ABSTRACT

Fish welfare, suffering and the perception of pain were debated, together with several factors reducing *infra vitam* welfare of farmed fish (genetic, environment, density, malnutrition, starvation, cataracts, deformities, vaccination side effects, transport, handling, confinement, crowding, harvesting, killing method). Behavioural and physiological stress responses were considered as indicators of welfare reduction. The effects of pre-slaughter management practices, and the most commonly used stunning/slaughtering methods on welfare and quality reduction of farmed fish were discussed. A number of indicators can be used to assess fish welfare-suffering, both in a scientific and practical context, such as behavioural, haematic, cellular, tissue *post mortem* fish stress and quality indicators, but none of them are optimal. The best strategy for a reliable assessment of fish welfare/suffering and their impact on product quality is a multidisciplinary approach that takes into account animal behaviour and the different biochemical and physiological *ante mortem* and *post mortem* processes involved: several components, all influenced in a similar way by the same condition, suggest real welfare and quality reduction.

Key words: Farmed fish, Welfare, Stress indicators, Welfare reduction, Quality indicators.

RIASSUNTO

**Valutazione del benessere/sofferenza del pesce allevato e impatto sulla qualità del prodotto**

Benessere, sofferenza e percezione del dolore sono stati discussi insieme a diversi fattori che possono ridurre il benessere *infra vitam* del pesce allevato (genetica, ambiente, densità, malnutrizione, digiuno, cataratta, deformità, vaccinazione, trasporto, manipolazione, confinamento, affollamento, raccolta, metodo di uccisione). Le risposte comportamentali e fisiologiche agli stressors rappresentano indicatori di riduzione del benessere nel pesce. Sono stati esaminati gli effetti delle pratiche prima della macellazione e dei metodi più comunemente usati per stordire/uccidere il pesce sulla riduzione del benessere alla morte e della qualità del prodotto finale. Numerosi indicatori possono essere utilizzati per valutare il benessere/sofferenza del pesce, sia in un contesto scientifico che pratico, ma nessuno di loro è perfetto. La migliore strategia per una valutazione affidabile dello stato di benessere/sofferenza del pesce e del suo impatto...
sulla qualità del prodotto è un approccio multidisciplinare che prenda in considerazione il comportamento animale ed i diversi processi biochimici e fisiologici ante mortem e post mortem implicati: diversi componenti influenzati tutti in modo simile dalla stessa condizione suggeriscono una reale riduzione di benessere e di qualità.

Parole chiave: Pesce allevato, Benessere, Indicatori di stress, Riduzione di benessere, Indicatori di qualità.

Introduction

The concept of fish welfare is rather complex as for the other animals. Huntingford (2002) chose the welfare definition related to aquaculture as the opposite of sufferance, where sufferance is the complex of negative physical, physiological and behavioural conditions, and, over all, the individual subjective experience of the animal in these conditions. Recently Huntingford et al. (2006), in an exhaustive review on the current issue in fish welfare, discussed welfare as the absence of suffering. In any case, it can be stated that 1) different definitions of welfare are neither correct nor incorrect: they each focus on different issues relating to animal welfare and 2) depending on the species, the factors impacting on the way we think of and define welfare can be very different.

Farmed fish welfare was not considered as important as that of other farmed vertebrates (ex hot-blooded animals) because of the lack of tradition in considering fish as sentient animals. Fish do not evoke compassion/preoccupation in humans, as in other taxa, as they are not able to emit sounds when stressed. Moreover, there is yet no agreement among researchers on fish capability of pain/suffering perception. The FAWC report (1996) suggested that the principles of “humane” slaughter, already applied to farmed birds and mammals, should be applied also to fish, to avoid unnecessary stress and pain to the animal during the slaughtering process. Ethical aspects regarding food production, seafood production included, are gaining increasing importance. The problem in the fish sector emerged mostly for aquaculture where the industrialized rearing system on a large scale is relatively recent. Marine fisheries have in fact reached a ceiling in terms of output, while the increase in aquaculture has confirmed an increasing role in fisheries supply. According to the last FAO data, in 2004 aquaculture accounted for more than 43% of the total world food fish supply (106 million tonnes) from capture fisheries and aquaculture, versus the 36% of four years earlier (FAO, 2006). Nowadays the Organic Certification Disciplinary of several organizations (IFOAM - AIAB, I - BIO GRO, NZ - DEBIO, N - CC REPAB, F) also includes a welfare recommendation. Some fish producer associations have also developed accreditation schemes including fish welfare as a criterion (www.britishtrout.co.uk; www.scottishsalmon.co.uk).

The “five freedoms” postulated by the Brambell Committee Report in 1965 to render acceptable the animal rearing system in relation to welfare, could be applied also to farmed fish (FAWC, 1996). The first freedom could be applied to fish with the provision of an adequate and nutritionally complete diet according to the species and age requirements, with the starvation before slaughter and transport as short as possible. The second freedom could be applied to fish by supplying different species with adequate water quality parameters (Dissolved Oxygen, NH₃, pH, contaminants), flow rates and temperatures, appropriate light intensities and other needs. The third freedom could be applied to the fish by avoiding injuries,
through careful and gentle procedures, and preventing infections and diseases, through good sanitary conditions and eventually vaccination, avoiding malformation. The fourth freedom - to express normal behaviour by providing sufficient space, proper facilities and company of the animal’s own kind - suit the fish without any change. In Table 1 values range of welfare requirements for water quality parameters, nutrition and fish density of the most common farmed species are reported to give some reference point. The fifth freedom - from fear and distress by ensuring living conditions which avoid physical and mental suffering - could be applied to fish by ensuring gentle and adequate handling procedures, stunning procedures before slaughter for the animal immediate insensitivity and human slaughter methods. The latter - freedom from mental and physical sufferance in animals different from humans - cannot yet be applied to fish, because of the lack of research and scientific evidence in this area. The central nervous system of mammals and poikilothermic vertebrates are very different (Rose, 2002), but higher level cognitive ability cannot be inferred from neuro-anatomy alone. Even if less well developed than in other farmed animals (neo cortex lack), other parts of fish

Table 1. Values range of welfare requirements for water quality parameters, nutrition and stocking density of the most common farmed species.

| Parameter | Atlantic salmon | Rainbow trout | European eel | Common Carp | Sea bass | Sea bream |
|-----------|-----------------|---------------|--------------|-------------|---------|---------|
| Water quality: |
| Temperature | 10-18°C | 12-17°C | 22-26°C | 25-30°C | 11-28°C | 13-30°C |
| Salinity (mg/l) | <40 | <40 | <40 | <40 | <40 | <40 |
| Oxygen | 6.0-7.0 | >5.0 | >4.0 | >3.0 | >5.0 | >5.0 |
| CO2 | <10 | <10 | <6.0 | <6.0 | <35 | <35 |
| pH | 6.5-8.5 | 6.5-8.5 | 6.0-9.0 | 6.0-9.0 | 7.5-8.3 | 7.5-8.3 |
| N-NO3 (mg/l) | <3.0 | <3.0 | <3.0 | <3.0 | <3.0 | <3.0 |
| P-PO4 (mg/l) | <3.0 | <3.0 | <3.0 | <3.0 | <3.0 | <3.0 |
| Diet: |
| Crude Protein (%) | 45-50 | 43-47 | 45-48 | 31-38 | 45-50 | 40-45 |
| PD/ED (mg/kj) | 17-20 | 17-19 | >20 | 17-20 | 18-22 | 18-20 |
| Essential fatty acids | C18:3 n-3 | C18:3 n-3 | C18:2n-6; C18:3n-3 | C18:2n-6; C18:3n-3 | EPA | EPA |
| +DHA | +DHA |
| Requirements (% diet) | 1.0-2.5 | 0.8-1.7 | 0.5; 0.5 | 1.0; 0.5-1.0 | 1.0 | 1.0 |
| Stocking density (kg/m³) | 22 | 25 | <25 | <25 | 14-25 | 14-25 |
brain are as well developed and are used to produce complex behaviour: fish have sense organs able to detect noxious stimuli, sensory pathways for processing such stimuli and brain mechanisms that process this information and generate behavioural responses to positive and negative conditions (Kestin, 1994; Wendelaar Bonga, 1997; FSBI, 2002; Huntingford, 2002). In particular, Southgate and Wall (2001) and Sneddon et al. (2003) demonstrated the presence of nociceptors (capable of sensing noxious stimuli) in fish. On the other hand, Rose (2002) argued that nociceptive-based behaviour of fish can occur in the absence of pain and that these behaviours are not dissimilar to the adaptable reflex behaviour of all the animals, including protozoa, which simply avoid noxious stimuli. So the evidence for the cognitive ability of fish to interpret noxious stimuli like pain remains equivocal and subjective (Rose, 2003; Braithwaite and Huntingford, 2004). However, as underlined by Rose (2003) “the improbability that fish can experience pain, in no way diminishes our responsibility for concerns about their welfare. Fish are capable of robust, unconscious, behavioural, physiological and hormonal responses to stressors, which, if sufficiently intense or sustained, can be detrimental to their health”.

