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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

glucose oxidase and BSA for 6 h. The sensors were soaked and stored in PBS (pH 6.5) overnight at 4 °C before use.

2.4. Sensor implantation and device immobilization

The sensor was connected to a wireless potentiostat (3102BP, Pinnacle Technology, Inc.) and covered with a waterproof polypropylene sheet. The waterproofed wireless potentiostat was attached to the dorsal and pectoral fins of the fish using nylon thread. The signal of the sensor output current signal was sent to a receiver (3100RX, Pinnacle Technology, Inc.) using a radio wavelength of 916.5 MHz The fishes were then transferred to an aquarium (45 cm × 45 cm × 30 cm). Blood samples were collected periodically from fish during the monitoring. Before the experiment, the water was maintained at 26 °C, aerated, and filtered. The glucose level was continuously monitored after stabilization of the sensor output current (over 12 h).

2.5. In vivo calibration

Continuous estimation of glucose levels was performed using a one-point or two-point *in vivo* calibration method. In this paper, we used the one-point calibration method represented by the following formula:

$$G(t) = \frac{I(t)}{S}$$

The sensor sensitivity (S), expressed in nA/(mg dl^{-1}), was determined from a single glucose level measurement in the plasma as the ratio between the concomitant sensor output current (I; expressed in nA) and the glucose concentration. Glucose concentration (G) was then estimated based on the sensor output current (I).

2.6. Stress response to transfer between aquariums

Stress in fish caused by physical disturbances encountered in aquaculture, such as handling and transport, evokes a variety of responses that may be adaptive or maladaptive (Barton, 2000, Hobby et al., 2000). To observe whether the transfer between the aquariums evoked fish stress and to test whether anesthesia suppressed the stress response to a certain extent, measurements were obtained during transfer of the fish between two aquariums with the same conditions (pH 6.8, water temperature 26 °C in fresh water). Two aquariums (A, B) with a water depth of 8 cm were prepared. In order to assure the measured significant variations of the glucose concentrations caused by the environment stressors, we stop feeding the test fish for 2 days to eliminate the influence from glucose absorption (following experiments are also applied). Tilapia (total length: 26 cm weight: 220.65 g, sensor immobilized) was used in the experiment after acclimation more than 15 h in aquarium A. Measurement was started once the fishes were returned to aquarium A and sensor output current become steady. We continuously monitored the blood glucose levels for 160 min. When the output current value of the sensor was stabilized (~30 min after returning to aquarium A), fishes were anesthetized and moved to aquarium B. Variations in the glucose levels were measured during and after the transfer.

2.7. Stress response to changes in dissolved oxygen

Because water is the respiratory medium for most fish, its dissolved oxygen content is the most critical and limiting water quality variable (Matthews and Berg, 1997, Kramer, 1987). In this experiment, fish stress from changes in dissolved oxygen was monitored using our proposed biosensor system. Two aquariums (A, B) with a water depth of 8 cm were prepared. Tilapia (total length: 21.5 cm weight: 170.00 g, sensor immobilized) was used in the experiment after acclimation more than 15 h in aquarium A. After insertion of the sensor in the test fish, measurement was started following return of the fishes to aquarium A and sensor output current become steady. Stopping the air pump in the aquarium and aerating nitrogen into the breeding water from a nitrogen cylinder dramatically reduced the levels of dissolved oxygen in the water. From that time we measured the stress of the test fish caused by changes in dissolved oxygen.

2.8. Stress due to water chemistry alterations

2.8.1. Stress response to pH changes in the breeding water

Water pH directly affects other water quality variables and thus fish health. At near-lethal or sub-lethal levels, such as pH 4.2–5.0 for various salmonids, H^+ acts directly on gill membranes, increasing ion permeability and generally causing a loss of Na^+ and Cl^- ions (Wendelaar Bonga, 1997). Glucose changes in the fish may not be readily detectable due to the short amount of time before the fish dies at near-lethal or sub-lethal levels. In addition, sometimes actual changes cannot be observed due to the sustained stress exposure caused by drawing blood. In this experiment, two aquariums (A, B) with a water depth of 8 cm were prepared and the pH set (aquarium A; pH 6.8, aquarium B; pH 4.5). Tilapia (total length: 26 cm weight: 250.46 g, sensor immobilized) was used in the experiment after acclimation more than 15 h in an aquarium. After stabilization of the sensor output current value, fishes were transferred to aquarium B for measurement of the variation of glucose levels. Exposure to the low pH in aquarium B continued for ~340 min.

