PathoGelTrap

New Blue Revolution through a pioneering pathogen-trapping technology based on bioselective hydrogel-forming proteins

H2020 – FET OPEN - Challenging Current Thinking

Deliverable No. 4.1

Fish welfare assessment

PathoGelTrap™
Sustainable Prevention in Aquaculture

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Executive Summary

Within the objectives of WP4 of the PathoGelTrap project, task 4.1 assesses the fish welfare during *in vivo* experiments. IZSVE developed several laboratory assays for stress markers detection to assess animal welfare. SW has constructed one device to measure water quality parameters, which will be used to control these parameters in IZSVE facilities during the *in vivo* experiments, providing relevant info to guarantee animal welfare. IZSVE started the *iter* to obtain the authorization from the Italian Ministry of Health to perform animal experiments.

List of acronyms/abbreviations

AWB= Animal Welfare Body
DL= Deliverable
EC= European Commission
EU= European Union
HNE= 4-Hydroxynonenal
HSP70= Heat Shock Protein 70
IHC= Immunohistochemistry
IZSVE= Istituto Zooprofilattico Sperimentale Delle Venezie
LLPS = Liquid-Liquid Phase Separation proteins
MDA= Malondialdehyde
NT= Nitrotyrosine
PGT= PathoGelTrap
Real Time PCR= Real-Time Polymerase Chain Reaction
SW: Smartwater Planet S.L.
VNNv= Viral Nervous Necrosis virus
VLP= Virus Like Particle
WP= Work-Package
YR= Yersinia ruckeri

Glossary of terms

Term	Explanation
Betanodavirus	Viral agent causative of Viral Nervous Necrosis, also known as viral encephalopathy and retinopathy, one of the target infective agents of the PGT project
Yersinia ruckeri	Bacterial agent causing Enteric Redmouth disease, one of the target infective agents of thePGT project
Pathogen	Organism able to cause disease
Antigen	Any substance that causes the immune system to produce antibodies against itself. In terms of pathogens, antigens are a protein (or part of it) exposed on its surface and capable of being recognized.
Liquid-liquid phase separation (LLPS)	Certain molecules (such as proteins) are rearranged into a dense phase that coexists with a dilute phase reminding liquid droplets.
Real Time PCR	A real-time polymerase chain reaction (real-time PCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR

HSP70	Heat shock proteins (HSP) are a family of proteins that are produced by cells in response to exposure to stressful conditions. The 70 kilodalton heat shock proteins (Hsp70s) are a family of conserved ubiquitously expressed heat shock proteins.
HNE	4-Hydroxynonenal is an α,β-unsaturated hydroxyalkenal that is produced by lipid peroxidation in cells. It's a widely used stress marker for the peroxidation of polyunsaturated fatty acids n-3 series instead generates compounds.
MDA	MDA is the most abundant aldehyde produced in lipid peroxidation, it is formed in cells in various forms, and it binds covalently to various compounds such as lysine residues or amines bound to phospholipids.
NT	Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species. Nitrotyrosine is identified as an indicator or marker of cell damage, inflammation as well as NO (nitric oxide) production
Catecholamines	Catecholamines are monoamine neurotransmitters secreted by cells mostly by the nervous and the endocrine systems. They are usually a healthy physiological response parameter.
Lactate	The conjugate base of lactic acid, is detected in blood to determine the status of the acid base homeostasis in the organism.
Cortisol	Cortisol, or hydrocortisone, is the major circulating glucocorticoid in Teleosts. It is also called *stress hormone* and is widely used as stress indicator in both short and long term exposure.
Lysozime	Lysozyme is bactericidal, hydrolysing b-[1,4] linked glycoside bonds of bacterial cell wall peptidoglycans resulting in lysis. The analysis of lysozyme activity is of diagnostic value for assessing the health of the fish.
Animal Welfare Body	The Animal Welfare Body serves as an interface with researchers, from whom it receives study projects involving the use of animals. The Animal Welfare Body liaises with the Ethics Committee of IZSVe to evaluate these projects and provide the opinions needed to apply for authorization from the Ministry of Health. The Body has the task of interfacing with the Ministry and then with the researchers in relation to project approval.