Factors reducing welfare of farmed fish

To better approach the factors reducing welfare of farmed fish, it has to be remembered that they are ectothermic animals, therefore they do not need to use energy to maintain a high body temperature and that the effects of the forced starvation are less marked than in mammals and birds. On the other hand, fish are in close contact with their environment through the huge superficial area presented by their gills, therefore they are particularly vulnerable to the poor quality and pollution of the water where they are living. It has to be remembered that also wild fish experience a variety of adverse conditions such as predator attack, injury, limited feed availability, parasite infestations and illness, natural changes in the environment. A wild fish welfare assessment can be a sensible index of the quality of the water they live in. However fish possess mechanisms (behaviour and stress response) to face these adverse natural situations, mechanisms which are carried out also during their interaction with humans.

Several conditions and activities used during aquaculture practices cause stress (species and context dependent, chronic or acute) and can involve a reduction of welfare. Some aspects of aquaculture practice impacting on fish welfare were reviewed by Conte (2004). The mainly relevant factors for the welfare reduction of farmed fish can be listed as following:

- Genetic factors
- Environmental factors
- Stocking density during growth
- Malnutrition
- Starvation
- Cataracts
- Deformities
- Vaccination-side effects
- Transport
- Handling, selection, overcrowding
- Harvesting management
- Slaughtering methods

Genetic factors

Great genetic variability exists in fish within species and it is possible to select and breed for faster growth, earlier age of sexual maturation, disease resistance and flesh quality. While genetic selection for high productivity may create serious welfare problems and undesirable side effects, the selection for higher stress resistance may diminish problems of welfare reduc-
tion. High responding (HR) and low responding (LR) lines of rainbow trout have been generated by selection for high or low cortisol response to a standard confinement stressor (Pottinger, 2003). LR also showed a quicker resumption of feeding when placed in a novel environment (Overli et al., 2004), grew larger when maintained in a mixed groups (Pottinger and Carrick, 2001) and were more aggressive and competitive than HR fish. It will be interesting to understand whether it is possible to select for stress responsiveness alone or as a part of a more complex pattern. In their exhaustive review on the evaluation of animal welfare by the exploration of the hypothalamic-pituitary-adrenal function (HPA) Mormède et al. (2007) also considered the specific features of hypothalamic-pituitary-interrenal cells axis function (HPI) in fish. The authors concluded that the use of HPI axis functioning as a tool to evaluate fish welfare is possible for genetic selection for stress resistance in fish, after a proper validation for each species and each type of stressor.

Environmental factors

Fish are in constant interaction with their environment through the gills and skin, therefore water quality (Dissolved Oxygen, salinity, NH₃, nitrites, pH, temperature, pollutants levels), is crucial for their welfare. In particular welfare has to be preserved by a constant and careful monitoring of the quantity and quality of water reserve and by the chemical, physical and microbiological parameters. These should be optimised by considering individual species requirements and development levels, in reference to the environment where the rearing farm is inserted. Dissolved oxygen exerts a determinant role in fish welfare and health maintenance. Different variables interact: undisturbed salmonids use 300 mg of oxygen per kg of fish per hour, but if they are disturbed the oxygen consumption may double. Low oxygen levels negatively influenced sea bass immunoglobulin levels (Scapigliati et al., 1999). The low water temperature over the winter periods were related to some winter bacterial diseases suffered by a number of different warm species. In particular research on gilthead sea bream (Sparus aurata) indicated an important role played by a severe immunosuppression in winter months (Tort et al., 1998a, 1998b). Light levels and periods can also be important welfare factors. Continuous light promotes increased growth in many species, but Atlantic salmon avoid bright light at the water surface except when feeding (Juell et al., 2003). Monitoring of fish behaviour and water quality would be the way to maintain good welfare conditions.

Stocking density during growth

Increasing fish densities means increasing the number and the types of interaction among fish. Because the biology of individual species is complex, the identification of a correct density is a challenging task: it is necessary to take into account age, size, environmental conditions and biological traits of the species. Fish density and water replacement have to be selected according to water resources management and to ensure the welfare of the cultured species, avoiding the excessive competition for feed and territory and aggressive behaviour. Poor welfare due to a given stocking density can be shown in one flow regime but not under other conditions (Ellis et al., 2002). Sea bass show high stress levels at high density (Vazzana et al., 2002). An intermediate level of density tends to increase growth efficiency and to decrease disease incidence. Several studies have shown a decrease of welfare in salmon and trout reared at very high densities but it is not clear if this is the result of poor water quality, of the high level of ag-
gressiveness, of simple physical damage or of other processes (Ewing and Ewing, 1995; Ellis et al., 2002). Fin damage or erosion can also occur as a result of aggressive interactions, which may increase susceptibility to a secondary infection (Turnbull et al., 1996; Ellis, 2002). North et al. (2006) reported that stocking density of 80 kg m\(^{-3}\) did not produce consistent effects on rainbow trout mean growth rate or physiological indicators of welfare, but fin erosion increased with increasing density (10, 40, 80 kg m\(^{-3}\)), although the cause of the erosion remains unclear. Fin damage in rainbow trout has been recently highlighted both as a welfare and quality issue, because it reduces the economic value of farmed fish (Hoyle et al., 2007). However, in the case of species living in large groups, a very low density could be more stressful than a high one. Fish density could also influence gene expression: genes coding for heat shock proteins are over-expressed in sea bass held at high densities (Gornati et al., 2004) and the enolase gene is up-regulated in sea bream held at high densities (Ribas et al., 2004). It is very hard to propose within species the maximum stocking density for all situations, so it has been suggested to study the specific and dynamic components of behaviour, water quality, health, stress physiology and gene expression (Ellis et al., 2002; Turnbull et al., 2005). Stocking density, the nature and level of food availability and social behavior act together to affect welfare (Ashley, 2007).

**Malnutrition**

Formulation of diet can have positive or negative effects on fish health and welfare. Fish meal and oil are the essential components of feed used in aquaculture to satisfy the essential fatty acids and amino acids requirements of reared fish. In fact they are characterised by high protein levels (Table 1), correct presence of the essential amino acids and high levels of C20: 5 n-3 EPA and C22: 6 n-3 DHA fatty acids. Following species diet requirements closely leads to high resistance towards stress and disease, can lower potential environmental impact and can improve the quality of the final product. Insufficient levels of high polyunsaturated fatty acids (HUFA) have a negative impact on the immune system and reproductive functions. However there are differences among species. Freshwater fish mainly need linoleic (C18:2 n-6) and linolenic (C18:3 n-3) acids as HUFA precursors while marine species need minimum levels (0.5-1% in diet) of n-3 ecosapentaenoic (EPA C20:5) and docosaoenoic (DHA C22:6) fatty acids (Table 1). The problem is that fish meal and fish oil are a limited resource, also due the fact that marine fisheries have reached a ceiling and their supply is constantly and markedly decreasing. Therefore it is necessary to find sustainable alternative protein and oil resources to be used in aquaculture. The most studied sources are plant derived. The problem is that plant sources often show unsuitable contents of amino acids and essential fatty acids. Moreover they often have anti-nutritional factors, themselves a problem for fish welfare. In fact they cause decreased nutrient digestion and absorbance efficiency and this, in turn, can cause severe pathologies and malfunctions in the digestive system, cataract manifestation, and malformations, thus decreasing fish welfare. However, the large number of experiments already carried out in the sector seems to confirm that diets with 30-50% substitution of fish meal with raw plant material (ex. Soya bean meal or mixtures of different plant sources), when corrected for the shortage of essential amino acids and fatty acids and checked for the absence of anti nutritional factors, are well tolerated in term of fish performances (Kaushik et al., 1995; Médale et al., 1998). Too high substitution levels, on the contrary,
could still cause alterations in protein and lipid metabolism, influencing health, stress resistance and product quality and causing malnutrition to the fish (De Francesco et al., 2004; Parisi et al., 2004). Juvenile gilthead sea bream (Sparus aurata) fed a Vitamin E deficient diet showed faster elevation of plasma cortisol levels in response to stress and a lower survival rate than control fish (Montero et al., 2001).

**Starvation**

Fish starving is a practice used before the transport to reduce metabolic rate, stress response, oxygen consumption and production of discard products. Fish starving is also used before harvesting at slaughter to eliminate the intestinal content and reduce contamination risk. Starving can be also a natural behaviour in fish according to the water temperature, the age, the species and the season (over winter). However, feed deprivation during not natural periods can bring to a reduction of welfare. Moreover, starving should not be longer than necessary. In Atlantic salmon, plasma glucose increased after 7 days of starving while other welfare indices were not influenced but when starved for long periods (up to 86 d) fish clearly lost weight and body condition, even this was stabilized after 30 days (Einen et al., 1998).

