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Considerations on psychophysical welfare of fish employed in scientific procedures and on Recommendation 2007/526/EC

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Summary. The use of fish for experimental purposes has seen a significant increase over the past years, consequently scientific findings on factors influencing welfare of these vertebrates are now available, as well as debates on their capacity of experiencing suffering are increasingly found in animal welfare discussions. Nowadays, in Europe, the use of these animals as experimental models is regulated by the Recommendation 2007/526/EC, where in the Section on the species-specific guidelines for fish, aspects such as the environmental characteristics of housing, the monitoring of animal health, the general care of subjects (*i.e.* feeding, handling, transport), and the killing procedures, are considered. In this manuscript, some aspects regarding the use of fish for human benefits will be discussed, and the suggestions provided by the European legislation are pointed out in order to identify limits and advantages.

Key words: fishes, laboratory animal science, ethology, ecology.

Riassunto *(Considerazioni relative al benessere dei pesci utilizzati in procedure scientifiche e raccomandazione 2007/526/EC)*. In questi ultimi anni il numero di pesci utilizzati per fini scientifici è notevolmente aumentato. Molta letteratura riguardante i fattori che influenzano il benessere di questi animali è attualmente disponibile, inoltre cresce il dibattito sulla capacità o meno di questi vertebrati di provare sofferenza. Oggi l'uso e il mantenimento dei pesci nei laboratori di ricerca e negli allevamenti ittici con fini sperimentali sono regolamentati dalla Raccomandazione europea 2007/526/EC. In questo lavoro di rassegna critica verranno presentate alcune considerazioni generali sull'uso dei pesci nella ricerca scientifica, inoltre verrà descritto e commentato quanto attualmente presente nella Raccomandazione europea. Tale legislazione è in fase di ulteriore revisione.

Parole chiave: pesci, scienza degli animali da laboratorio, etologia, ecologia.

INTRODUCTION

Nowadays, fish are considered a valid animal model in numerous research fields. For example, zebrafish *(Danio rerio)* is traditionally used as model in toxicological research, in studies on vertebrate development [1-3] and it has been recently suggested as an attractive model for the basic and clinically applied human skin research [4]. Then, fathead minnow *(Pimephales promelas)* is an organism with a consolidated history of experiments on metal exposure, and is commonly used in biological assays [5-7]. Additionally, rainbow trout *(Oncorhynchus mykiss)* and goldfish *(Carassius auratus auratus)* are considered standard fish species relevant in the studies of the neurodegenerative diseases [8]. In fact, while many rodent species are resistant to a variety of toxic compounds, including MPTP, these species of fishes show clear signs of central nervous system damage.

In Europe the use of fish as experimental animals is dealt with the Recommendation 2007/526/EC, on guidelines for the accommodation and care of animals used for experimental and other scientific purposes. This document represents a revision of the Appendix A of the European Convention ETS 123, for the protection of vertebrate animals used for experimental and other scientific purposes [9].

The Section on the species-specific guidelines for fish takes in account aspects such as the environmental characteristics of housing, the monitoring of animal health, the general care of subjects (*i.e.* feeding, handling, transport), and the killing procedures.

These recommendations represent a step toward an effective improvement of their well-being; nevertheless the great biological diversity of this vertebrate group makes difficult the drafting of compre-

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hensive normative and regulations for their effective protection. As a matter of fact, the number of species of fish is greater than all other vertebrate species combined; furthermore, they show a wide range of physiological and behavioural traits.

In this manuscript, we intend to tackle three aspects regarding the use of fish for human benefits. Firstly, we will briefly provide considerations on the use of fish in biomedical research as alternative animal model; the second section will discuss some of the issues concerning fish experience of pain and distress; finally, we will comment the current European Recommendation on husbandry and care of fish housed in the research laboratories and in the aquaculture systems.

FISH AS ALTERNATIVE ANIMAL MODEL

Fish species used for experimental purposes have seen an expansion over the past years. The maintenance of these animals in research laboratories can be considered an implementation of concept of "relative" replacement introduced by Russell and Burch [10]. In the 1959, these authors attempted to establish the general principles of the treatment of the animals during experimentation, taking into account the intimate relationship between humane treatment and "efficiency" in animal experimentation. These remarks, known as the principle of the ThreeRs – Replacement, Reduction and Refinement – are now embodied in laws and technical guidelines, and widely considered in the planning of animal experiments.

Replacement means the substitution of animal models with alternative methods, such as *in vitro* studies, molecular approaches, mathematical and computer models. In recent times the concept of "relative" replacement has been introduced, meaning the use of animals with lower neurophysiological development or the employment of animal tissues. This concept of "relative" replacement arises from the general idea that the characteristics of the nervous system, especially brain structural organization, provide a powerful insight into the nature of an organism, including its capabilities and limits.