2.8.2. Stress response to ammonia–nitrogen compounds

Nitrogen, acquired from food-based proteins, is continually turned over with the synthesis of new proteins, used for development and growth, and acts as an amino acid "sink" with excretion of nitrogenous wastes, such as urea and NH_3.

In this experiment, we monitored the fish stress response to total ammonia–nitrogen including ammonia, nitrite, and nitrate. Experiments to test the effects of these three factors were performed similarly with the same reagent concentration (25 mg L^{-1}), but only one substance was added at a time. In this experiment, two aquariums (A, B) with a water depth of 8 cm were prepared and the conditions set (A: normal breeding water B: normal breeding water with reagent added to 25 mgL^{-1}). Test fish (total length: 26 cm weight: 250.46 g, sensor immobilized) was used in the experiment after acclimation more than 15 h in normal breeding water (ammonia: 0.25 mg L^{-1}, nitrite and nitrate: 0 mg L^{-1}; all tested by Tetra test kits, Germany). After stabilization of the sensor output current value, fishes were transferred to aquarium B, and the variations in the glucose levels were monitored. Exposure was continued until the end of the experiment.

2.9. Stress response to interactions between individuals

Dominance hierarchies develop quickly in some species of fish, including Nile tilapia, and fish of lower status usually exhibit impaired feeding and poor growth as a result (Jørgensen et al., 1993). Because stress (i.e., with plasma cortisol increases) can reduce appetite, aggressive behavior may lead to cascading effects on fish growth and health. Aggressive interactions may also inhibit foraging time and activity, thereby reducing food intake (Brown et al., 1992, Ellis et al., 2002). Thus, it is important to determine fish status and eliminate aggressive behavior (isolate aggressive fish, *etc.*). It is not sufficient, however, to only observe fish behavior as it cannot be observed in the dark or from a distance. Therefore,

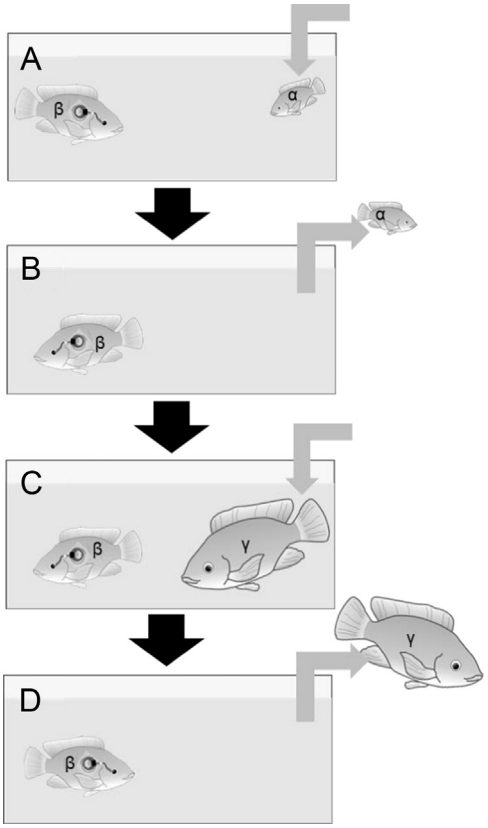

Fig. 1. Experiment design of stress response to interactions between individuals. Sensor was implanted in Test Fish β. (A) Test Fish α placed into the aquarium for ~5 h. (B) Test Fish α was picked up from the aquarium and Test Fish β occupied the aquarium for ~6 h. (C) Test Fish γ placed into the aquarium for 5 h. (D) Test Fish γ was picked up from the aquarium and Test Fish β occupied the aquarium for ~6 h.

a method that allows for both observation of fish behavior and physiologic changes is necessary. We therefore designed the experiment shown in Fig. 1. Three test fishes, arranged from α to γ (from small size to large size) were used in this experiment: Test Fish β (total length: 21.5 cm weight: 162.02 g) was immobilized by the biosensor and bred in the aquarium (normal breeding water) for over 2 days. When the sensor output current value was stabilized, Test Fish α (total length: 19.3 cm weight: 143.02 g) was transferred to aquarium and picked up after ~5 h. After a period of time (~6 h), Test Fish γ (total length: 25.5 cm weight: 247.12 g) was transferred to aquarium and picked up after ~4 h. Finally, Test Fish β was maintained in the aquarium for another period time and the changes in its glucose levels were monitored.