**Cataracts**

Factors related to the cataracts formation can include environmental conditions (temperature), nutrition (deficiency in histidine, methionine, zinc), toxins, genetic predisposition, parasites, tissue and skeletal deformity. Direct consequence of cataracts are changes in behaviour and decrease in growth, due to the difficulties in feeding. These factors can even lead to mortality (Wall, 1998). Cataracts have a negative impact on economy and quality, because they ultimately result in a considerable reduction in fillet quality, to the extent where it loses all its commercial value.

**Deformities (skeletal and tissue)**

Skeletal deformities have a negative impact both on welfare and on product value and quality, due to fish growth impairment, high mortality level and reduced fillet quality. Most farmed species present vertebral column (lordosis), head, mouth and opercular deformations. Factors responsible for the induction of malformations are generally genetic: fish from families showing a high incidence of deformities should not be used for breeding (Gjerde et al., 2005). Also environmental factors such as water temperature, Dissolved Oxygen, stream speed, light intensity, salinity or malnutrition - lower nutritive level in HUFA, Amino Acids, vitamin C and E or excess in vitamin A - especially during fast-growth periods, can induce deformities. Moreover, apparently, healthy fish of large size show problems in coping with normal aquaculture procedures, like transport, handling and grading, due to abnormally small and shaped hearts. A combination of sedentary lifestyle and breeding programs that do not take into account organ functioning could be the cause of this malformation.

**Vaccination – side effects**

Vaccination programmes have contributed significantly to decrease the losses due to disease outbreaks in fish farming and represent the major factor for the enormous reduction in antibiotics used in aquaculture. On the other hand, the more efficient vaccinations - the intra peritoneum with oil adjuvant - may present important side effects, such as appetite loss, tissue adhesions around the injection site, pigmentation and intra peritoneum granuloma (Midtlyng, 1997; Sorum and Damsgård, 2004). Severe lesions can disturb and interfere with the
normal functions of the affected organs, resulting in reduced growth and in worsening of animal welfare.

**Transport**

Transport generally induces stress that requires prolonged recovery times (Iversen et al., 1998; Sandodden et al., 2001; Chandroo et al., 2005). Moreover, the transport of living fish often implies high densities, after gathering and netting or pumping procedures. During transport there could also be an inadequate water exchange with depleting of dissolved oxygen and accumulating of CO₂ and NH₃. All these factors contribute to a significant increase of stress condition and to impairment of fish welfare. A water temperature decrease during transport is useful to induce a sedation effect. The Scientific Panel on Animal Health and Animal Welfare (March 2004) gave a number of recommendations to assure animal welfare during transport:

- sufficient available dissolved oxygen
- fish air exposition avoiding during the loading and unloading procedures
- starving time before transport adequate to the species, size and water temperature
- vehicle well equipped and assuring minimum contact with the animals
- adequate monitoring of water quality and fish condition.

**Handling, selection, confinement and overcrowding**

The physical disturbance and confinement evokes stress responses (Pickering, 1998), cortisol and glucose increases and can alter immunological activity disease resistance in several farmed species. Crowded fish are more sensitive to an additional acute stressor (Ruane et al., 2002). Good crowding management includes a slow and gentle technique, assessment of water quality, the addition of oxygen to the water if levels fall below a critical 6 ppm and the close monitoring of the fish behaviour and activity (HSA, 2005).

**Harvesting**

The interest in fish welfare, including the time of slaughter, is nowadays increasing both at consumer and at producer levels and it is important both from an ethical and from a product quality point of view. The pre-mortem procedures are critical for animal welfare and must be carried out in a adequate way, avoiding unnecessary pain, fear and sufferance. A correct feeding strategy before harvesting that imply a starving period of about 2-3 days is considered a priority. Fish density must be compatible with not too stressing situations. With regards to the quality of water a high concentration of dissolved oxygen combined with an average not high temperature is essential. After transport to the slaughter area, a resting period has to be allowed, when density, constant temperature and oxygen supply must be under control. It is important to avoid excessive and prolonged crowding before killing, but the density at capture generally reaches 70-100 kg m⁻³ and interferes in a complex way with other factors such as water quality; mainly in relation to the hypoxic condition due to overcrowding (Reddy and Leatherland, 1998; Wall, 2001). The harvesting process (length, intense handling, crowding during most of the catching protocols and related decrease of oxygen) could be a very traumatic time for the farmed fish, involving the onset of a stress status which can compromise the organoleptic, merchantable and sanitary quality of the final product. The stress and exercise before death are linked to a reduction of flesh quality and this is associated mainly with the pre-slaughter distress. In fact, the relative endocrine responses imply alterations before death.
starting processes of recall and intense consumption of glucose reserves which cause modifications of normal post mortem processes and higher susceptibility to microbial attack. Stress during harvesting and death times plus relative endocrine response can hardly influence post mortem biochemical processes such as the ATP degradation rate, rigor mortis onset and release, freshness involution rate. The result is that capture and slaughter phases management can significantly influence fish quality expression and relative changes along with preservation and shelf life. On the other hand it is difficult to distinguish the real “resting period before death” (Jerrett et al., 1996; Milligan, 1996; Pottinger, 2001).

Slaughtering management

The killing method has to be chosen according to fish species aiming to stun the fish until death with avoidable stress throughout all the operations based on practical issues. Killing methods that provoke a long agony in fish are very stressing and must be avoided. Stressors intensity and lasting determine the level of the acute stress exposure at death time. From the fish welfare point of view, the killing method should be fast, efficient in getting fish stunned and slaughtered without avoidable stress and pain (Southgate and Wall, 2001; HSA, 2005). The killing method can also be considered suitable if it is able to cause gradual unconsciousness without pain and stress. Most killing methods used for farmed fish were developed to meet merchantable needs, for easy operations, suitable quality maintenance and low costs. In fact until 1996 (FAWC, 1996) no particular concern about fish welfare at death was considered. It is true that all killing methods are stressful, but some more than others. Many of the methods used at present at European levels are quite unsatisfactory. There can be fast or slow stunning/slaughtering methods. Fast methods are fast in getting insensibility and unconsciousness: knocking, shot, spiking, coring, ike-jime, electric stunning. They are less aversive both for the animals and for the product quality, but cannot be used for lots of small-sized fish. Slow methods are slow in producing insensibility and unconsciousness: asphyxia in air, in ice, in water and ice, bleeding, gutting, anaesthetic, narcosis in CO₂, electrocution. They are generally more stressful and some of them, as has been suggested, should be avoided.

The Opinion of the EFSA Scientific Panel on Animal Health and Welfare (EFSA, 2004), on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals, acknowledged that many existing commercial killing methods expose fish to substantial suffering over a prolonged period of time and recommend that criteria for human application of percussive stunning, spiking and electrical stunning should be made available to the industry and in any case it should be mandatory that stunning/killing step is incorporated before exsanguinations or any processing of fish commences e.g. gutting, desliming, etc... More recently, the EC Council Decision (of the 5 December 2005 and legally adopted as from June 2006) extends animal welfare regulations to the EU fish-farming sector (these developments could eventually influence imported fish). Its article 19 (emergency killing) acknowledges that the killing methods to be used depend on farming system, on the species, on the size and on the number of fish to be killed and that the need for rapid killing of large batches of fish should be also considered. In any case, the methods used should either: cause immediate death or rapidly render the fish insensitive until death, or cause the death of fish which has been anaesthetised or effec-
tively stunned. Indicators of effectiveness of the procedures can be 1) the immediate and irreversible cessation of respiratory movements (rhythmic opercular activity) and 2) the immediate and irreversible loss of eye-roll (vestibule-ocular reflex –VOR), that is the movements of the eye when the fish is rocked from side to side (in a stunned/dead fish the eye does not move). The last article of the EC Council’s Decision begins explaining that this recommendation shall be reviewed within five years of coming into force and ends saying that it shall be completed by an appendix providing a description of certain emergency killing methods “as soon as adequate scientific knowledge, or practical experience, is available”. The lack of annexes is surely related to the numerable assumptions that still now are not based on scientific evidence and are therefore contentious. So it is clear that empirical and scientific research in this area is lacking. There is a need for an increase in scientific knowledge on which to base future guidelines and potential legislation.

