA to functions) is a complex mechanism by which cells perform several processes and respond to different physiological, pathological, and environmental stimuli. Therefore, also, a stressful stimulus or a deviation from a welfare status can elicit a modification in the expression of a given set of genes. Such genes can be utilized as biomarkers for the study and for the evaluation of the welfare status of an organism. In this context, in the last years, several fish genes whose expression is modified by stress factors or by

perturbations of the allostatic optimum have been studied mainly with the aim to have molecular biomarkers capable of diagnosing a stress condition at its earliest onset to allow an immediate corrective intervention in an aquaculture facility (Gornati et al. 2005a, b).

The search for these candidate genes can be approached looking for them among those genes whose expression is already known to be modified by stress conditions. In this case, the orthologs of the genes of interest are cloned in each fish species and sequenced in order to obtain specific primers for PCR-based quantification. With this approach, several gene transcripts have been sequenced and tested by semi-quantitative and quantitative PCR. As examples of this approach, we will discuss overcrowding, a stressful condition often encountered in intensive farming and therefore well studied (Santos et al. 2010), and environmental perturbations such as hypoxia and hypercapnia.

In sea bass (*Dicentrarchus labrax*), the expression of genes such as HSP70, CYP4501A, and Metallothioneines is up-regulated following an increase in stoking density (Gornati et al. 2004). The glucocorticoid receptor, whose expression is known to be modified by cortisol (Sathiyaa and Vijayan 2003; Vazzana et al. 2010), is on the other hand down-regulated (Terova et al. 2005). Atlantic cod (*Gadus morhua*) subjected to short-term overcrowding displayed up-regulation of glucose transporter, pro-inflammatory cytokines, and antibacterial genes (Caipang et al. 2008). The increase of glucose transporters occurs in response to the increase of glucose production to meet the stress augmented energy demand as shown in salmonids (Wiseman et al. 2007). Up-regulation of pro-inflammatory cytokines and antibacterial genes are phenomena which set themselves in the frame of a well-known general enhancement of the immune response following short-term stress (Fast et al. 2008).

Aquatic hypoxia is a common event, and fish often have to contend with hypoxic stress in order to survive in environments with low or variable oxygen levels. Abrupt decreases in water oxygen concentrations may occur in intensive fish farming, especially when fish are reared at high densities. Acute and chronic hypoxia dramatically affects mRNA levels of hypoxia inducible factor-1, and this gene has been proposed as a reliable biomarker of exposure (Terova et al. 2008).

A quite subtle threat to fish welfare is hypercapnia (elevated blood CO_2). Hypercapnia often develops under conditions related to intensive aquaculture, and, paradoxically, it occurs frequently in well-oxygenated waters. Rimoldi et al. (2009) have shown that, in sea bass, NHE-1 and c-Fos genes are involved in the response to hypercapnia and that they may be good biomarkers capable of providing useful information about fish welfare.

As an alternative to the candidate gene approach, we can search for genes whose expression was not previously suspected to be modified by a stress condition by means of genomic techniques, such as DNA microarrays or differential display, capable of identifying altered gene expression at the mRNA level.

DNA microarray-based biomarker discovery in fish has been widely used, and this approach allowed for instance to highlight a clear up-regulation of CYP1A enzyme in rainbow trout gill under an oxidative stress condition (Leguen et al. 2010). Using differential display approach, Gornati et al. (2004) selected six genes differentially expressed in sea bass kept at different population densities. One of these genes resulted to be HMG-CoA reductase, a key enzyme of the cholesterol synthesis (Gornati et al. 2005a, b).

With few exceptions such as cortisol receptor expression analyzed in the brain of selected trout line expressing reduced anxiety and enhanced memory retention (Johansen et al. 2011), these genes, and several others activated or inhibited by the most disparate stress conditions, are not utilized to explore a more psychological status taking into consideration individually variable emotional states such as anxiety and discomfort. Brain-derived neurotrophic factor (BDNF), in this respect, could also represent a quite interesting molecule, in fact, it appears to have an essential role in mediating aversive social experiences (Berton et al. 2006), depression, and anxiety (Martinowich et al. 2007). In the zebrafish, although the control of its expression involves multiple tissue and cell-specific promoters (e.g., skeletal muscle) that are differentially regulated (Heinrich 2003), the early onset and cellular sites of its expression suggest that BDNF mainly functions in nervous system and fin development (Hashimoto and Heinrich 1997) acting as a pro-survival primary sensory neuron factor (Diekmann et al. 2009). Very recently, Tognoli

