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Fish stress become visible: A new attempt to use biosensor for real-time monitoring fish stress

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ABSTRACT

To avoid fish mortality and improve productivity, the physiological conditions including stress state of the cultured fish must be monitored. As an important indicator of stress, glucose concentrations are monitored using *in vitro* blood analysis. The physiological processes of fish under environmental conditions are harsher in many ways than those experienced by terrestrial animals. Moreover, the process of anaesthetizing and capturing the fish prior to analysis may produce inaccurate results. To solve these problems, we developed wireless biosensor system to monitor the physiological condition of fish. This system enables artificial stress-free and non-lethal analysis, and allows for reliable real-time monitoring of fish stress. The biosensor comprised Pt–Ir wire as the working electrode and Ag/AgCl paste as the reference electrode. Glucose oxidase was immobilized on the working electrode using glutaraldehyde. We used the eyeball interstitial sclera fluid (EISF) as the *in vivo* implantation site of the sensor, which component concentration correlates well with that of blood component concentration. In the present study, we investigated stress due to alterations in water chemistry, including dissolved oxygen, pH, and ammonia–nitrogen compounds. Stress perceived from behavioural interactions, including attacking behaviour and visual irritation, was also monitored. Water chemistry alterations induced increases in the glucose concentration (stress) that decreased with removal of the stimulus. For behavioural interactions, stress levels change with avoidance, sensory behaviour and activity. We believe that the proposed biosensor system could be useful for rapid, reliable, and convenient analysis of the fish physiological condition and accurately reflects the stress experienced by fish.

1. Introduction

Physiologic responses to stressors generally serve an important survival function, and the stress response pattern is highly conserved in vertebrates. Repeated and chronic exposure to stressors has detrimental effects on many aspects of an organism's physiology, including changes in nervous system function, metabolism, growth, and development, reproductive function, and immune system function.

In response to a stressor such as handling or crowding, fish exhibits a series of biochemical and physiologic changes in an attempt to cope with the stress. The stress response in fish has been broadly categorized into primary, secondary, and tertiary responses (Mazeaud et al., 1977). Primary responses include the rapid release of stress hormones, such as catecholamine and cortisol, into the circulation. Various biochemical and physiologic effects subsequently occur in association with stress and are mediated to a large extent by the stress hormones mentioned above. Stress hormones activate a number of metabolic pathways that alter blood chemistry and hematology, such as carbohydrate metabolism by stimulating glucose production through gluconeogenesis, resulting in an elevation of plasma glucose (Barton and Iwama, 1991; Nakano et al., 2014). If the fish cannot acclimate or adapt to the stressor, whole-animal changes may occur as a result of energy repartitioning by the diversion of energy substrates to cope with the enhanced energy demand associated with stress and away from vital life processes such as reproduction and anabolic processes such as growth.

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Fishes are exposed to stressors in natural as well as artificial conditions, such as aquaculture or laboratory conditions. Stressors such as collecting, handling, sorting, holding, and transporting are routine practices in aquaculture that can significantly impact fish physiology and survival. Under more crowded conditions typical of intensive aquaculture systems, physiologic challenges from water quality and interactions between fish are added to the normal demands of the aquaculture environment. Water quality is one of the most important contributors to fish health and stress. Fish may be able to tolerate adverse water quality conditions, but when combined with other stressors, fish may quickly overcome the physiologic challenges (Barton et al., 2002). Temperature, dissolved oxygen, ammonia, nitrite, nitrate, salinity, pH, carbon dioxide, alkalinity, and hardness are the most common water quality parameters affecting physiologic stress (Portz et al., 2006). In addition, fish held for a relatively short duration are also influenced by negative interactions associated with intraspecific and interspecific competition, such as cannibalism, predation, and determination of nascent hierarchies (Bass and Gerlai, 2007; Pickering and Pottinger, 1989, Vijayan and Leatherland, 1988). These interactions can be lethal (*i.e.*, predation) or may act as a vector for pathogens to enter (*i.e.*, bites and wounds).

To avoid fish mortality and improve productivity, the physiologic conditions of cultured fish, including the stress state, should be monitored. An understanding of the stressors affecting fish holding can lead to practices that reduce stress and its detrimental effects. To accurately determine the health status of fish, monitoring fish blood has become a recent focus in fish physiology. Measuring fluctuations in blood glucose in addition to cortisol levels is now one of the most widely used methods of monitoring stress in fish (Pickering and Pottinger, 1989, Barton and Iwama, 1991), especially acute stress because blood glucose levels return to basal levels within 24 h (Carmichael et al., 1984). Several studies have demonstrated that blood glucose levels closely correlate with stress levels in fish (Mazeaud et al., 1977, Silbergeld, 1974). Nowadays, the fish farmer draws blood from various individual randomly in fish farm for monitoring fish health. But it requires techniques and skills and fish blood glucose levels are currently measured using clinical laboratory test kits designed for humans. Blood-sampling procedures are invasive and may add unnecessary artificial handling stress to fish. Furthermore, as each sample must be obtained separately, testing is both time- and labor-intensive. Thus, behavioral changes in fish can be observed in real-time, but the physiologic indicators cannot be measured over time due to stress caused by handling the fish.