**Behavioural and physiological stress responses as indicators of welfare reduction in fish**

Stress can generally be defined as the condition in which the dynamic balance of an animal organism, the so-called homeostasis, is threatened or troubled as a consequence of an intrinsic or external factor, commonly defined the stressor (Chrousos and Gold, 1992; Pickering and Pottinger, 1995; Wandelaar Bonga, 1997). As in other vertebrates, fish stress is not necessarily considered detrimental: it could be merely a form of an adaptive response to the stressor and it could be considered a condition of eustress (good stress), which may even be regarded as beneficial and stimulating because it prepares fish for the activity (Moberg, 1999) or, on the contrary, a condition of distress (bad stress), a response to more threatening challenges which may lead even to a pathological state (Wandelaar Bonga, 1997). The magnitude and the type of behavioural and physiological response to stress as a coping strategy can vary among fish species, but also at strain and individual levels (Pottinger and Pickering, 1997; Schjolden et al., 2005). After an attack by another fish of the same species, fish may flee and hide or take up a submissive posture, often with an altered body colour (Sutor and Huntingford, 2002). After an attack by a predator, fish may respond by shoaling (Pitcher and Parrish, 1993), freezing (Goodey and Liley, 1985) or taking shelter (Brown and Warburton, 1999), may change colour (Endler, 1986) and then may avoid areas in which they have been attacked (Lima, 1998). Fish stress physiology is related to the endocrine responses to stressors directly comparable to that of superior vertebrates (Sumpter, 1997; Wandelaar Bonga, 1997), while there is not enough knowledge as regards to the emotional implications of stressors in fish that could be important and critical to create a link between physiological stress and sufferance. Physiological stress responses can be divided into primary, secondary and tertiary responses. The primary responses to stress imply 1) the rapid release of catecholamines (adrenaline and noradrenaline) from the chromaffin tissue (homologous to the mammalian adrenal medulla) and 2) the release of the hypothalamic releasing factor of the pituitary adrenocorticotrophic hormone which, in turn, promotes the secretion of cortisol by interrenal tissue (homologous to the mammalian adrenal cortex) (Sumpter, 1997). The secondary responses are characterised by the immediate reactions to those hormones at haematic and tissue level. These adaptive responses to short term (acute) stress could not cause particular sufferance and need some hours
to recover (Pickering et al., 1982). The tertiary responses determined by prolonged (chronic) stress could, on the contrary, induce a significant reduction of animal welfare and cause long term responses influencing in a negative way, appetite, growth, reproduction and immune responses (Pickering and Pottinger, 1989). Stress responses, as a natural animal reaction to a risk factor for survival, are often used as indicators of welfare reduction. However it is important to recognise that physiological stress is not a synonym of sufferance. The monitoring of stress and its effects at a physiological level could contribute to a part of the general picture of fish welfare. The main stress indicators could be useful to signal the risk, in this way allowing a quick intervention to prevent the most severe consequences of distress. Though generally considered “inferior” animals, fish are very complex cold blooded vertebrates. Recent empirical studies show that painful stimuli are strongly aversive to fish and that fish can experience fear-like states and avoid situations in which they have experienced adverse conditions. There are analogies with mammals in the basal structure of neurons and of neuro-hormonal biochemistry, in stress responses and in behaviour, which seem to indicate that also fish are able to feel pain and sufferance, mainly at the end of their life, when they are harvested and slaughtered (Kestin, 1994; Verheijen and Flight, 1997; Lambooj et al., 2002b; Braithwaite and Huntingford 2004; Chandoo et al., 2004). The behavioural responses, altered to maintain the homeostasis in the same way as the physiological ones, are an important part of the stress response because they allow the animal to avoid or to overcome the stressor (destabilizing stimulus). An altered behaviour is an easily observable response which produces immediate information on physical and biochemical changes taking place in response to stress, with no need for the use of invasive tools. Specific behavioural responses could be used as indicators of stressing conditions able to endanger welfare.

**Fish welfare-suffering, stress and quality indicators**

Indicators can be used to assess fish welfare-suffering, stress and product quality both in a scientific and in a practical context, but none of them are perfect. Fish stress during rearing would cause short time physiological changes (acute stress), mainly due to the action of some hormones (catecholamine and cortisol) and long time related physiological changes (chronic stress conditions). Indicators of welfare reduction during rearing in response to stress condition are: behaviour and physiological parameters, related to the struggle undertaken by the fish to maintain their homeostasis, in addition to their general condition (scales loss, damaged fin, growth and reproduction worsening, immunity defence and health). Oxidative stress as a welfare index could also be used as a new approach to fish quality evaluation (Bagni et al., 2007). Cells also can react to severe stress by synthesis of particular proteins, called “Heat Shock Proteins” (HSPs) which have been described in all organisms (Feder and Hofmann, 1999), including fish (Iwama et al., 1998). They represent fundamental molecules in some aspects of protein metabolism in order to maintain cell homeostasis (Fink and Goto, 1998). Moreover, cellular biomarkers of genetic expression modification, such as up regulation of particular genes, can be used as indexes to diagnose stress related genes and improve welfare in the future (Gornati et al., 2004; Ribas et al., 2004). When slaughtered, the key issue in fish welfare/suffering evaluation is mainly animal suffering. This can be evalu-
imated by acute stress responses and related changes in physical parameters measured after death. It has to be underlined that, even if the physiological stress expression is not synonymous with suffering (Dawkins, 1998), the stress response monitoring may give part of the complex outline of animal welfare. In particular, several stress aspects, all influenced in a similar way by the same condition, will suggest real welfare reduction and be a cause of concern about fish welfare (FSBI, 2002). For example, Turbull et al., 2005 used PCA to integrate four commonly used indicators of fish welfare reflecting different functional systems (body and fin condition and glucose and cortisol plasma concentration) into a single welfare score for farmed Atlantic Salmon. This score had a significant and negative relationship with fish stocking density and cage position. The best strategy for a reliable assessment of fish welfare /suffering and their impact on product quality is a multidisciplinary approach that takes into account the main relative changes of significant indicators of behaviour, of the biochemical and physiological ante mortem and/or post mortem processes involved and of the quality changes (Poli et al., 2005).

**Behaviour indicators**

Often animals respond rapidly to the environment and changes in social relations so behaviour can be a good early and non-invasive indicator of fish welfare/suffering, even if this can rarely be an exhaustive approach. A general way to assess welfare reduction could be the comparison between normal behaviour and induced one. The behavioural responses that demonstrate anxiety and fear can be evaluated by freezing (in the presence of a predator) or, on the contrary, by struggle, attempt to escape, muscular spasms, pupil dilatation, external stimuli reaction, etc.

During rearing the general visible changes that can be observed are the following:
- *skin or eye colour change,* due to a complex neural and hormonal background following social stress or subordinate status (Sutor and Huntingford, 2002);
- *respiratory frequency increase,* due to a higher oxygen demand following to stress or exposure to pollutants (Handy and Depledge, 1999);
- *cutaneous mucus, wounds, fin damage presence* due to adverse event;
- *body condition loss,* due to loss of weight, change of shape and illness (reduced feeding and mobilisation of reserves are secondary responses to stress);
- *speed and direction swimming changes* and excessive activity, different body positions, attempt to escape or immobility, due to adverse conditions (Morton, 1990; Kristiansen et al., 2004);
- *loss of appetite and slow growth,* due to acute or chronic stress such as reduced dissolved oxygen in rearing water (Kramer, 1987).

At slaughter the most frequent observations which indicate self initiated changes in behaviour with different strength and persistence are:
- *swimming motility;*
- *gill movement.*

Fish also give responses to external stimuli (Marx et al., 1997; Tobiassen and Sørensen, 1999; Van der Vis et al., 2001) such as:
- *capability to maintain the equilibrium* when fish is turned upside down;
- *movement of eye* following changes in body postures in the longitudinal axis;
- *reaction to the needle puncture* on head or tail;
- *handling along the lateral line;*
- *electricity application* of low voltage electricity.

Fish behaviour at slaughter changes and gives macroscopic indication of the presence
or absence of consciousness. However these indicators only supply a rough estimate of the stress suffered by the animal. In fact, a paralysis can be obtained without unconsciousness (ex. electrical stunning) (Close et al., 1996; Lambooij et al., 2002a, 2002b). Objective methods able to indicate the presence and the duration of a sensitive state of consciousness exists but they are generally of difficult execution, such as the Electroencephalogram (EEG), which requires the positioning in fish of 4 electrodes, the Electrocardiogram (ECG), 2 electrodes plus a ground electrode, the Visual Evoked Responses (VERs), with a light flashed directly on the eyes and the Somatosensory Evoked Responses (SERs), (Kestin et al., 1991; Kestin et al., 1995; Robb et al., 2000; Van der Vis et al., 2001; Lambooij et al., 2002a, 2002b; Robb and Roth, 2003; Van der Vis et al., 2003). However, more simply, behaviour can in part indicate if the fish is conscious or not, by autonomous behaviour such as branchial movements and vestibule-ocular reflex (VORs), the latter being the movements of the eye when the fish is rocked from side to side (in a stunned/dead fish the eye does not move). Their absence indicate unconscious and insensible fish.