et al. (2010), after providing a comprehensive characterization of BDNF gene in sea bass, have studied its transcriptional translational and post-translational regulation after acute stress, and they observed a significant increase in proBDNF levels and reduction in mature BDNF suggesting altered regulation of proBDNF proteolytic processing. They also showed that the proBDNF/totBDNF ratio is a highly predictive novel quantitative biomarker to detect stress in fishes, a process that is likely to be a central mechanism in adaptation to stress conserved throughout evolution of vertebrates. This study brings the first experimental data indicating involvement of genes regulating behavioral activity in the stress response. However, most of the studies on regulation of gene expression are related to physiological functions and to the analysis of stress response. These physiological descriptors may not be sufficiently reliable when studying chronic stress or animal welfare, and more studies on genes related to emotional responses still need to be developed.

Functional genomics studies and fish welfare

While the analysis of candidate genes has provided useful information on particular mechanisms underlying the physiology of stress response and could lead to description of biomarkers for stress responses, genomic screening approaches using microarray technology have been recently applied to the study of the stress responses in fish (see review Prunet et al. 2008). These new approaches provide a system-wide assessment of alterations of gene expression which should generate both overviews of the biological pathways involved in the stress responses (Cossins et al. 2006). When reviewing the literature related to the genomic analysis of the biological responses to stressors, most of the studies were related to physiological responses, and very limited data have been published on behavioral and social responses. This means that most of the functional genomics studies so far published are related to the stress responses and thus are only partly illustrating fish welfare. However, these studies on the effects of biotic and abiotic stressors related to aquaculture can be reviewed, and analysis of genomic responses will be presented under 3 topics: aquaculture stressors, environmental changes, and responses related to behavior. From

these studies, we will mainly highlight new biological processes and biological pathways, but it is clear that such information may give rise to new molecular biomarkers of stress (see also paragraph 1).

Genomic responses to aquaculture stressors

Aquaculture practices frequently lead to a range of unnatural stressors which are related to rearing practices. These include overcrowding, handling, and transport. Thus, gene expression analysis of a large set of genes in various tissues of rainbow trout *Oncorhynchus mykiss* led to identification of a complex network of genes involved in responses to handling or confinement (Krasnov et al. 2005). This study identified in the brain genes related to binding and transport of metal ions, chaperone and heat shock proteins, cytoskeleton and microtubules, whereas in the kidney genes related to mitochondria functions, extracellular structures and peptidases were highlighted. Liver transcriptome has been also studied, and significant changes in various functions including inflammation (acute phase proteins), immune response, gluconeogenesis and glycogenolysis, energy metabolism, and protein degradation have been observed (Cairns et al. 2008; Wiseman et al. 2007; Mommoda et al. 2007). Interestingly, some of these functions have been also modified in vitro in rainbow trout hepatocytes treated with cortisol, the stress-indicator hormone (Aluru and Vijayan 2007). A similar study carried out in the liver of gilthead sea bream *Sparus aurata* exposed to confinement led to the characterization of four major temporal gene expression profiles that cover rapid metabolic readjusment followed by tissue repair and remodeling processes and finally re-establishment of cellular homeostasis and regulation of the immune system (Calduch-Giner et al. 2010).

Interestingly, gene expression analysis following confinement or handling stress also indicated involvement of cellular mechanisms not previously associated with stress responses. Thus, a transient change in immune-related genes and a prominent acute phase protein, haptoglobin, was observed in rainbow trout liver exposed to confinement (Wiseman et al. 2007; Mommoda et al. 2007; Cairns et al. 2008). Transcriptome analyses are also suited for the identification of new biomarkers of acute or chronic stress responses. This can be found in the gene expression

studies of acute stress responses in rainbow trout and sea bass (Krasnov et al. 2005; Mommoda et al. 2007; Wiseman et al. 2007; Sarropoulou et al. 2005) but appear more clearly in the time-course analysis of confinement stress (acute and chronic) carried out in trout (Cairns et al. 2008).