In the vertebrate taxa, relevant anatomical and neurochemical differences in the structure and complexity of the brain have been found [11, 12]. In particular, fish are lacking of neocortex, a layered type of cortex present only in mammals [13, 14], which appears to be associated with the sensation of pain [15, 16] (however for comparative neurology contributions among vertebrate taxa see, Avian Brain Nomenclature Consortium 2005 [17]). The absence of this anatomical nervous structure would make fish unable of having suffering experiences [18]. Therefore, the behavioural and physiological responses (*i.e.* alteration in the normal feeding, in the swimming pattern, increase in the ventilation rate) to the mechanical, thermal, and chemical noxious stimuli would represent only a strategy to re-establish a disturbed homeostasis [18]. Those statements, however, are a matter of increasing critical discussion.

On account of this, why should we be then interested in welfare of fish?

WHY THE WELFARE OF FISH MATTERS TO US

It is generally assumed that the animal suffering is an issue to be considered in the use of animals to study biological mechanisms of human diseases or to improve medical care. In the 1986, the British ethologist Patrick Bateson suggested that a balance between costs (in terms of pain and suffering to the animals) and benefits (in terms of new knowledge on the etiology of the disease and potential advantages for human health) should be always carried out before to start any scientific experiment involving living animals [19]. Before him, Russell and Burch (1959) suggested the need to refine the experimental procedures to guarantee to the non-human animal a humane treatment during the experiment [10].

In these last years, the concept of "Refinement" has been progressively expanded and completed, including the attention and care of the animals before, during, and after any experimental procedure, as well as the attention for the housing and general husbandry of the animals [20-22].

Today, the concepts of animal welfare and suffering experience are too often overlapping incorrectly. In fact, the tendency to misunderstand the need to guarantee the welfare of the animals living under human influence and the capacity of these subjects to experience physical and/or psychological discomfort is increasing.

The principal mistake is due to the fact that, when the concepts of suffering or discomfort are taken in account, a generally underlying assumption, both of the scientific and no scientific environment, is these feelings to be thought in a manner similar to that experienced by humans [23, 24].

This anthropomorphic thinking undermines our responsibility of care of other species, besides it can easily induce to the idea that the existence of physiological, physical and behavioural animal needs is somehow correlated with the consciousness of themselves, more than with their biology and natural history. At the same time, the term "sentient" should be carefully used, not only to avoid scientifically incorrect assertions, but especially because the risk of undervaluing the importance of pure biological aspects may be particularly high. Finally, it should be borne in mind that the human tendency to attribute mental states to other animals may be strongly counterproductive to guarantee the well-being of themselves, causing as final result the protection of just a limited number of species. Fish are vertebrates which have undergone a consistent evolutionary process, resulting in complex and different anatomical, physiological and behavioural adaptations [25, 26]. The aquatic life, the physical and chemical differences of living environments, the different biological characteristics of surrounding habitat, have led to a pronounced specialization in the physiological and behavioural mechanisms. In particular, the great diversification in the sensory capacities, their diverse swimming and feeding behaviours, their specific social organizations and their mating systems (sometimes very sophisticated) permit to occupy particular niches [26, 27]. For example, some species, such as silurid catfish *(Apodoglanis furnessi),* have taste receptors across their entire body surface [27]; conversely, goldfish *(Carassius auratus auratus)* and some carps have evolved an extremely specialized taste system, represented by intraoral food-sorting apparatus, expression of a particular development of brain structure [28].

Furthermore, feeding behaviour is extremely diverse and specialized in the sensory modalities used to locate food and in the oral motor specializations employed in food capture or ingestion. Additionally, the reproductive behaviour can be represented by social displays involving elaborate courtship; moreover, most species show little or no parental care, whereas some (*i.e.* cichlids or males of fathead minnows) exhibit active protection of young [26].

The consideration of this ecological perspective is critical, because these evidences tend to demonstrate that, independently from capacity of feeling a human-like experience of pain, the fish have numerous basic demands, whose dissatisfaction can determine stress conditions.

The stress can be defined as a condition in which the dynamic equilibrium of animal organisms (homeostasis) is disturbed by intrinsic or extrinsic stimuli, commonly defined as stressors [29]. The fish exhibit a coordinated set of behavioural and physiological adaptive responses, which enable them to compensate the aversive stimulus. Nevertheless, when the stress persists over time (chronic-type), the response may lose its adaptive value and become dysfunctional [30].

The identification of stress in fish under field, aquaculture and laboratory conditions may be complicated. Firstly, because the stress responses in these animals, as in all vertebrates, are characterized from complexity and flexibility, in fact different factors operate to determine the response to a stressor. For example, the features of stressors (*i.e.* type, duration, controllability) affect the specific nature of response (primary, secondary and tertiary) that the organism will perform [31]. Additionally, it may be hard to identify the borderline between a mild form of stress (eustress) and a more intense, potentially life-threatening, stress (distress).