1. Introduction

PathoGelTrap proposes to provide the industry with two different pathogen-removal solutions, using the current knowledge on self-assembling properties of the Liquid-Liquid Phase Separation proteins, that will efficiently recognize and trap fish pathogens (both viruses and bacteria) directly in the water and inactivate them.

i) PathoGelTrap Liquid
ii) PathoGelTrap Filter

The final objective of the PGT project is to have operational products for the treatment of rearing water that remove the target pathogens from the water. Hence, it is important that the criteria needed for these products are fed into the models from the start.

During last years, fish welfare is receiving increasing attention in public and therefore in the world of research, industry and governments. In general, stress could be defined as the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state. In reference to fish species, "stress" means the condition in which the dynamic equilibrium of an organism, called homeostasis, is threatened or disturbed by the action of internal or external stimuli, commonly referred to stressful events. Measures of physiological stress response naturally feature prominently in studies of welfare. However, stress response is an adaptive function in the face of a perceived threat to homeostasis and, as suggested above, stress physiology does not necessarily equate to suffering and diminished welfare. In the short term, stress responses serve a very important function to preserve the individual. Welfare measures in aquaculture are, therefore, largely associated with tertiary effects of stress response that are generally indicative of prolonged, repeated or unavoidable stress. There is no single measure of welfare and although a wide range of physiological, biochemical and behavioural measures are used to assess welfare, none of these are considered reliable in isolation and multiple measures need to be taken. Indicators associated with chronic stress response provide a potential source of information concerning the welfare of the fish and are important because they allow the development of protocols that reduce stress. Behavioural and physiological measures are intrinsically linked and are dependent on one another for correct interpretation with regard to welfare. The most realistic assessment of welfare, however, is obtained through a series of information measures combined together through an appropriate statistical approach.

In aquaculture, stress is caused by physical disturbances, such as handling, weighing, crowding, grading, transportation, temperature, water quality which determine a variety of responses. Many of these responses can be used as quantitative indicators of stress. The overall effect of stress is the activation of the hypothalamic–pituitary–interrenal axis (HPI) and the release of corticosteroid hormones such as cortisol. Cortisol, or hydrocortisone, is the major circulating glucocorticoid in Teleosts. It is a fat-soluble hormone whose production is performed by steroidogenic interrenalic cells, which are localized in the anterior region of the kidney (head-kidney), mainly along the posterior cardinal veins and their branches. Due to its fat-soluble nature, cortisol can not be accumulated within the cells that produce it, so it passes into the bloodstream, through which it reaches the target tissues upon which exerts its action. Cortisol, also called stress hormone, is widely used as stress indicator in

both short and long term and is the primary indicator of stress in fish. Moreover, multiple-stress conditions amplify its production. ACTH can also stimulate the release of catecholamines and chronic elevation of cortisol can affect their storage and secretion in trout. Because both the interrenal and chromaffin tissue, by which the catecholamines are secreted, are located in the front of the kidney, it is conceivable that in fish there is a paracrine control of the regulation of stress hormones.

At the cellular level, the stress response is characterized by the heat shock proteins (HSPs), a family of highly conserved proteins that are present in all cells in all life forms. Among the numerous HSPs families that have been investigated, the 70 kDa protein family (HSP70) has been most largely employed as a biomarker due to its rapid and significant increase in response to various stressors. In fish, expression of HSP70 mRNA has been examined during diapause and after exposure to pesticides, virus, metals and other toxic compounds. An increased expression of HSP70 mRNA has been observed in sea bass subjected to overcrowding and transport stress. Moreover, transport stress influences the expression of inducible HSP70 protein in skeletal muscle of sea bass and in the epithelia of renal tubules, gills and skin of carp.