Despite these transcriptome studies, we are far from having a comprehensive view of the molecular mechanisms that underlie stress responses to a standard stressor such as confinement or handling. Although more genomic analysis should be carried out on a larger variety of tissues, such studies should also be completed by transcriptomic analysis of target cells in primary culture exposed to corticosteroids, the major endocrine factor that is released after exposure to acute stressors. Such in vitro models should help to better distinguish between primary causes and secondary consequences of the stressors.

Genomic responses to environmental stress

Stress in aquaculture may also come from sudden or sufficiently extreme changes of physical characteristics of the aquatic environment. At a cellular level, such environmental stress leads to defensive or immediately adaptive responses followed by a second type of cellular response which restores cellular homeostasis when changes in environmental conditions become permanent (Kültz 2005). Recent genomic studies of environmental stress brought new information on the regulation of the physiological responses to such stressors.

Thus, following exposure to stressful temperature changes, a time-course analysis of gene expression in seven tissues has been carried out in carp and showed a large set of genes that were affected by cold acclimation (Gracey et al. 2004; Gracey 2007). Such studies led to the identification of the major molecular functions that are modified by such stressful situations and highlighted the importance of lipid for a proper function of the central nervous system and the importance of modification of protein folding required for cold acclimation. These studies also highlighted the importance of genes related to thermotolerance and the complexity of the molecular responses to temperature acclimation through several tissues. Another original study was aiming to analyze trancriptome during acclimation to three constant temperatures or to daily fluctuations in killifish

(Podrabsky and Somero 2004). Such situations correspond to natural temperatures to which this species is exposed. The transcriptome analysis in liver revealed effects of temperature on several cellular pathways including the high mobility group B1 (HMGB1) which would act as a compensatory modulator of transcription, thus acting as a temperature sensor for global gene expression. Microarray profiling of the gill transcriptome in zebrafish also revealed that the expression of hmgb1 mRNA was strongly up-regulated by cold treatment along with ionoregulation-related genes (Chou et al. 2008). Short-term effects of temperature on cellular pathways have also been studied in gobies (Gillichthys mirabilis) exposed to ecologically relevant situations (Buckley et al. 2006). This transcriptome analysis indicated that several different cellular mechanisms are regulated by temperature changes including those related to molecular chaperones. Analysis of correlation between gene expression and protein levels indicated that induction of mRNA production is correlated to increase in protein levels with, however, variation in timing and magnitude of the protein increase.

Hypoxia is an important stress factor to which aquacultured fish are exposed. Several transcriptome studies have been devoted to this stressor in different fish species. Thus, study in muscle and liver of gobies (Gracey et al. 2001) suggested a coherent view of the mechanisms activated following hypoxia exposure: A rapid shut-down of the major energy-requiring processes is followed by a strong induction of genes involved in anaerobic ATP production and gluconeogenesis at the level of liver associated with suppression of cell growth and proliferation and with a down-regulation of genes encoding protein involved in the translation machinery. In medaka Oryzia latipes exposed to short-term hypoxia, analysis of gill, liver and brain transcriptomes showed dysregulation of two important cellular functions, ubiquitin–proteasome and phosphatidynositol pathways. Interestingly, such transcriptome analysis of hypoxia carried out in carp by Fraser et al. (2006) allowed the discovery of a myoglobin expression in non-muscle tissues, probably to facilitate oxygen delivery to the tissue when exposed to hypoxia. Such study was a pertinent example to illustrate the perspective offered by such genomic studies, i.e., the discovery of new mechanisms.