2.10. Stress response to visual stimuli

Fish can sometimes be stressed by visual stimuli (Olla and Davis, 1989). Over a long period of time, behavioral changes can be detected in fish in real-time, but the physiologic indicators cannot be measured using conventional methods due to the blood collection process. In this experiment, we examined tilapia without handling each individual. This was an uninterrupted experiment and we not only observed behavioral changes due to stress caused by visual stimuli, but we also measured internal glucose levels using our sensor system. We prepared an aquarium (60 cm × 30 cm × 35 cm) with a partition plate to separate the two sides of the aquarium. After immobilization of the sensor, Test Fish δ (total length: 21.5 cm weight: 170.02 g) was bred on one side of the aquarium for more than 15 h. After the sensor output current value was stabilized, we placed Test Fish ε (total length: 26.5 cm weight: 250.24 g), which had a bigger size than Test Fish A, on the other side of the aquarium. Then, we measured the variations in the output current value of the sensor to estimate the change in glucose levels in the fish and thus the stress experienced by the fish.

3. Results and discussion

3.1. Transfer between aquariums

The findings from real-time stress monitoring of the effects of fish transfer between aquariums are shown in Fig. 2. The fabricated sensor was inserted into a living fish for approximately 15 h prior to the measurement to assure the accuracy of the sensor and applied in the experiments. Blood glucose levels were continuously monitored for 160 min. The calibration curve was used which introduced in our published paper (Endo et al., 2009) to calculate from sensor output current into estimated glucose levels. To obtain an estimate of the precision and accuracy of the sensor readings, we applied the one-point calibration method. One-point calibration was used as calibration value (G1, I1) in which G1 was the initial blood glucose concentration (48 mg dL^{-1}) and I1 was the subsequently obtained sensor output current (16.93 nA). The theoretical background current calculated using the one-point calibration method is important for determining accurate sensor sensitivity (*S*) for short-term *in vivo* glucose monitoring. Because the sensor was performed under a stable background current, the sensor-estimated glucose based on one-point calibration method can be applied. The stability and reproducibility of the sensor were discussed in our previous study (Endo et al., 2009) and actual glucose levels were obtained from the conventional method.

The calibrated blood glucose level showed changes similar to those of the actual blood glucose level. Neither of the methods induced a large change in the blood glucose levels. These findings indicate that the transfer of anesthetized fish between aquariums with the same water conditions does not cause a large change in the glucose levels. Further, the fish stress status could be monitored under a relatively stress-free condition using our proposed biosensor system after an overnight adaptation period.

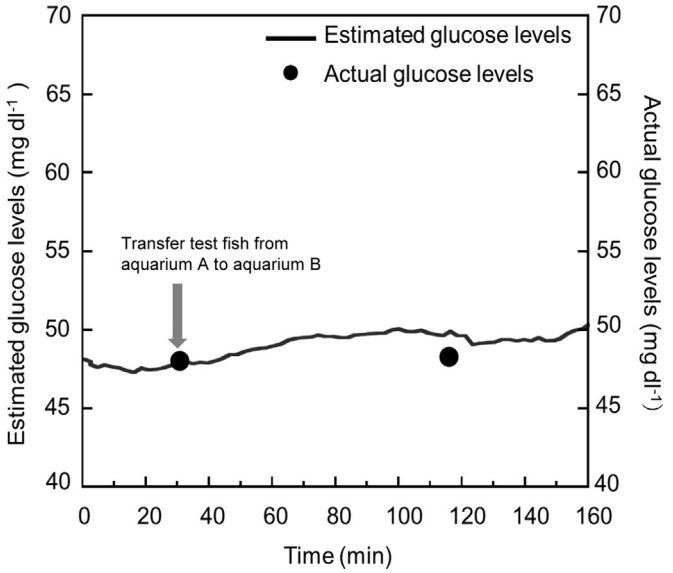

Fig. 2. Stress monitoring of the effects of fish transfer between aquariums. Glucose concentrations in the blood ~30 min, plotted as black circles, were used for the one-point calibration method.