From all of these issues it follows that the welfare decisions on fish should be taken through careful examinations of their species- sex- and age-specific biological and ecological lifestyle, leaving out the considerations about potential suffering experience.

WELFARE ITEMS FOR FISH

The European document, Recommendation 2007/526/EC, which now provides advice on accommodation, housing and care of fish used for experimental purposes, presents a specific fish section based on proposals made by expert groups, and comprising the followed paragraphs: "Environment and its control", "Health", "Housing, enrichment and care" and "Transport"[9].

Environment and its control

It is generally recognized that the rearing and housing environment may influence the validity of experimental data [32, 33]. For primary aquatic animals as fish, the chemical and physical characteristics of the water represent the more important factors of the captive environment to be cared for. This is mainly caused by the very intimate relationship between the body fluids in the gills and the ambient water. Therefore, the quality of water, comprising aspects such as the oxygen and carbon dioxide concentrations, nitrogen compounds, pH and salinity levels, must be carefully considered.

The oxygen concentration results in affecting the fish activity, altering the swimming behaviour [34, 35], and the metabolism [36], with related effects on many aspects of fish life. For example, at severe levels of hypoxia, alterations of fish growth and survival rates have been found [37]. Furthermore, Domenici and co-workers found an effect of hypoxia on fish escape responses and schooling behaviour [38].

The level of oxygen required for the survival depends on the species, and on the ecological adaptation to the hypoxia condition [35]. Nevertheless, we must consider that factors including density, handling, water flow and temperature, have a profound impact on the level of available and demanded gas in captive conditions [39].

The presence of chemicals in the water can have effects at the cell and tissue levels, as well as to act as stressors. In captivity, the levels of nitrite and ammonia, very toxic agents for fish, represent relevant limiting factors for breeding success. Ammonia and the converted nitrite derive from feed and faeces, as well as from dissolved urea. Adequate water flow and supplementary aeration are indicated as effective strategies to control these chemical compounds [9, p. L197/85].

Furthermore, the effects that the presence of metals in the water environment has on the physiology, behaviour and development of fish, are well-known [40, 41]. Recently, the waterborne copper has been found to affect gene expression and the physiological endpoints of chronic exposure to metals in zebrafish *(Danio rerio)* [42].

The introduction of air or pure gas could be a strategy to maintain adequate concentration of oxygen. Nevertheless, an accumulation of carbon dioxide, with consequently decrease of the value of pH can result as indirect effect. High levels of carbon dioxide are not considered a problem under normal conditions of housing, therefore only a general indication of monitoring is suggested in the current European normative [9, p. L197/86]. Conversely, the value of pH should be kept stable by daily monitoring so that other pH-related parameters do not change.

With respect to the level of salinity, the Recommendation 2007/526/EC notes that the alterations of salinity are tolerable for some species of fishes (euryhaline), even if the changes in this parameter must be gradual to permit the adaptation of the individuals to the new environmental condition [9, p. L197/86].

With regard to the temperature, fish have the same body temperature of the surrounding environment. Therefore, the maintenance of this physical parameter within an opportune range is particularly important for the well-being of these animals. In the literature a standard environmental temperature (SET), defined as the temperature that the fish would prefer if given them the possibility to choose, has been suggested [39]. Lower temperatures involve a decrease of metabolic rate, and in the presence of higher temperatures a reduction in feed conversion rate has been found [43]. Furthermore, the temperature of water has been found to affect aspects of the behaviour of fish, such as swimming behaviour [44] and feeding pattern [43], as well their physiology [45] and morphology [46].

Physical characteristics of the housing environment, such as lighting and noise, are considered two important elements affecting the welfare of fish. Lighting strongly influences the physiology of fish. For example, the maturation and the feeding behaviour are two aspects consistently related to the photoperiod [30]. Furthermore, it should be considered that some species avoid the direct light in the wild and, therefore, the absence of covered areas in the tank may represent a source of stress for these individuals [47].

In order to guarantee the validity of data obtained, all of these aspects should be taken in account. For example, the potential habituation of individuals to noise or vibrations (efficiently transmitted in the water), in the evaluation of the external variability of an experimental design, should be considered.

Health

In the Recommendation 2007/526/EC only general suggestions on the hygiene of experimental facilities are provided. No instructions on what investigations should be performed before the starting of a trial are indicated. Furthermore, health monitoring is usually limited to statements about the prevalence of diseases, and the reporting on the health of fish employed in research is often sparse and not stated in the papers.