Haematic indicators

The evaluation of the physiological reactions to the stressors in fish during rearing and at death is related to primary response to stress, i.e. to the above mentioned immediate massive release of catecolamines from chromaffin cells and the gradual release of cortisol from interrenal tissue into the blood stream, followed by the switch of metabolism towards a catabolic trend. Catecolamins levels are not used as stress indicators, because they are not easy to determine and quickly disappear from the blood (Wendelaar Bonga, 1997), while the increase in plasmatic cortisol production has been widely employed both as a short term and a long term stress condition index. Also the effects of the high levels of cortisol, represented by blood cell numbers, glycaemia and plasmatic lactate level modifications could be used as stress indicators. However it should be underlined that haematic parameters are not always ideal as stress markers because the blood sampling activity itself can be source of stress. It is important to have a suitable premises and equipment and well trained technicians in order to avoid the induction of stress in animals when taking blood samples.

Cortisol

Plasmatic levels of cortisol increase quickly after exposure to an acute stress and the standard conditions are restored in few hours. When the stress event becomes chronic, the cortisol levels can be maintained at a high value for days or weeks, even if they are gradually decreasing (Wendelaar Bonga, 1997). Cortisol (evaluated by RIA) is widely used both as a long term and as a short term stress condition index, even if it may be influenced by species, feeding, reproductive cycles, seasonal cycles, photoperiod, husbandry condition and sampling (Pickering and Pottinger, 1985; Audet et al., 1986; Pickering et al., 1987; Thorpe et al., 1987; Lowe et al., 1993; Vijayan and Moon, 1994; Pickering and Pottinger, 1995; Reddy et al., 1995; Wendelaar Bonga, 1997; Barton, 2002). Multiple stress condition seems to amplify the cortisol response (Ortuño et al., 2002). However because blood sampling itself can be stressful, it could be also useful to test cortisol in less invasive alternative biological matrixes such as in mucus and faeces, especially during rearing (Oliveira et al., 1999; Turner et al., 2003; Bertotto et al., 2007).

Glucose

The secondary response involve disorders of the metabolism due to the endocrine re-
responses and are an increase in heart-beat, higher oxygen intake, energy source mobilisation and increase of plasma glucose. This last is easy to determine (colorimetric method) so it is frequently used as a stress indicator, although some authors have found a delay in its release (Barry et al., 1993).

**Lactic acid**

Higher energy mobilization and utilisation due to the increase in muscular activity, imply anaerobic glicolysis in white muscle which imply a related increase of lactic acid in blood (colorimetric method). The plasma lactate increase is also used as a stress index (Lowe et al., 1993; Erikson et al., 1999), even if most fish store lactate in muscle tissue. Sea bass killed by chilling in iced water had lower plasma glucose and lactate levels and showed less marked behavioural responses than those killed by other methods, in particular by asphyxia and electro-stunning (Skjervold et al., 2001; Poli et al., 2002, 2004).

**Haematocrit**

The increase in heart-beat and the need for a higher oxygen intake cause an increase in the number of moving erythrocytes and of haematocrit value. This is also used as stress index because it is very simple to determine, even if standard values have to be validated for each species before they can be correctly used (Reddy and Leatherland, 1998).

**Free fatty acids**

Energy source mobilisation includes the fatty reserve, so changes in plasma free fatty acids could be a stress condition index, but these parameters generally did not show a clear response and they are seldom used.

**Reactive oxygen metabolites (ROMs) and antioxidant power (AOP)**

Stress could also expose fish flesh to possible oxidation of PUFAs which can result in the production of reactive oxygen metabolites (ROMs), which, in turn, can induce severe alteration in nucleic acid, protein and lipids (Tappel, 1973; Halliwell and Gutteridge, 1984). The ROMs production can be counteracted by an adaptive response such as the activation of the endogenous detoxification pattern in terms of anti-oxidant power mechanism (AOP). The determination of oxidative stress by Reactive Oxygen Metabolites (ROMs) and AOP aims to detect the early products of oxidation (hydro-peroxides) due to the presence of reactive oxygen species and the correspondent failure of the anti-oxidant power mechanism. Micro methods based on the Fenton reaction can be used for the oxidative stress assessment (Alberti et al., 1999).

In a research on the effect of routine stressful pre-slaughter and killing procedures on sea bass and sea bream, Bagni et al. (2007) reported ROMs and AOP values were relatively low, in comparison to other farmed (non aquatic) animals previously tested by the same methods (Brambilla et al., 2002). However, in sea bream ROMs values were higher than in sea bass and in both species a negative correlation between ROMs and AOP was observed in crowded fish, whereas a positive correlation was recorded in non crowded fish. Animals in good welfare conditions usually show a proportional and positive AOP response to the ROMs release, while animals forced to cope with a prolonged oxidative stress show a non proportional and positive AOP response and animals with a major impairment show a negative correlation.

**Cell indicators HSP70 and HSP90**

HSP family are constitutive and useful to assist the folding of nascent polypeptide chains, to mediate the repair and degradation of altered or denatured proteins, to sup-
port various components of the cytoskeleton, enzymes and steroid hormone receptors. All these proteins are also “induced” in cells in response to a variety of stressors and enhance survival by protecting vital cellular functions, especially from insults that affect protein machinery. The evaluation of the expression of constitutive and inducible forms of HSPs in conditions such as overcrowding and selection, which represent the important sources of stress during rearing, could be an innovative way to measure fish stress condition. The expression of constitutive and inducible forms can be evaluated for HSP70 and HSP90 by means of molecular biology as well as immuno histochemistry. RT-PCR and immuno histochemistry (specific antibodies) can be used in order to examine the marker tissues that represent the samples in which inducible forms are highly expressed (stress condition). A quantitative real time-PCR approach, could be used on the marker tissues to obtain the expression of inducible form in relation to different stress conditions (overcrowding, selection and oxygenation) (Iwama et al., 1998).

**Tissue post mortem fish stress and quality indicators**

Pre-slaughter and slaughter stressful practices can have an important effect on the flesh quality in fish as in mammals and poultry. Improvements in killing methods offer a chance to better the quality of the final product. In fact less stressful pre-slaughter practices and killing methods produce less intense physical exercise in fish and reduce the stress response, in this way minimising changes in the normal post mortem processes and development and involution of quality traits. Severe stress due to the pre-slaughter practices can be so aversive to the fish that it masks the benefits of good slaughter practices. Slaughter methods mostly have an effect on the physical properties of flesh. Stressful pre-slaughter and killing methods exhaust muscular energies, produce lactic acid, reduce muscular pH and increase the rate of rigor mortis onset (Poli et al., 2005).

**Lactic acid and pH**

The vigorous swimming during crowding implies an intense use of white muscles and will increase muscle lactic acid production and lowering of pH, due to the related anaerobic glycolysis. When fish are slaughtered in a short time the recovery is not possible and the muscle pH will remain low and will be further decreased by the post mortem glycolytic activity. The marked muscle acid lactic increases and the pH decreases within the first day after death are generally good early stress and muscular activity indices (Oka et al., 1990; Lowe et al., 1993; Marx et al., 1997; Robb and Warris, 1997). Sometimes there is an absence of correlation between lactic acid and pH because glycolysis is not the only source of energy and there is an accumulation of H⁺ from alternative metabolic patterns. From the third day of storage to the end of shelf life differences are generally less marked (Azam et al., 1989; Lowe et al., 1993; Marx et al., 1997; Robb and Warris, 1997; Sigholt et al., 1997; Clarke, 1999; Marx et al., 2000; Ottera et al., 2001; Ruff et al., 2002).

**ATP, ATP/IMP, AEC, K value**

Stress effect from pre slaughtering and slaughtering distress can be also identified by the concentration of muscle phosphocreatine and ATP, ADP, AMP, ATP/IMP, inosine (HxR) and hypoxanthine (Hx). Another way to express the cellular energy charge could be the AEC (Adenilate Energy Charge)=(0.5ADP+ATP)/(AMP+ADP+ATP). All these indices can be used as early stress indices. During the whole shelf life the K values could be used = [(Hx+HxR)/(Hx+HxR+IMP+AMP+ADP+ATP)]*100.
Rigor mortis phases
Together with the gradual depletion of the ATP reserves there is the gradual onset of rigor mortis. Therefore the measure of the rigor mortis onset rate in the first hours after death and particularly the length of the pre rigor phase can give good information on the stress status of the fish before death, together with important information from a commercial point of view. On the contrary with reduced pre-slaughter stress and activity, fish will take longer to go into rigor giving the possibility of handling the fish while being processed and packed before they enter into rigor, increasing the yield of the fillets and reducing the damage of the flesh.