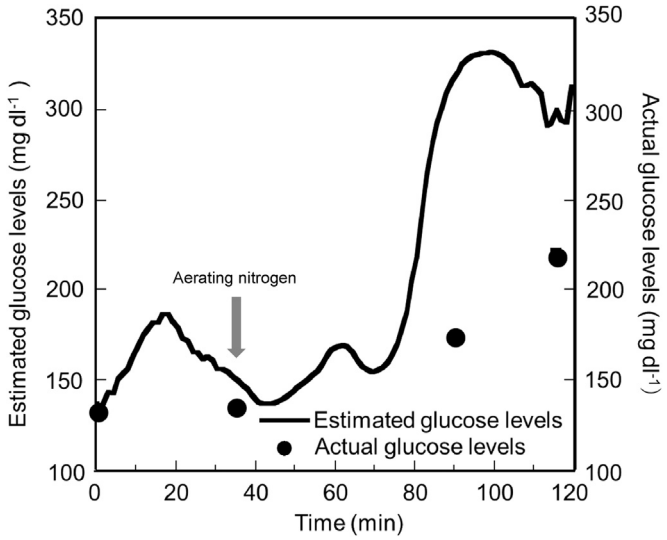

Fig. 3. Stress monitoring of the effects of change in dissolved oxygen. Glucose concentrations in the blood at 0 min, plotted as black circles, were used for the one-point calibration method. Dissolved oxygen concentration changed from 7.8 ppm to 3.1 ppm.

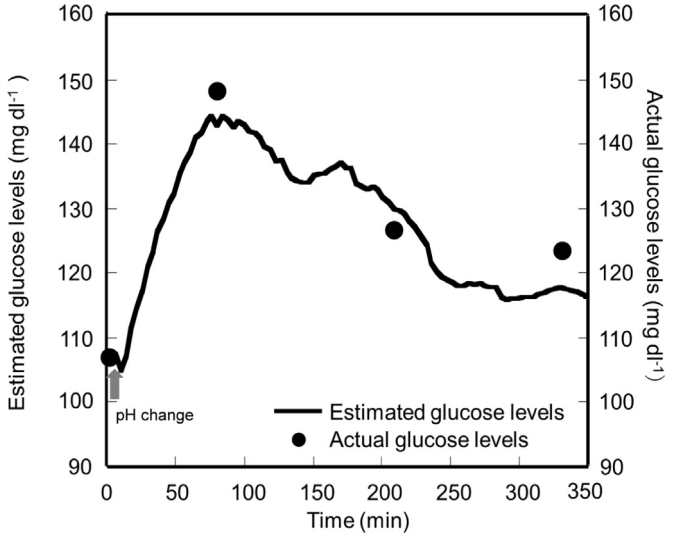

Fig. 4. Stress monitoring of the effects of change in pH. Glucose concentrations in the blood at 0 min, plotted as black circles, were used for the one-point calibration method. pH changed from 6.8 to 4.5.

3.2. Change in dissolved oxygen

For organisms that live in air, the relatively high concentration of oxygen means that oxygen is likely limited only during periods of intense demand; in general, activity is more likely to be limited by food than by the oxygen supply. Oxygen, however, is much more likely to be limiting for aquatic organisms than for terrestrial organisms, due in part to the lower availability of oxygen in water and the high density and viscosity of water, which increase the costs of extraction. When the oxygen supply is insufficient to meet the minimal energy demands of essential functions, suffocation occurs. Carlson et al. (1980) reported that channel catfish, *Ictalurus punctatus*, appeared "stressed" after feeding under anoxic conditions. The temporal changes in glucose levels according to changes in dissolved oxygen over 120 min are shown in Fig. 3. Calibrated blood glucose levels exhibited the same trend as actual glucose levels. When dissolved oxygen was artificially decreased, we observed similar trends in the blood glucose levels of Nile tilapia, reported by Evans et al. (2003). The calibrated blood glucose level increased with a decrease in the oxygen concentration, demonstrating that anoxic conditions with the addition of nitrogen stressed tilapia and the wireless monitoring successfully reflected the stress level experienced by fish in real-time.