Exposure to salinity changes is a frequently studied example of stress situation which led to a wide literature on the osmoregulatory mechanisms developed by euryhaline species to withstand hyper- or hypo-osmotic choc. Numerous studies have identified the major ion transporters involved in freshwater or seawater osmoregulation (see review by Evans et al. 2005), but recent transcriptome analysis brought new information on the molecular mechanisms involved in responses to salinity changes. Thus, an extensive study of the transcriptome in the major osmoregulatory organs (gill, kidney, intestin) and in brain revealed the major cellular processes involved in acclimation of eel Anguilla anguilla to seawater (Kalujnaia et al. 2007). This included functions such as cell protection and immune response, energy metabolism, detoxification, transport functions, structural proteins, regulation of gene transcription and translation. Such study both confirms previous known cellular functions associated with response to hyperosmotic environment and suggests new functions which now need to be confirmed. Using both transcriptomic and proteomic approaches Kültz et al. (2007), identified biological functions, cellular pathways, and molecular functions associated with osmotic stress responses. Kinetic analysis of gene expression changes following transfer to hyperosmotic environment indicated that most of these genes showed rapid and transient increase and have all characteristics of immediate-early genes originally described in neuronal tissues: These genes notably control the transcriptional regulation of effector genes necessary for the adaptation to hyperosmotic medium (Fiol et al. 2006; Kültz et al. 2007; Lanahan and Worley 1998). Protein quantification using specific antibodies allowed to confirm the major roles of several candidate genes such as osmotic stress transcription factor 1 (OSTF1) that is likely to mediate osmotic adaptation by means of transcriptional regulation (Fiol and Kültz 2005).

Genomic responses related to behavior

Understanding the molecular mechanisms underlying fish behavior using genomic approach has been engaged during these last years with the objectives to bring new light on various behaviors as diverse as social dominance, mate choice, reproduction, or migration (see review by St-Cyr and Aubin-Horth

2009). The main way to approach these questions is to associate study of changes in gene expressions with various individual behaviors and to relate these changes to variation at other phenotypic levels. Such approach has produced interesting information on dominance associated with maturation in the Daffodil, an African cichlid fish (Neolamprogus pulcher) where high expression of AVT in the brain was associated with dominance in females and males (Aubin-Horth et al. 2007). Moreover, this study also suggests that dominant females had masculinized brains at the molecular level and that large molecular changes occurred in the brain of females with a gain of social dominance rank. A similar study was also carried out in another African cichlid fish, the Tilapia burtoni (Astatotilapia burtoni) to study gene expression in dominant and subordinate males as well as in brooding females (Renn et al. 2004). Candidate genes as well as specific functional gene ontology (GO) categories associated with dominance or reproductive state have been revealed by such study which also highlighted high degree of individual variation. In rainbow trout, Sneddon et al. (2005) applied a microarray approach to study gene expression profiles in the brain of fish expressing divergent social status. The results show that the expression of as many as 1,165 genes differed between fish of different social status. Given the link between social rank and food intake, feed conversion ratio, and growth rate, it is no surprising that the majority of genes found to be differentially expressed in that study were related to processes like protein turnover and metabolism. However, a number of genes related to stress responses and behavior, which could serve as candidates for future studies, were also identified (Sneddon et al. 2005).

Despite these genomic studies on fish behavior, very limited information has been gained by such approaches on fish welfare. However, Reilly et al. (2008) studied gene expression changed in the forebrain of rainbow trout and carp exposed to nociceptive stimulus. Among the numerous genes that are differentially regulated in the two species in response to this stimulus, several genes are involved in mammalian nociception, but new candidates are also identified. In particular, the Van Gogh-like 2 (vangl2) gene, a member of the non-canonical Wnt signaling/planar cell polarity pathway, was identified, and further studies confirm its involvement in nociceptive processing in the adult fish brain (Reilly et al. 2009) where

it participates in mechanism that integrates signaling between the facial branchiomotor neuron and its environment, at least during neuron migration (Sitt-aramane et al. 2009). Interestingly, *vangl2* has also been shown to govern the zebra fish hair-cell planar polarization during development and regeneration (López-Schier and Hudspeth 2006), the orientation of which ultimately determines the sensitivity of hair cells to directional mechanical stressors. Recently, analysis of brain transcript expression in dominant, subdominant and submissive trout showed large differences and experimentally induced transitions from subdominant to dominant position also led to significant changes (Sneddon et al. 2011). Finally, that study also revealed the important role of brain ependymin in the control of aggressive behavior in trout.

Altogether, these first genomic studies illustrated the potential of genomic approach to characterize the complexity of the molecular mechanisms that underlie behavioral response in relation to fish welfare. These genomic approaches are just starting and combination of behavioral and physiological studies should be very helpful for characterizing welfare state in fish exposed to challenging situation.