The stress related post mortem changes can have significant negative effects on flesh quality, and in particular on the keeping quality of fish. So most of the stress indexes can be also quality indexes. In fact many quality traits can change as affected by conditions at slaughter time (severity of the pre-slaughter and slaughter stresses) and during storage (handling and storage temperatures). The relative quality changes can be indicated by:

- **fish and fillet appearance**: physical injuries, flesh gaping and colour;
- **technological properties of the fish and fillet**: rigor evolution, texture (firmness, cohesiveness, elasticity), water holding capacity and fillet shrinkage, rigor mortis onset and texture in particular are important for flesh processing;
- **freshness indicators**: dielectric properties or impedance, K value, and spoilage indicators such as biogenic amines, lipid oxidation products such as malonaldehyde;
- **sensory qualities**: raw fish (appearance of skin, rigor status, eye, gills colour, smell, mucus, condition of flesh), the shelf life and, even if less frequent, cooked fillets (texture, taste, flavour, odour).

Minimising pre-slaughter stress and the use of humane slaughter methods also improves product quality (Southgate and Wall, 2001; Skjervold et al., 2001; Robb and Kestin, 2002; Poli et al., 2005)

Conclusions
Like other farmed animals, good fish rearing practices and welfare, both during rearing and at slaughtering, have to be guaranteed, according to the last European regulations. Even if it is improbable that fish are able to feel pain in the same way as humans or mammalian animals, this does not decrease in any way the responsibility that we have to worry about their welfare. Fish are able of strong and unconscious behavioural, physiological and hormonal responses to the stressor which, if intense and lasting enough, can be detrimental for their health. Therefore the aim to reach should be to minimise and keep under control the infra vitam, pre-slaughter and slaughter stress. An optimal slaughter method should render fish unconscious until death without avoidable excitement, pain or suffering prior to killing. Further research is required for more humane and less stressful slaughter practices. It is important to study methods that would be useful when the achievement of instantaneous induction of insensibility is not possible, the objective being that the animal should be rendered unconscious and insensitive until death. In particular, the improvement of pre-slaughter and slaughter management has to be achieved not only from an ethical point of view, but also because there is a close relationship between fish welfare, even at death, and quality of the final product. Slaughtering methods can be evaluated by the measuring of stress, condition of uncociousness, insensibility...
and trauma. A longer period of time before rigor onset and resolution from pre-slaughter cooling gives a potential for filleting pre-rigor and has the added benefit of improving flesh quality. The aquaculture industry can then provide high quality fish to processors and consumers by using improved pre-slaughter handling and slaughter methods. No single index is perfect in fish welfare or sufferance evaluation, therefore the best strategy is to use as many methods as possible, both in scientific and in practical contexts and to try to focalise on the most significant results.

REFERENCES

Alberti, A., Bolognini, L., Macianfelli, D., Cerratelli, M., 1999. The radical caption of N,N-dieethyl-para-phenylenediamine: a possible indicator of oxidative stress in biological samples. Res. Chem. Intermediat. 26:253-267.

Ashley, P.J., 2007. Fish welfare: Current issue in aquaculture. Appl. Anim. Behav. Sci. 104:199-235.

Audet, C., Fitzgerald, G.J., Guderley, H., 1986. Photoperiod effects on plasma cortisol levels in Gasterosteus aculeatus. Gen. Comp. Endocr. 61:76-81.

Azam, K., Mackie, M., Smith, J., 1989. The effect of slaughter method on the quality of rainbow trout (Salmo gairdneri) during storage on ice. Int. J. Food Sci. Tech. 24:69-79.

Bagi, N., Civitareale, C., Prioro, A., Ballerini, A., Finoia, M., Brambilla, G., Marino, G., 2007. Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (Dicentrarchus labrax) and sea bream (Sparus aurata). Aquaculture 263:52-60.

Barry, T.P., Lapp, A.F., Kayes, T.B., Malison, J.A., 1993. Validation of a micro titre plate ELISA for measuring cortisol in fish and comparison of stress responses of rainbow trout (Oncorhynchus mykiss) and lake trout (Salvelinus namaycush). Aquaculture 117:351-363.

Barton, B.A., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 42:517-525.

Berton, D., Poltronieri, C., Radaelli, G., Negrato, E., Simoncetti, C., Poli, B.M., Tibaldi, E., 2007. Welfare and quality of farmed trout fed high plant protein diets. 3. Alternative indicators to evaluate stress in fish. Ital. J. Anim. Sci. 6 (Suppl.1):789 (abstr.).

Braithwaite, V.A., Huntingford, F.A., 2004. Fish and welfare: do fish have capacity for pain perception and suffering? Anim. Welfare 13:587-592.

Brambilla Committee, 1965. Report of the Technical Committee to enquire into the welfare of animals kept under intensive husbandry systems. Command report 2836. Her Majesty’s Stationery Office, London, UK.

Brambilla, G., Civitareale, C., Ballerini, A., Fiori, M., Amadori, M., Archetti, L.I., Regini, M., Betti, M., 2002. Response to oxidative stress a welfare parameter in swine. Redox. Rep. 7(3):159-163.

Brown, C., Warburton, K., 1999. Social mechanism enhance escape responses in shoals of rainbow fish (Melanotaenia duboulayi). Environ. Biol. Fishes 56:455-459.

Chandroo, K.P., Cooke, S.J., Mckinley, R.S., Moccia, R.D., 2005. Use of electromyogram telemetry to assess the behavioural and energetic responses of rainbow trout, Oncorhynchus mykiss to transportation stress. Aquacult. Res. 36:1226-1238.

Chandroo, K.P., Duncan, I.J.H., Moccia, R.D., 2004. Can fish suffer? Perspectives on sentience, pain, fear and stress. Appl. Anim. Behav. Sci. 86:225-250.

Chrousos, K.P., Gold, P.W., 1992. The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. J. Am. Med. Assoc. 267:1244-1252.

Clarke, J., 1999. The Effects of slaughter method on rigor mortis development in farmed turbot (Scophtalmus maximus, Rafinesque, 1810). Degree Diss., National University of Ireland College Cork, Cork, Ireland.

Close, B., Banister, K., Baums, V., Bernoth, E-M., Bromage, N., Bunyan, J., Erhardt, W., Flecknell,
P., Gregory, N., Hackbart, H., Morton, D., Warwick, C., 1996. Recommendations for euthanasia of experimental animals: Part 1. Lab. Anim. 30:293-316.

Conte, F.S., 2004. Stress and the welfare of cultured fish. Appl. Anim. Behav. Sci. 86:205-223.

Dawkins, M.S., 1998. Evolution and animal welfare. Q. Rev. Biol. 73:305-328.

De Francesco, M., Parisi, G., Medale, F., Lupi, P., Kaushik, S.J., Poli, B.M., 2004. Effect of long term feeding with a plant protein mixture based on growth and body/fillet quality traits of large size rainbow trout (Oncorhynchus mykiss). Aquaculture 236:413-429.

Einen, O., Waagan, B., Thamassam, M.S., 1998. Starvation prior to slaughter in Atlantic salmon (Salmo salar). Effects on weight loss, body shape, slaughter- and fillet-yield, proximate and fatty acids composition. Aquaculture 166:85-104.

Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationship between density and welfare in farmed rainbow trout. J. Fish Biol. 61:493-531.

Endler, J.A., 1986. Defence against predators. In: M.E. Feder and G.V. Lauder (eds.) Predator-Prey Relationship. University of Chicago Press, Chicago, IL, USA, pp 109-134.

Erikson, U., Sigholt, T., Rustad, T., Einarsdottir, I.E., Jorgensen, L., 1999. Contribution of bleeding to total handling stress during slaughter of Atlantic salmon. Aquacult. Int. 7:101-115.

European Food Safety Authority, 2004. Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. The EFSA Journal 45:1-29.

Ewing, R.D., Ewing, S.K., 1995. Review of the effects of rearing density on the survival to adulthood for Pacific salmon. Prog. Fish. Cult. 57:1-57.

FAWC, 1996. Report on the welfare of fish. Home page address: http://www.fawc.org.uk

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annu. Rev. Physiol. 61:243-282.

Fink, A.L., Goto, Y., 1998. Molecular Chaperones in the Life Cycle of Proteins: Structure, Function, and Mode of Action. Marcel Dekker, New York, NY, USA.

Fisheries Society of British Isles, 2002. Fish welfare. Briefing Paper 2, FSBI, Granta Information System, Sawston, Cambridge, UK.

Food and Agriculture Organization, 2006. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations ed. FAO 2007, Roma, Italy.

Gjerde, B., Pante, M.J.R., Baeverfjord, G., 2005. Genetic variation for a vertebral deformity in Atlantic salmon (Salmo salar). Aquaculture 244:77-87.

Goodey, W., Liley, N.R., 1985. Grouping fails to influence the escape behaviour of the guppy (Poecilia reticulata). Anim. Behav. 33:1032-1033.

Gornati, R., Papis, E., Rimoldi, S., Terova, G., Saroglia, M., Bernardini, G., 2004. Rearing density influences the expression of stress-related genes in sea bass (Dicentrarchus labrax L.). Gene 1:111-118.

Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219(1):1-14.

Handy, R.D., Depledge, M.H., 1999. Physiological responses: their measurements and use as environmental biomarkers in ecotoxicology. Ecotoxicology 8:329-349.

Hoyle, I., Oidtmann, B., Ellis, T., Turnbull, J., North, B., Nikolaidis, J., Knowles, T.G., 2007. A validated macroscopic key to assess fin damage in farmed rainbow trout (Oncorhynchus mykiss). Aquaculture 270:142-148.

Humane Slaughter Association, 2005. Humane Harvesting of Salmon and Trout., HSA ed., Wheathampstead, UK.

Huntingford, F., 2002. Welfare and Aquaculture. Proc. Int. Conf. Aquaculture Europe on Sea farming today and tomorrow, Trieste, Italy. European Aquaculture Society Special Publ. 32: 52-54.

Huntingford, F.A., Adams, C., Braithwaite, V.A., Kadri, S., Pottinger, T.G., Sandee, P., Turnbull, J.F., 2006. Current issues in fish welfare. J. Fish Biol. 68:332-372.
Iversen, M., Finstad, B., Nilssen, K.J., 1998. Recovery from loading and transport stress in Atlantic salmon (Salmo salar L.) smolts. Aquaculture 168:387-394.

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. Rev. Fish. Biol. Fisher. 8:35-56.

Kaushik, S., Cravedi, J., Lalles, J., Sumpter, J., Faconneau, B., Laroche, M., 1995. Partial or total replacement of fish meal by soybean protein on growth, protein utilisation, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout (Oncorhynchus mykiss). Aquaculture 133:257-274.

Kestin, S.C., 1994. Pain and stress in fish. Report of the Royal Society for the Prevention of Cruelty to Animals. Causeway, Horsham, West Sussex, UK.

Kestin, S.C., Wootton, S.B., Adams, S., 1995. The effect of CO₂, concussion or electrical stunning of rainbow trout (Oncorhynchus mykiss) on fish welfare. Proc. Int. Conf. Aquaculture Europe on Quality in Aquaculture, Gent, Belgium. European Aquaculture Society Special Publ. 23: 380-381.

Kestin, S.C., Wootton, S.B., Gregory, N.G., 1991. Effect of slaughter by removal from water on visual evoked activity in the brain and reflex movement of rainbow trout (Oncorhynchus mykiss). Vet. Rec. 128:443-446.

Kramer, D.L., 1987. Dissolved oxygen and fish behaviour. Environ. Biol. Fish. 18:81-92.

Kristiansen, T.S., Ferno, A., Holm, J.C., Trivitera, L., Bakke, S., Fosseidengen, J.E., 2004. Swimming behaviour as an indicator of low growth rate and impaired welfare in Atlantic halibut (Hippoglossus hippoglossus L.) reared at three stocking densities. Aquaculture 230:137-151.

Jerrett, A.J., Stevens, J., Holland, A.J., 1996. Tensile properties of white muscle in rested and exhausted king salmon (Oncorhynchus tshawytscha). J. Food Sci. 61:527-532.

Juell, J.E., Oppendal, F., Boxaspen, K., Taranger, G.L., 2003. Submerged light increases swimming depth and reduces fish density of Atlantic salmon (Salmo salar L.) in production cages. Aquacult. Res. 34:469-477.

Lambooij, E., Van Der Vis, J.W., Kloosterboer, R.J., Pieterse, C., 2002a. Evaluation of head-only and head-to-tail electrical stunning of farmed eels (Anguilla anguilla, L.) for the development of a humane slaughter method. Aquacult. Res. 33:323-331.

Lambooij, E., Van Der Vis, J.W., Kloosterboer, R.J., Pieterse, C., 2002b. Welfare aspects of live chilling and freezing of farmed eel (Anguilla anguilla L.): neurological and behavioural assessment. Aquaculture 210:159-169.

Lima, S.L., 1998. Predator induced stress and behaviour. Adv. Stud. Behav. 27:215-290.

Lowe, T., Ryder, J.M., Carrager, J.F., Wells, R.M.G., 1993. Flesh quality in snapper, Pagrus auratus, affected by capture stress. J. Food Sci. 58:770-773.

Marx, H., Brunner, B., Weinzierl W., Hoffman, R., Stolle, A., 1997. Methods of stunning freshwater fish: impact on meat quality and aspects of animal welfare. Z. Lebensm. Unters. Forsch. 204:282-286.

Medale, F., Boujard, T., Vallee, F., Blanc, D., Mambri, M., Roem, A., Kaushik, S.J., 1998. Voluntary feed intake, nitrogen and phosphorus losses in rainbow trout (Oncorhynchus mykiss) fed increasing dietary levels of soy protein concentrate. Aquat. Living Resour. 11:239-246.

Midtlyng, P.J., 1997. Vaccinated fish welfare: Protection versus side-effects. Dev. Biol. Stand. 90: 371-379.

Milligan, C.L., 1996. Metabolic recovery from exhaustive exercise in rainbow trout. Comp. Biochem. Physiol. A 113:51-60.

Moberg, G.P., 1999. When does an animal become stressed? Lab. Anim. 23:22-26.

Montero, D., Tort, L., Robaina, L., Vergara, J.M., Izquierdo, M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (Sparus aurata) juveniles. Fish Shellfish Immun. 11:473-490.

Mormède, P., Andanson, S., Auperin, B., Beerda, B., Guéméné, D., Malmkvist, J., Manteca, X., Manuteufel, G., Prunet, P., Van Reenen, C.G., Richard, S., Veissier, I., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to eval-
Poli, B.M., Parisi, G., Scappini, F., Zampacavallo, G., 2005. Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquacult. Int. 13:29-49.

Poli, B.M., Scappini, F., Parisi, G., Zampacavallo, G., Mecatti, M., Lupi, P., Mosconi, G., Giorgi, G., Vigiani, V., 2004. Traditional and innovative stunning/slaughtering methods for European sea bass compared by the complex of the assessed behavioural, plasmatic and tissue stress and quality indexes at death and during shelf life. pp 58-63 in Proc. 34th WEFTA Conf., Lubeck, Germany.

Poli, B.M., Zampacavallo, G., Iurzan, F., De Francescco, M., Mosconi, M., Parisi, G., 2002. Biochemical stress indicators change in sea bass influenced by slaughter methods. Proc. Int. Conf. Aquaculture Europe on Sea farming today and tomorrow, Tri-

Oka, H., Ohno, K., Ninomiya, J., 1990. Changes in texture during cold storage of cultured yellowtail meat prepared by different killing methods. Nippon Suisan Gakk. 56:1673-1678.

Oliveira, R.F., Canario, A.V.M., Bsharry, R., 1999. Hormones, behaviour and conservation of littoral fishes: current status and prospects for future research. In: V.C. Almada, R.F. Oliveira and E.J. Goncalves (eds.) Behaviour and conservation of littoral fishes. Instituto Superior de Psicologia Apilcada publ., Lisboa, Portugal, pp 149-168.

Ortuño, J., Angeles Esteban, M., Meseguer, J., 2002. Lack of effect of combining different stressors on innate immune responses of sea bream (Sparus aurata L.). Vet. Immunol. Immunopathol. 84:17-27.

Ottera, H., Roth, B., Torrison, O.J., 2001. Do killing methods affect quality of Atlantic Salmon? In: S.C. Kestin and P.D.Warris (eds.) Farmed Fish Quality. Blackwell Science Ltd., Oxford, UK, pp 398-399.

Overli, O., Korzan, W.J., Hoglund, E., Winberg, S., Bollig, H., Watt, M., Foster, G.L., Barton, B.A., Overli, E., Renner, K.J., Summers, C.H., 2004. Stress coping style predicts aggression and social dominance in rainbow trout. Horm. Behav. 45:235-241.

Parisi, G., De Francesco, M., Médale, F., Scappini, F., Mecatti, M., Kaushik, S.J., Poli, B.M., 2004. Effect of total replacement of dietary fish meal by plant protein sources on early post mortem changes in the biochemical and physical parameters of rainbow trout. Vet. Res. Commun. 28:237-240.

Pickering, A.D., 1998. Stress responses in farmed fish. In: K.D. Black and A.D. Pickering (eds.) Biology of farmed fish. Sheffield Academic Press, Sheffield, UK, pp 222-255.

Pickering, A.D., Pottinger, T.G., 1985. Factors influencing blood cortisol levels of brown trout under intensive culture conditions. In: B. Lofts and W.N. Holms (eds.) Current Trends in Endocrinology. Hong Kong University, Hong Kong, China, pp 1239-1242.
este, Italy. European Aquaculture Society Special Publ. 32:429-430.

Pottinger, T.G., 2001. Effects of Husbandry Stress on Flesh Quality Indicators in Fish. In: S.C. Kestin and P.D. Warriss (eds.) Farmed Fish Quality. Fishing News Book, Oxford, UK, pp 145-160.