This state of art may cause a widespread variability in the results obtained from very similar research programs. In fact, even if no infectious agents or diseases are detected, the health status of different fish may consistently vary. Following that, the comparability of the data obtained is not achieved and the concept of harmonization is unsatisfied. In this respect, Johansen and collaborators suggested the introduction of a health card system similar to that used in livestock production in some countries [39]. Generally, vaccination programs should be performed before fish are approved for research, and specific health monitoring should be conducted during the whole experiment. Furthermore, the influence that potential diseases may have on the scientific results should be evaluated and considered in the data discussion.

Housing, enrichment and care

A common problem of captive environment is the density of the animals in the enclosures. In the case of fish, this parameter results to have effects on many factors, strongly affecting the well-being of these animals. For example, level and demand of oxygen and other gas levels, the ammonia concentration, are all variables stock density-dependent.

When the density of fish is relevant, an adequate water supply and efficient recirculatory system are suggested by European Recommendation 2007/526/EC as effective tools to facilitate the maintenance of oxygen and other gas within acceptable levels [9, p. L197/85].

A relationship between feeding activity and stocking density in European sea bass (*Dicentrarchus labrax*) has been found. The juveniles of this species restrict their feeding activity to specific hours of day, when they are housed in low density in the tanks [48]. Therefore, density manipulation may be used to increase the feeding behaviour and stimulate weight gain in these animals.

Additionally, ecological aspects such as fecundity, species-specific territorial behaviour (particularly for male subjects) and predation are also deeply influenced by density of fish group [26]. High density can cause stress, even if it should be noted that different species show different preferred density. For example, in fish that normally form shoals, low density may lead to stress condition [31, 39]. Therefore, the evaluation of the behavioural and ecological aspects plays a key role in the assessment of the opportune population density.

With respect to housing, generally the available space, more than its furnishing, is to be considered relevant for fish well-being. These vertebrates should have sufficient space for normal swimming and to perform territorial behaviour. In this latter sense, the presence of physical elements, acting as barriers and covers, or sand for some flatfish, may facilitate both the recognition of different individual areas and to minimise the aggressive encounters.

Hygiene routine procedures are important to run the sources of infectious fish agents, such as personnel, feed, and water. Nevertheless, the use of products for the disinfection should be carefully evaluated to minimise the risk to introduce substances directly toxic for animals themselves.

Experimental fish should be fed with commercial dried feed, both prior and during the trials. The introduction of frozen or fresh fish material frequently represents the major source of infection with *Mycobacterium* spp. and several parasites [49].

The procedures of handling can be another potential source of stress. When the catching is needed, it should be carried out by nests with appropriate frame and mesh size (for an anthropological interaction with other warm-blooded vertebrates see Alleva and Francia, 2007 [50]).

Finally, the European legislation briefly provides indications on the transport. In particular, attention to food supply, loading and temperature changes, are suggested.

CONCLUSIONS

The current European Recommendation 2007/526/EC on guidelines for the protection of fish employed for experimental purposes, surely represents a relevant improvement for the guarantee of well-being of fish housed in laboratory or aquaculture conditions. Nevertheless, it should be noted that in these guidelines, the wide biological diversity of fish, and their consequently different physiological and behavioural needs, are sparsely and incompletely considered. In particular, general recommendations for all fish species in all types of research are provided about aspects such as housing, monitoring of health and handling, with few or no considerations for the species-specific characteristics.

Furthermore, a relevant limit of this document is the absence of indications for the standardization of procedures, both with respect to how to measure the parameters of interest and what specific measurements should be taken in account. The lack of these aspects does not permit the matching with the concept of harmonization, leading to incomparable results among different laboratories, and finally, bringing about the use of large number of fish in research.

It seems highly relevant to reach an European consensus on the characteristics of the present Recommendation, although this effort is a challenge, given different country-specific sensitivities of the 27 European Member States, with different traditions, local ethics, zooanthropological cultures and deontology of the various scientific communities.

Furthermore, it is important to understand the relevance of refinement and improvement of the current normative, as the employment of fish as animal model in biomedical research may represent a good example of positive interplay between the Three Rs – Replacement, Reduction and Refinement – suggested by Russell and Burch [9] (for concept of interplay between Replacement, Reduction and Refinement see, de Boo *et al.* 2005 [51]). In fact, the substitution of vertebrates of higher neurophysiological sensitivity, such as mammals, with lower neurophysiologic developed animals, can be considered a positive "relative" Replacement strategy. Furthermore, the use of animals with a potential lower capacity for suffering must also be considered a Refinement, because the experience of harm may also be reduced. Finally, the international harmonization of protocols and of legally required standard conditions of housing would represent an effective strategy for reducing the number of animal experiments that must be carried out in individual European countries.

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Conflict of interest statement

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