Proteomic studies of fish welfare

Critical evaluation of current proteomic studies of fish welfare

Although a great deal of knowledge has been gained by transcriptomic studies, they do not provide information about either the subcellular localization of gene products or post-translational protein modifications that may be essential for its function, transport, or activation. Additionally, mRNA abundance and protein levels are not necessarily correlated (Gygi et al. 1999). To this end, the discipline of proteomics has been initiated to often complement functional genomic research. Proteomics, the study of global changes in proteins, can be defined as the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes. Proteomics has emerged as a field in which technology is being developed to enable proteins to be analyzed in a high-throughput, automated way similar to how genes are being studied. This technique can thus provide a critical link between the transcriptome and

the metabolome, the latter of which is the end result of transcriptional and proteomic activity. Up to now, very few studies have been performed regarding proteomic investigations aiming to evaluate fish welfare. Hereafter, we critically discuss on the results obtained by current proteomic analysis of fish physiology that can be related to welfare.

Salinity exposure

For this stressor analyzed on fish cells in primary culture, proteomic analysis has led to unexpected results for which further confirmation could be needed. Smith et al. (2005) investigated the in vitro effects of hypo-osmotic environment on the resistance of rainbow trout gill epithelium. Primary cultures of gill cells were exposed to simultaneous contact with apical hypotonic and basolateral isotonic solutions. Five proteins were positively modulated in response to hypo-osmotic environment, and only one of them was correctly identified as pre-Apolipoprotein A-I (ApoA-I), which in mammals is the major protein component of high-density lipoprotein (HDL) in plasma notably promoting cholesterol efflux from tissues to the liver for excretion. In fish, although it seems to have crucial role during embryonic development (Babin et al. 1997), the function of this HDL protein in relation to gill resistance is, however, difficult to interpret. In fish, it has been observed that HDL constitutes one of the most abundant plasma proteins. It has been observed that ApoA-I is involved in the regulation of fish complement and displays antimicrobial activity in the micromolar range against gram positive and gram negative bacteria (Villarroel et al. 2007). Evidence also accumulated that ApoA-I is involved in diverse protective roles in mammals including anti-athero-sclerotic, anti-oxidant, anti-inflammatory, and anti-thrombotic activities (Villarroel et al. 2007). In order to test the physiological relevance of such result, Smith et al. (2005) conducted additional experiment during which trout gill cells were treated with a dose of human ApoA-1. It was found that trans-epithelial resistance increased consequently, thus indicating that ApoA-I may really be involved in such process.

Hypoxic conditions

Zhang et al. (2009) reviewed genomic and proteomic studies associated with hypoxic conditions in fish. To

date, two proteomic approaches have been applied to study the effects of hypoxia in fish, especially on zebrafish and medaka species. For the zebrafish study, proteomic analysis of skeletal muscle revealed that one protein decreased while five proteins increased in response to hypoxia (Bosworth et al. 2005). However, due to their respective low abundance, none of them were identified with MS (MALDI-TOF). It must be stated here that 2D-PAGE coupled with silver staining methods has some limitations such as low sensitivity, reduced dynamic range, and gel to gel variability. Regarding evaluation of medaka brain proteome, it has been observed that some proteins were up-regulated following exposure to hypoxia: two hemoglobin β-subunits, four carbonic anydrase forms, calbindin, aldolase, sucinate dehydrogenase, and gluthatione-S-transferase (Oehlers et al. 2007). From previous studies, all these proteins have been associated with hypoxia/ischemia in other animals, representing good source of potential hypoxia markers.

In conclusion, few studies have indirectly evaluated fish welfare through assessment of exposure to stressors so that more investigations are required to go further in the mechanisms involved in such processes. Proteomic may be an interesting technique to highlight without a priori potential biomarkers that could be related to animal welfare as some identified proteins were proven in relation with stimuli-specific response pathways. It is also true that proteomic often reveals non-specific and ubiquitous stress proteins. But these may also be informative as response of animals to stress involved both cellular stress response (CSR) and cellular homeostasis response (CHR) (Kültz 2005). CSR is characteristic of all cells and involves proteins that are conserved in all organisms. These proteins share their responsiveness to diverse stresses probably arising from the most striking and common impact of stress: damages to macromolecules, membranes, lipids, proteins, and DNA (Kültz 2005). Some specificity may arise because the type of lesions and damages somewhat depends on the type of stress. In that way, CHR is aimed to restore homeostasis with specific regard to the particular environmental variable that has changed as it is not triggered by macromolecular damage or oxidative burst (such as for CSR) but by stressor-specific sensors. Discrimination between markers of general stress syndrome and more specific markers of fish welfare (that can be subsequently used as operation welfare indicators—OWI) is also needed,

but this problem can be highlighted for all techniques used.