Oxidative stress is a condition due to the production of reactive oxygen species (ROS). ROS, such as superoxide radical (O2-), hydrogen peroxide (H2O2), the hydroxyl radical (.OH) and the radical nitric oxide (NO.), are generally produced during normal metabolism. To minimize the toxic effect on cellular components, organisms have developed antioxidant defence mechanisms. Under conditions of oxidative stress, it is altered the balance between ROS production and availability of antioxidant defences, so the defensive action become ineffective. This imbalance results in enzyme inactivation, protein degradation, lipid peroxidation and severe damage to nucleic acids. Oxidative stress is increasingly considered one of the major upstream components of the signalling cascade involved in many cellular functions, such as the inflammatory response, stimulating the adhesion molecules and the production of chemoattractive substances. Under conditions of oxidative stress, many effects of cellular dysfunction are mediated by products of non-enzymatic reactions, such as oxidation of proteins and polyunsaturated fatty acids. The lipids peroxidation derived from a reaction with free radicals and lipidic hydroperoxides, these are the major initial reaction products. Studies on the production of cytotoxic molecules led to the discovery of a group of conjugated aldehydes with toxic potential. Within this group, the most abundant member was identified as 4-hydroxy-2-nonenal (HNE). HNE is also an endogenous lipid mediator and can induce a variety of cellular processes that represent a program of cell response to oxidative stress conditions. Based on these results, it is believed that HNE may be involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues. The breaking of the carbon chain of fatty acids produces a wide range of smaller fragments of different lengths. Based on their structural characteristics, the short chain reactive aldehydes generated by lipid peroxidation can be mainly classified into three families: 2-alkenals, 4-hydroxy-2-alkenals and ketoaldehydes.

Another important reactive aldehyde family arising from lipid peroxidation includes ketoaldehydes, such as malondialdehyde (MDA), glyoxal and 4-oxo-2-nonenal (ONE). MDA is the most abundant aldehyde produced in lipid peroxidation, it is formed in cells in various forms and it binds covalently to various compounds such as lysine residues or amines bound to phospholipids.

The innate immune system is also of primary importance in combating infections in fish. The reason is basically the intrinsic inefficiency of the acquired immune response of fish due to its evolutionary

status and poikilothermic nature. This results in a limited antibody repertoire, affinity maturation and memory and a slow lymphocyte proliferation. The acquired immune response of fish is therefore sluggish (up to 12weeks) compared to the instant and relatively temperature independent innate immune response.

The innate immune system is also important in activating an acquired immune response. Various lytic enzymes, acting either singly or in a cascade, are important defence elements especially against bacteria. These are hydrolases like lysozyme and chitinase, the cathepsins, the lytic pathway of the complement system and other bacteriolytic/haemolytic enzymes found in tissues and body fluids of fish.

Lysozyme is an important parameter in the immune defence of both invertebrates and vertebrates. Lysozyme is bactericidal, hydrolysing b-[1,4] linked glycoside bonds of bacterial cell wall peptidoglycans resulting in lysis. Although primarily associated with defence against Gram positive bacteria, Gram negative bacteria can also be lysed by this enzyme. Lysozyme is also known to be an opsonin and activate the complement system and phagocytes. It is present in mucus, lymphoid tissue, plasma and other body fluids of most fish species. The analysis of lysozyme activity may be of diagnostic value for assessing the health of the fish. In fact, the lysozyme molecule is an important defence of the innate immune system and has been shown that, due to stressful events, the concentration of lysozyme in the blood is altered. It is seen that after an acute stress, there is a temporary increase in the levels of lysozyme, followed by the well-known immunosuppressive effect of chronic stress. In Teleost fish, the analysis of lysozyme activity is usually carried out on serum, since the method is more practical and less variable.

The objective of this deliverable is to assess the fish welfare during the in vivo trials to be performed in DL 2.6 and DL 3.5, in order to evaluate PathoGelTrap safety to fish.

2. Requirements for fish welfare assessment

2.1. Stress markers evaluation

IZSVE developed several laboratory assays to monitor the stress response of fish during the in vivo trials. In details, IZSVE developed:

- Real Time PCR to monitor the expression of HSP70 in sea bass. The analogue technique will be developed also for rainbow trout in the very next future. (Ref. Fiocchi et al., 2020)

- IHC stainings to detect and localize the most common oxidative stress related markers (HNE, MDA, NT, HSP70). (Ref. Pascoli et al., 2011; Fiochhi et al., 2020)

- Detection of different stress markers (Cortisol, Catecholamines, Lactate dehydrogenasis, Lysozime) by chromatographic assay on plasma. (Ref. IZSVE PDP ACC 043; IZSVE PDP ACC 072)

2.2. Animal experiment authorization

The protection and welfare of animals is an area covered by a wide range of EU legislation. This includes the protection of wildlife, zoo animals, farm animals, animals in transport and animals used for scientific purposes. Animal studies, whether for the development or production of new medicines, for physiological studies, for studying environmental effects or for the testing of chemicals or new food additives, has to be carried out in compliance with EU legislation.