3.3. Alterations of water chemistry

3.3.1. Change in pH

Exposure to chemicals may also affect the stress response by interfering with specific neuroendocrine control mechanisms. External factors, such as water pH, mineral composition, and ionic calcium levels, significantly impact stressor intensity. To better understand the effects of water pH on the fish stress response, we measured temporal changes in the glucose levels according to pH variation using our proposed biosensor system (Fig. 4; arrow indicates point at which test fish was transferred from normal breeding water [pH 6.8] to weak acid water [pH 4.5]). The results confirmed that glucose levels increased sharply within 20 min of exposure to the stress cause by a sudden change in pH. After ~70 min, the blood glucose levels gradually decreased even when the fishes were bred in the same low pH water. After a short exposure during breeding, the fish may adapt to changes in the water pH as well as to the stress caused by the change in pH.

3.3.2. Change in ammonia–nitrogen compounds

Under natural conditions, total ammonia–nitrogen (including ammonia, nitrite, and nitrate) is added to the water from fish waste, the accumulation of dead organisms, and other debris from anthropogenic sources such as industries and agriculture. Short-term exposure to an elevated ammonia concentration leads to increased gill ventilation, erratic and quick movements, lack of foraging, and even mortality, in fish (Meade, 1985). Nitrite is an intermediate product between ammonia and nitrate *via* the nitrification process. Nitrite is toxic to fish, especially if allowed to accumulate or when fishes are chronically exposed to low nitrite levels, and the impact is influenced by body size, species tolerances, and by other water quality variables (Lewis and Morris, 1986). Nitrate is the final product of the nitrification process. Although this compound is the least toxic of the three, it may affect fish gas exchange. In this experiment, we monitored all three of these ammonia—nitrogen compounds and observed their effects on fish stress. As shown in Fig. 5(A, B), ammonia and nitrite, which have a relatively high toxicity, are strong stress factors for fish. Based on Fig. 5(C), nitrate is unlikely to be much of a stressor for fish.

3.4. Monitoring stress effects on fish behavior physiology

3.4.1. Interactions between individual fish

Fish display altered and agonistic behaviors as a result of stocking density and confinement. Behaviors to indicate social rank and aggressive intent include visual stimuli, such as darkened body coloration, raised fins, and increased swimming activity, as well physical threats, biting, nipping, and charging (Brown et al., 1992; Ellis et al., 2002). Male tilapia have very strong territory behavior and we took advantage of this characteristic to observe the test fish behavior following stress induced by another individual, and examine the correlation between the stress response and the glucose levels measured using our sensor system in real-time (Fig. 6(A)). When Test Fish α (smallest size) was placed into the aquarium, Test Fish β (middle size, sensor implanted) did not exhibit a marked change in glucose levels. We observed that if a threat is not acknowledged (Test Fish α), then the attacker (Test