Proteomic studies of mammal welfare and possible extrapolation to fish

As afore mentioned, proteomic studies on fish welfare are very scarce, but, based on several comparative studies, it can be assumed that data obtained from mammals may be partly extrapolated to fish or at least may turn research toward similar fish molecules, pathways, or systems. Actually, comparative approaches of animal physiology afford a better understanding of general molecules, functions, and pathways (Singer 2003; Takei et al. 2006). Likewise, Owen et al. (2007) argued that gathering experimental data in fish toxicology may be done more efficiently by considering mammalian data. Therefore, we suggest that similar comparative approaches can be carried out with regard to stress and welfare issue in fish species. Proteomic studies conducted on mammal species may also be an interesting source of information to reveal potential indicators of fish welfare as some modulated and identified proteins were proven in relation with the imposed stimuli. However, information in various mammalian models is scarce, and studies especially focus on rodent models and particularly on the brain proteome response in relation to anxiety and depressive status. In this context, extrapolation of the data from rodents to fish should be carried out with caution particularly when dealing with proteomic studies on specific brain regions as fish do not have the same brain structure and functionality as in mammals (Chandroo et al. 2004; Ashley 2007). Hereafter, the current proteomic studies more or less directly related to animal welfare are summarized, followed by a critical discussion on possible data extrapolation from mammals to fish.

Major depression is a disorder associated with increased morbidity, and it is suggested that such disease may be derived from genetic, biochemical, psychological, and environmental elements. Evidence suggests that adverse events in life such as stress or trauma may predispose individuals to develop depression in later life. In addition, elevation of cortisol has been reported for 43% of patients with major depression. Skynner et al. (2006) used subcutaneous implants of corticosterone pellets to mimic such depression situation in mice and evaluate its potential effects on brain areas with 2D-DIGE technique and subsequent MALDI-TOF analysis. A total of 150 protein spots

were altered, and 117 spots were identified, corresponding to 51 different proteins. Proteins were implicated in alteration of cell morphology and death, glycolysis, and gluconeogenesis as well as changes in phenylalanine, glutamate, and nitrogen metabolism.

Constitutive stress response leads to a number of psychiatric disorders, including depression, Alzheimer's disease, post-traumatic stress, and anxiety disorders. Kim and Kim (2007) exposed some rats to a restraint stress and compared their brain proteome response to a control un-stressed group. It appeared that one interesting protein, the hippocampal cholinergic neuro-stimulating peptide precursor protein (HCNP-pp), was down-regulated in stressed rats. This result was also confirmed by HCNP-pp mRNA analysis. However, it is mentioned that further studies are needed to provide more significant information about correlations between HCNP-pp and neurogenerative diseases caused by stress.

Mammalian hippocampus plays critical roles in memory, learning, neuro-plasticity, and emotion. Increasing evidence suggests that hippocampus and particularly decrease in neurogenesis plays a role in major depression disorder (Mu et al. 2007). In animal model, elevated glucocorticoid levels associated with major depression disease negatively regulate neurogenesis, reduce the levels of neurothrophins, and cause excitotoxic damage in the hippocampus. In this way, Mu et al. (2007) examined the alterations of protein expression patterns in the hippocampus of rats depressed by submission to unpredictable chronic stressors. Expression of 27 proteins was altered, and identified proteins ($n = 15$) play a role in neurogenesis, oxidative metabolism, transcription, and signal transduction. One interesting protein, mitogen-activated protein kinase (MAPK), appeared to be down-regulated in depressed rats MAPK plays a pivotal role in the development of the nervous system by mediating neurogenesis and neuronal differentiation. Another study aimed to identify the molecular changes induced in rat hippocampus by repeated exposure to psychosocial stress (social defeat stress against dominant male rat) with proteomic approach (Carboni et al. 2006). A total of 69 spots were differentially expressed, and 21 were unambiguously identified. Proteins were related to chaperoning, signal transduction, synaptic plasticity, cytoskeleton regulation, and energy metabolism. Several proteins have already been associated with stress-related responses while some of them were described for the first time in relation to stress.