Pottinger, T.G., 2003. The selection of trout for high and low responsiveness to stress: progress and prospects. In: Centre for Environment, Fisheries & Aquaculture Science (ed.) Trout News 36:14-16.

Pottinger, T.G., Carrick, T.R., 2001. Stress responsiveness affects dominant-subordinate relationships in rainbow trout. Horm. Behav. 40:419-427.

Pottinger, T.G., Pickering, A.D., 1997. Genetic basis to the stress response: selective breeding for stress-tolerant fish. In: G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck (eds.) Fish Stress and Health in Aquaculture, University Press, Cambridge, UK, pp 171-194.

Reddy, P.K., Leatherland, J.F., 1998. Stress Physiology. In: J.F. Leatherland and P.T.K. Woo (eds.) Fish Diseases and Disorders. Vol.2: Non-infectious Disorders, CAB International, Wallingford, UK, pp 279-301.

Reddy, P.K., Vijayan, M.M., Leatherland, J.F., Moon, T.W., 1995. Does RU486 modify hormonal responses to handling stressor and cortisol treatment in fed and fasted rainbow trout? J. Fish. Biol. 46:341-359.

Ribas, L., Planas, J.V., Barton, B., Monetti, C., Bernardini, G., Saroglia, M., Tort, L., Mackenzie, S., 2004. A differentially expressed enolase gene isolated from the gilthead sea bream (Sparus aurata) under high-density conditions is up-regulated in brain after in vivo lipopolysaccharide challenge. Aquaculture 241:195-206.

Robb, D.H.F., Kestin, S.C., 2002. Methods used to kill fish: field observation and literature reviewed. Anim. Welfare 11:269-282.

Robb, D.H.F., Roth, B., 2003. Brain activity of Atlantic salmon following electrical stunning using various field strengths and pulse duration. Aquaculture 216:363-369.

Robb, D.H.F., Warriss, S.P.D., 1997. How killing methods affect salmonid quality. Fish Farmer 6:48-49.

Robb, D.H.F., Wotton, S.B., Mckinstry, J., Sarensen, N.K., Kestin, S.C., 2000. Commercial slaughter methods used on Atlantic salmon: determination of the onset of brain failure by electroencephalography. Vet. Rec. 147:298-303.

Rose, J.D., 2002. The neurobehavioural nature of fishes and the question of awareness and pain. Rev. Fish. Sci. 10:1-38.

Rose, J.D., 2003. A critique of the paper, “Do fish have nociceptors: evidence for the evolution of a vertebrate sensory system” by Sneddon et al. 2003. In: H.S. Erikson (ed.) Information resources on fish welfare, 1970-2003. Animal Welfare Information Center Resource Series N. 20, pp 49–51.

Ruane, N.M., Carballo, E.C., Komen, J., 2002. Increased stocking density influences the acute physiological stress response of common carp (Cyprinus carpio L.). Aquacult. Res. 33:777-784.

Ruff, N., Fitzgerald, R.D., Cross, T.F., Teurtrie, G., Kenny, J.P., 2002. Slaughtering method and dietary α-tocopherol acetate supplementation affect rigor mortis and fillet shelf life of turbot Scophthalmus maximus L. Aquacult. Res. 33:703-714.

Sandodden, R., Findstad, B., Iversen, M., 2001. Transport stress in Atlantic salmon (Salmo salar L.): anaesthesia and recovery. Aquacult. Res. 32:87-90.

Scapigliati, G., Scalia, D., Marras, A., Meloni, S., Mazzini, M., 1999. Immunoglobulin levels in the teleost sea bass (Dicentrarchus labrax L.) in relation to age, season and water oxygenation. Aquaculture 174:207-212.

Schjolden, J., Stokeshus, S., Winberg, S., 2005. Does individual variation in stress responses and agonistic behaviour reflect divergent stress coping strategies in juvenile rainbow trout?. Physiol. Biochem. Zool. 78:715-723.

Sigholt, T., Erikson, U., Rustad, T., Johansen, S., Nordtvedt, T.S., Seland, A., 1997. Handling stress and storage temperature affect meat quality of farm-raised Atlantic salmon (Salmo salar). J. Food Sci. 62:898-905.

Skjervold, P.O., Fjaera, P.B., Ostby, P.B., Einen, O., 2001. Live-chilling and crowding stress before
slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 192:267-282.

Sneddon, L.U., Braithwaite, V.A., Gentle, M.J., 2003. Do fish have nociceptors: evidence for the evolution of a vertebrate sensory system. P. Roy. Soc. Lond. B Bio. 270:1115-1121.

Sørum, U., Damsgård, B., 2004. Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar*). Aquaculture 232:333-341.

Southgate, P., Wall, T., 2001. Welfare of farmed fish at slaughter. In Practice 23:277-284.

Sumpter, J.P., 1997. The endocrinology of stress. In: G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck (eds.) Fish Stress and Health in Aquaculture. Cambridge University Press, Cambridge, UK, pp 95-118.

Sutor, H.C., Huntingford, F.A., 2002. Eye colour in juvenile Atlantic salmon: effects of social status, aggression and foraging success. J. Fish Biol. 61:606-614.

Tappel, A.L., 1973. Lipid peroxidation damage to cell components. Fed. Proc. 32:1870-1874.

Thorpe, J.E., Mceonway, M.G., Miles, M.S., Muir, J.S., 1987. Diet and seasonal changes in resting plasma cortisol levels in juvenile Atlantic salmon, *Salmo salar* L. Gen. Comp. Endocrinol. 65:19-22.

Tobiassen, T., Sørensen, N.K., 1999. Influence of killing methods on time of death of Atlantic salmon (*Salmo salar*) and rainbow trout (*Salmo gairdneri*) as measured by behavioural indices of sensibility and reflexes. Proc. Int. Conf. Aquaculture Europe on Towards Predictable Quality, Trondheim, Norway. European Aquaculture Society Special Publ. 27: 244-245.

Tort, L., Padros, F., Rotllant, J., Crespo, S., 1998a. Winter syndrome in the gilthead sea bream *Sparus aurata*. Immunological and histopathological features. Fish Shellfish Immun. 8:37-47.

Tort, L., Rotllant, J., Rovira, L., 1998b. Immunological suppression in gilthead sea bream *Sparus aurata* of the north west Mediterranean at low temperatures. Comp. Biochem. Physiol. A 120:175-179.

Turnbull, J.F., Bell, A., Adams, C.E., Bron, J., Huntingford, F.A., 2005. Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. Aquaculture 243:121-132.

Turnbull, J.F., Richards, R.H., Robertson, D.A., 1996. Gross, histological and scanning electron microscopic appearance of dorsal fin rot in Atlantic salmon *Salmo salar* L. J. Fish Dis. 19:415-427.

Turner, J.W., Nemeth, R., Rogers, C., 2003. Measurements of fecal glucocorticoids in parrotfishes to assess stress. Gen. Comp. Endocrinol. 133:341-352.

Van Der Vis, H., Kestin, S., Robb, D., Oehlenschlager, J., Lambooij, B., Munkner, W., Kuhlmann, H., Kloosterboer, K., Tejada, M., Huidobro, A., Ottera, H., Roth, B., Sørensen, N.K., Akse, L., Byrne, H., Nesvadba, P., 2003. Is humane slaughter of fish possible for industry? Aquacult. Res. 34:211-220.

Van Der Vis, H., Oehlenschlager, J., Kuhlmann, H., Munkner, W., Robb, D.H.F., Schelvis-Smit, A.A.M., 2001. Effect of the Commercial and Experimental Slaughter of Eels (*Anguilla anguilla* L.) on Quality and Welfare. In: S.C. Kestin and P.D. Warriss (eds.) Farmed Fish Quality. Fishing News Books, Oxford, UK, pp.234-248.

Vazzana, M., Cammarata, M., Cooper, E.L., Parriello, N., 2002. Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. Aquaculture 241:371-386.

Verheijen, F.J., Flight, W.F.G., 1997. Decapitation and brining: experimental tests show that after these commercial methods for slaughtering eel *Anguilla anguilla* (L.), death is not instantaneous. Aquacult. Res. 28:361-366.

Vijayan, M.M., Moon, T.W., 1994. The stress response and the plasma disappearance of corticosterone and glucose in a marine Teleost, the sea raven. Can. J. Zool. 72:379-386.

Wall, A.E., 1998. Cataracts in farmed Atlantic salmon (*Salmo salar*) in Ireland, Norway and Scotland from 1995 to 1997. Vet. Rec. 142:626-631.

Wall, A.J., 2001. Ethical Considerations in the Handling and Slaughter of Farmed Fish. In: S.C. Kestin and P.D. Warriss (eds.) Farmed Fish Quality. Fishing News Book, Oxford, UK, pp 108-115.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77:591-625.