Since 1986, the EU has had in place specific legislation covering the use of animals for scientific purposes. On 22 September 2010 the EU adopted Directive 2010/63/EU which updates and replaces the 1986 Directive 86/609/EEC on the protection of animals used for scientific purposes. The aim of the new Directive is to strengthen legislation, and improve the welfare of those animals still needed to be used, as well as to firmly anchor the principle of the Three Rs, to Replace, Reduce and Refine the use of animals, in EU legislation. Directive 2010/63/EU will take full effect from 1 January 2013. In Italy, this regulation was received and adopted under the Italian Law Decree 2014/26 of 04/03/2014.

IZSVE own an experimental aquarium authorized by the Italian Ministry of Health (Auth. Min. n. 19/2019-UT of 24/6/2019), with the permission to work with pathogens (BSL-2). Beside this authorization, the Italian Ministry requires that every experimental trial has to be authorized singularly before performing (art. 31 of LD 2014/26). That means that for any in vivo trial, we need to fulfil several documents regarding the experiments set up, the measures implemented to cope with the 3R principles (Reduce, Replace and Refine) and the statistical analyses performed to support the results.

For the *in vivo* trials to be performed in PGT project (DL 2.6 and DL 3.5), IZSVE draft the Annex 6 (Art. 31 of LD 2014/26) in order to get the Authorization from the Italian Ministry of Health. The document was sent to the Animal Welfare Body on 05/03/2021.

On 08/03/2021, AWB together with the Ethical Committee asked more details regarding the composition and the *in vitro* effect of LCR-AFB chimera, before approving the request. Since the chimera is not available at the moment, the authorization request was stopped, waiting for the test results, that will be performed as soon as the chimera will be delivered to IZSVE from the partners.

2.3. Water quality monitoring

Water is of far greater importance to fish as an environmental medium than air is to terrestrial animals. Not only does it support the fish physically, but it is also a source of oxygen, electrolytes and nutrients. It acts as a medium for osmoregulation and for the dilution of toxic metabolic wastes. Water quality therefore plays a critical role in all fish experiments.

SmartWater has developed an advanced sensor Platform called Medusa, a rechargeable plug&play multifunctional IoT device continuously measuring water quality, both in marine and freshwater. There are options for 85 different parameters, with a current configuration of 5 sensors in the platform (O2, Temperature, Conductivity, NH4 and Ph). These are critical water quality parameters in aquaculture production operations, both in marine and freshwater (See D 4.2).

IZSVE agreed with SmartWater for the supply of Medusa multiparametric probes to monitor the quality of the water during the *in vivo* experiments (D 2.7).

3. Overall monitoring and evaluation of results

- IZSVE developed several assays for stress markers detection to assess animal welfare during *in vivo* experiments.

- IZSVE prepared the documentation to seek the approval from Italian Ministry of Health to perform the animal experiments in DL 2.6 and DL 3.5. At the moment, the Animal Welfare Body requested some *in vitro* experiments to determine the effect of chimera (LCR-AFB) on cell lines prior to accepting the request.

- IZSVE agreed with SmartWater for the supply of Medusa multiparametric probes to monitor the quality of the water during the *in vivo* experiments.

4. Conclusion

Animal welfare is of great importance when performing experimental trials and when a new product is tested through those experiments.

To guarantee the fish welfare, IZSVE draft the request for animal experiment authorization to be submitted to the Italian Ministry of Health. The document at the moment is waiting for the *in vitro* testing results, and it will be updated as soon as the tests will be performed.

To assess the fish welfare, IZSVE developed several assays for stress markers detection and agreed with SmartWater for the supply of Medusa multiparametric probes to monitor the quality of the water during *in vivo* experiments.

Overall, 80% of the DL duties during first year are covered.

5. References

- Fiocchi, E., Civettini, M., Carbonara, P. et al. Development of molecular and histological methods to evaluate stress oxidative biomarkers in sea bass (*Dicentrarchus labrax*). Fish Physiol Biochem 46, 1577–1588 (2020).

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- IZSVE internal document: PDP ACC 043 Detection of the biochemical profile by Cobas C501

- IZSVE internal document: PDP ACC 072 Chemiluminescent analysis by Cobas e601