Further investigation is needed to elucidate these pathways and identified novel biomarkers in fishes. It should, however, be mentioned that the fish telencephalon contains areas homologous to the mammalian hippocampus (Northcutt 2006; Vargas et al. 2009; Broglio et al. 2010), and studies of neurogenesis and neural plasticity in fish suggest that the underlying molecular mechanism for negative affective states (recognized as clinical depression in humans) is conserved by evolution. For instance, brain cell proliferation was found to be reduced in the telencephali of rainbow trout exposed to chronic social stress (Sørensen et al. 2011). Also similar to mammals, a higher rate of telencephalic cell proliferation was demonstrated in zebrafish kept in enriched aquaria compared to conspecifics kept in a barren environment (von Krogh et al. 2010).

All in all, these aforesaid studies illustrate the interest of proteins characterized in mammals as potential candidates in fish which would deserve detailed analysis. Indeed, given the compelling molecules and systems similarities among the vertebrate group, it can be supposed that data from mammals can be somewhat inferred based on fish results and vice versa. Consequently and with some caution, mammal comparative approaches may be beneficial for highlighting the main mechanisms and potentially new biomarkers that can be exploited toward the assessment of fish welfare.

Perspectives: what future for analyzing genomic data to characterize fish welfare?

In the introduction, we have proposed to associate the definition of fish welfare with adaptive physiological, behavioral, and cognitives/emotional responses to stressful situations and define the concept of welfare as a functional integration of these elements. According to these definitions, keys for understanding fish welfare will rely on our knowledge of all components of allostatic responses to environmental perturbations. As a consequence, analyses of these responses to stress have been difficult to dissect because they require the activation of multiple genes and pathways.

Genomics technologies have emerged during the past decade and are useful in addressing, in an integrated fashion, the complexity of the studied biological mechanisms. Thus, development of such genomic approaches within the field of fish welfare illustrated in the present review by the genome-wide analysis of genes and their expression led to the discovery of new aspects of adaptative responses. This led to the discovery of new candidate genes that are part of the biological responses to stressful situations but, also to highlight specific functional gene ontology (GO) categories corresponding to major molecular processes (Ashburner et al. 2000). Taking into account the growing number of stress-related studies, it can be stated that genomic information will quickly offer a large overview on expression of many genes associated with these adaptative responses within which the role of defined biological pathways or processes and their underlying regulation can be interpreted. Finally, those studies have also yielded a raft of new promising findings that highlight the complex relationship between behavioral and physiological responses when analyzed at the individual level (MacKenzie et al. 2009).

However, this overview also indicates that a majority of the genomic information is targeted toward specific aspects of the physiological or behavioral response to stress and gives only a fragmented picture of fish welfare, i.e., the complexity related to the biological integration of a multitude of separate responses is usually not addressed. Comparative-genomics by pooling results over a growing body of studies analyzing transcriptional response to stress may help to identify common (and specific) response(s) across fish species. Additionally, this field is now opened for another round of significant advances as integrative-genomics research begins to incorporate protein and metabolite profiles and their dynamic changes as well as the effects of DNA polymorphisms. In this context, several new developments should be encouraged to improve our ability to have an integrated view of fish welfare. Thus, the following points should be considered:

(1) One of the first practical issues to be resolved for productive use of transcriptomic or proteomic studies is the use of laser microdissection laser that would allow gene expression analysis at the level of a few cells (Reilly et al. 2004).

This dissection technique allows isolation of small population of homogenous cells among a heterogeneous tissue. This might be a significant problem with genomic analysis of gene expression in multi-functional tissues such as brain or gill where it is quite important to have the gene expression signature of only the cells associated with specific functions. This approach has already been producing interesting data in the analysis of fish reproduction and immune response (Haugarvoll et al. 2008; Jørgensen et al. 2009), and a detailed functional genomic analysis of specific area of the brain in relation to specific physiological or behavioral responses to stressors should be of major interest for understanding fish welfare.