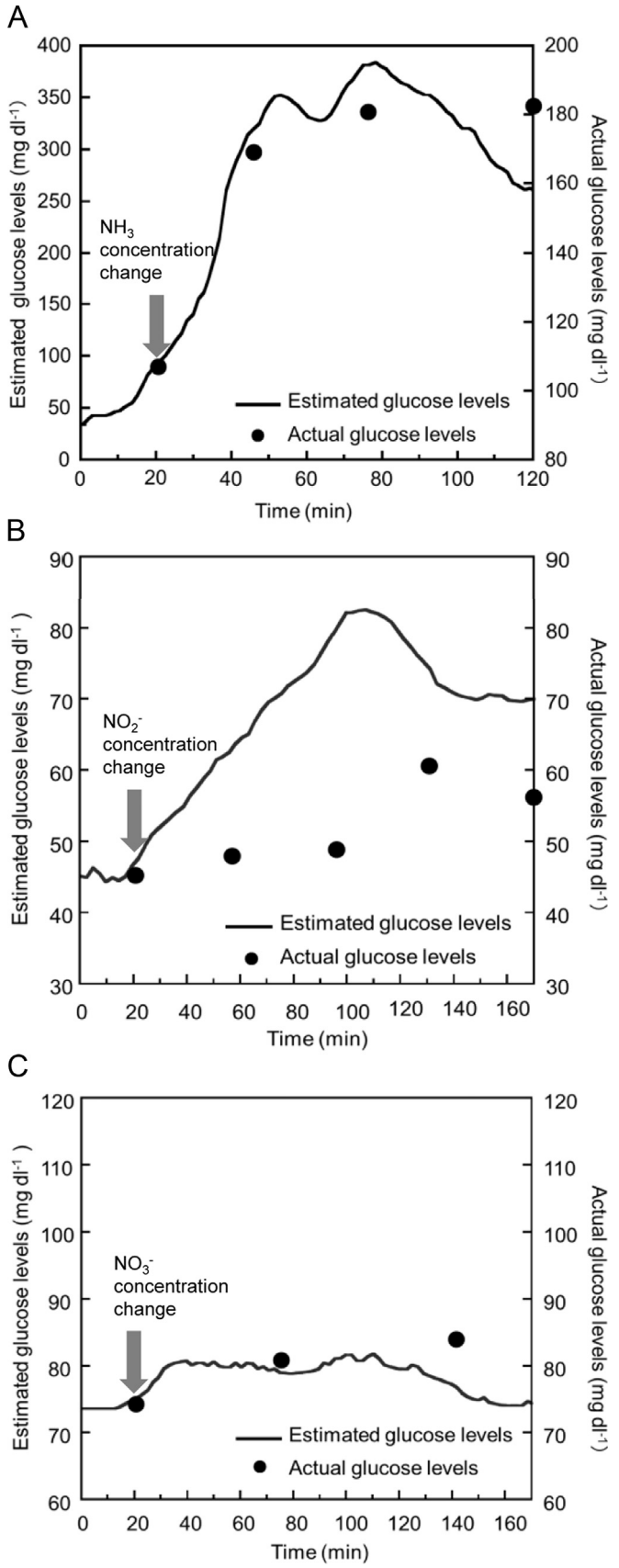

Fig. 5. Stress monitoring of the effects of change in ammonia–nitrogen compounds concentration. Glucose concentrations in the blood ~20 min, plotted as black circles, were used for the one-point calibration method. (A) Ammonia concentration changed from 0.25 mg L^{-1} to 25 mg L^{-1}. (B) Nitrite concentration changed from 0 mg L^{-1} to 25 mg L^{-1}. (C) Nitrate concentration changed from 0 mg L^{-1} to 25 mg L^{-1}.