To address this problem, we developed a wireless monitoring biosensor system that allows for easy and rapid assay of blood substances in free-swimming fish (Endo et al., 2009) and we believe it will also be effective as conventional method in fish farm but in easier and rapid way. The wireless monitoring system comprises an enzyme biosensor and wireless transmission device. Different from the conventional method of obtaining blood samples, we focused on the fluid under the scleral surface of the eyeball (EISF) in fish as an implantable site, which strongly correlates with blood glucose levels (Yonemori et al., 2009). This needle-type enzyme biosensor-based monitoring system allows for continuous measurement of glucose levels in fish and provides a rapid response, good linearity, and good reproducibility (Takase et al., 2012; Takase et al., 2013). Further, the transmission device is so light that fish can swim freely during monitoring.

The present paper describes a new approach of monitoring "actual stress" in real time using our proposed biosensor system induced by various stress factors experienced by fish. It also shows a potentiality of our proposed biosensor system in fish physiology and fishery in practice. In free-swimming fish, we monitored changes in blood glucose levels during exposure of fish to various physical, biochemical, and behavioral physiologic stressors in real time to evaluate the different physiologic states accompanying each stressor and to clarify the relationship between stress factors and behavioral changes.

2. Materials and methods

2.1. Reagents

Glucose oxidase (from *Aspergillus niger*; E.C. 1.1.3.4, type VII-S; 147,000 unit g^{-1}) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Ammonia solution (25%), acetic acid, sodium nitrite, sodium nitrate, and 2-phenoxy ethanol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). D (+)-Glucose was dissolved in 0.1 M phosphate buffered saline (PBS) and allowed to mix by the rotation for 24 h before use. All other reagents used for the experiments were commercial-grade or laboratory-grade.

2.2. Plasma collection and glucose analysis using the conventional method

We used Nile tilapia (*Oreochromis niloticus*) as the test fish, cultured at Tokyo University of Marine Science and Technology. Blood glucose levels were measured. Each fish (body weight; 106.35–300.65 g, body length; 15–27 cm) was netted from the preserve and anesthetized with 400 ppm 2-phenoxy ethanol by bath exposure. Blood samples were collected from the caudal vein along the backbone by inserting a heparinized syringe fitted with a 23 G needle (0.65 mm × 25 mm) into the basal anal fin. The blood samples (100–200 μl) were then centrifuged (550 × *g*) for 10 min to separate the plasma. To minimize measurement error, the collection procedure was performed within 10 min for each fish. Each collected sample was transferred to a test tube and stored at −80 °C until analysis.

Glucose levels were determined as actual glucose concentrations using an enzymatic colorimetric method (C-II glucose test; Wako Pure Chemical Industries). When each sample (20 μl) was mixed with 3 ml PBS (pH 7.1) containing enzymes (glucose oxidase, horseradish peroxidase) and the color-producing reagent, the glucose was oxidized, producing gluconolactone and hydrogen peroxide. The hydrogen peroxide was reacted with 4-aminoantipyrine and phenol to produce a red color. Glucose concentrations were measured using a UV/vis spectrophotometer (JASCO Co., Tokyo, Japan).

2.3. Enzyme sensor preparation

The system comprised a Pt–Ir wire working electrode and an Ag/AgCl reference/counter electrode. As a working electrode, 15-mm Pt–Ir cylinders with a 178-μm radius was prepared by cutting strips of Teflon-coated wire. A sensing cavity was prepared by stripping the Teflon along the wire to expose 2.0 mm of metal. Copper wire was wrapped around the Teflon-coated surface as a lead wire. Ag/AgCl paste was applied to the Teflon wrapped around the copper wire, which was then used as a reference electrode/counter electrode. The tip of the wire was sealed with epoxy resin, leaving a 0.7 mm-long sensing cavity. The sensing cavity was dipped in 5% Nafion® solution and dried for 10 min. An enzyme solution containing 2.5 mg glucose oxidase and 6 mg BSA dissolved in 0.25 ml 0.1 M phosphate buffer (pH 7.8) was freshly prepared. The Nafion-coated electrode was dipped in the enzyme solution and air-dried for 10 min; this procedure was repeated twice. The sensor was placed in a Petri dish, and 0.05 ml glutaraldehyde (25%) was added to induce cross-linking between the