(2) A quick look at the list of published functional genomic studies related to fish welfare indicates that, within a near future, it should be possible to have access to numerous sets of transcriptome data characterizing various aspects of physiological, behavioral, and cognitives/emotional responses to stressful situations. This will represent a very interesting opportunity for extracting genomic-relevant information using meta-analysis methods (Moreau et al. 2003) and for improving our understanding of fish welfare. Thanks to the existence of gene homology between species (Baron et al. 2011), such meta-analysis analysis would include not only integration of multiple microarray analysis applied to specific situation in a fish species but also comparisons between different species as demonstrated in mammals by several independent studies (see for instance Chalmel et al. 2007). By using relevant hierarchical clustering methods (Eisen et al. 1998), one should be able to detect within different sets of genomic data clusters of genes showing correlated expression. Carrying out such analysis within several sets of experimental data coming from the same species or from inter-species comparisons (Chalmel et al. 2007) will give rise to robust clusters of genes and GO functional categories and also to new genes that would not be detected in individual experiments. Such an approach has already been used in medical research and for example allowed identification of sets of genes involved in tumor development and metastasis

processes common to different cancers (Segal et al. 2005). In the meta-analysis of genomic data from several primary solid tumors, a new gene expression signature associated to metastasis has been proposed by Ramaswamy et al. (2003). An interesting objective of these meta-analyses is also the identification of bio-markers specific for different biological processes. This would be very interesting for characterizing common responses to different stressful situations, and this approach has been successfully applied to the characterization of a pronostic meta-signature for breast cancer (Shen et al. 2004; Hughes et al. 2000) In addition to specific genes, meta-analysis can also improve our understanding of the functional role of the genes involved in a specific response. Thus, in yeast, using more than 400 genomic analysis of numerous treatments or mutations, Hughes et al. (2000) have been able to identify the biological functions of unknown genes by comparing their expression profiles with cluster of genes involved in known physiological processes. Another benefit of the meta-analysis is the possibility to dissect complex genomic signatures obtained in tissues containing a mixture of different cell types. The characterization of these different cell populations through their respective genomic signature is possible by comparison with gene expression data obtained with isolated cells. This approach that is called "virtual microdissection" has been used by Alizadeh et al. (2000) for the identification of 2 new subtypes of lymphoma within the diffuse large B-cell lymphoma that was previously considered as homogenous. Such kind of analysis can also be achieved by integration of numerous datasets related to the same biological process as demonstrated for the signatures of the immune response by Shaffer et al. (2001). Finally, identification of specific signature and genes co-regulation (Tavazoie et al. 1999) give rise to the characterization of transcriptional regulation network which could be further validated by ChIP-chip studies (see, e.g., Blais et al. 2005; Ren et al. 2000; Bartel 2004).

(3) Such integrative studies may also be performed for proteome data in near future. Indeed, high-throughput proteomics is a rapidly developing field that offers the global profiling of proteins from a biological system. Numerous novel and powerful technologies have been developed around mass spectrometry and proteomics over the past 20 years to globally study complex proteomes (e.g., plasma). These high-throughput technological advances are fueling a revolution in biology, enabling analyses at the scale of entire systems (e.g., whole cells, tissues, or environmental communities) so that as for transcriptomics, large amount of data will be soon available for the scientific community. The same can be noticed for metabolomics (Blow 2008; Dunn et al. 2011; Weckwerth 2010) that is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind. Besides, in view of the rapid progress in these techniques, exploring fish welfare integrating the different approaches (e.g., both transcriptomics and proteomics) may be an added/complementary value to better understand stressor impact on large collections of tissues. Finally, welfare in fish can also be considered as a multifactorial problem. In addition to environmental factors, genetic factors are also to largely contribute to extensive inter-individual variation in how stress affects individuals (Øverli et al. 2005; Prunet et al. 2008). To this end, it would be invaluable to integrate genotyping data to transcriptome data in order to facilitate the discovery of genes affecting stress susceptibility (Jansen and Nap 2001) because genes affected by a same polymorphism locus are likely to share similar biological function. A more integrative-genomics view will combine the power of genetics, transcriptomics, and proteomics in a way that is likely to become instrumental in the further unraveling of metabolic, regulatory and developmental pathways relevant to welfare in fish.

In conclusion, development of these new approaches in the study of fish welfare using genomic tools should be as productive for our understanding of the biological processes underlying the responses to stressful situation as it has been for the progress of pathophysiological research.

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