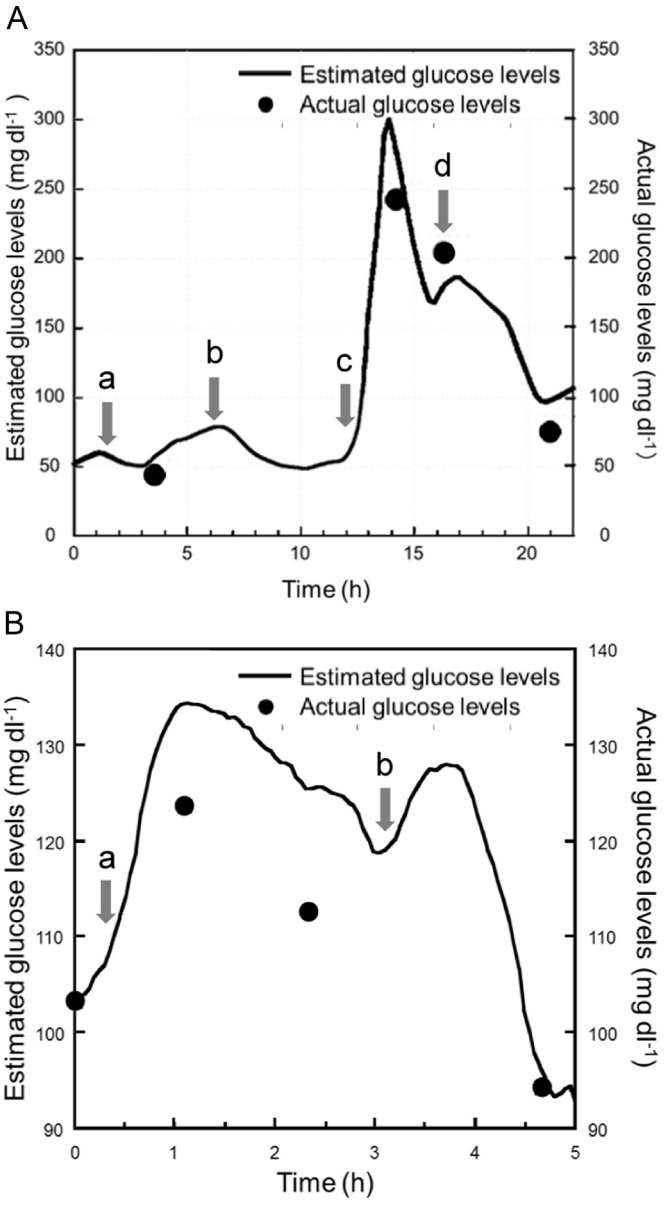

Fig. 6. Stress monitoring of the effects on fish behavior physiology. (A) Interactions between individual fish. The curve shows the change of glucose concentration of Test Fish β. (a) Test Fish α was placed into the aquarium. (b) Test Fish α was taken out from the aquarium. (c) Test Fish γ was placed into the aquarium. (d) Test Fish γ was taken out from the aquarium. Glucose concentrations in the blood ~3.5 h, plotted as black circles, were used for the one-point calibration method. (B) Visual stimuli between individual fish. The curve shows the change of glucose concentration of Test Fish δ. (a) Test Fish ε was placed into the aquarium. (b) Test Fish ε was taken out from the aquarium. Glucose concentrations in the blood at 0 min, plotted as black circles, were used for the one-point calibration method.

Fish β) will proceed to nip and bite until the submissive fish is displaced or signals its subordinate status. This observation indicated that Test Fish β had a higher social rank in this situation and did not experience the stress of the other smaller individual, thus the glucose levels change only in accordance with normal physiologic needs and occasional attack behavior. Before Test Fish γ was placed into the aquarium, Fish β existed in a relatively stress-free condition and thus showed no significant difference in glucose levels. Following placement of Test Fish γ (*big*gest size) into the aquarium, however, Test Fish β exhibited a marked rise in glucose levels. Test Fish γ was very aggressive and occasionally attacked Test Fish β. Based on these observations, aggressive social

interactions cause physiologic stress responses in subordinate fish but not in dominant fish. Aggressive social interactions cause physiological stress responses, can reduce appetite, aggressive behavior can result in cascading effects on fish growth and health (Ellis et al., 2002).Therefore, not only chemical environment but also social interactions should be focused on modern fishery. In most holding containers, each stressor is controllable to some degree. If fish physiological stress can be monitored in real-time, we can assume that behavioral impairments which has a strong relationship with physiological stress will be effectively minimized by improving the living environment in an early stage.

3.4.2. Visual stimuli

Vision is the dominant sense of many types of fish. Whether this sense dominates can often be predicted by the large size of the eyes of a species (Guthrie, 1986). In this experiment, we placed two fish of different sizes in one aquarium. When the bigger fish (Test Fish ε) was placed into the aquarium, the relatively smaller fish (Test Fish δ) exhibited markedly increased glucose levels (Fig. 6(B)). Removal of the bigger fish also induced an increase in the glucose levels of the smaller fish until a period of time which the reason we do nott know. From this we can understand that tilapia always have a stress response to what they have seen by their eyes and until the threat gone, they may keep in stress and show escape behavior.

In this experiment we have used a partition plate to separate the two sides of the aquarium but share the same breeding water. Based on this, tilapia may appear to perceive stress visually as well as through residual odors from other individuals in the water though the further discussion is necessary. Due to it is a possibility associated with odor, we cannot find the answer by our eyes but the actual real-time change of physiological indicator levels in fish.

4. Conclusions

In the present study, we investigated stress due to alterations in biochemistry, including dissolved oxygen, pH, and dissolved ammonia–nitrogen compounds. Stress perceived from behavioral interactions, such as physical contact and visual stimuli, was also monitored. Biochemical alterations induced increases in EISF glucose levels. Stress levels changed with behavioral interactions, such as avoidance, sensory stimuli, and activity. The present findings revealed that blood glucose levels in fish as an indicator of stress can be rapidly and conveniently monitored using our proposed biosensor in real-time. This is a novel and ideal method for the fish biologist for non-lethal monitoring of fish physiology, especially when handling and sampling techniques can have greater effects than the holding practice under study. Using the proposed wireless sensor system, stress in fish can be accurately evaluated and related to fish performance. Results obtained by this novel methodology may provide new knowledge in the field of fish behavior, culture biology, and fish physiology. The proposed biosensor system will be useful for rapid, reliable, and convenient analysis of “actual stress” in fish and accurately reflects the stress experienced by fish. In addition, cortisol concentration, which is also a stress indicator; blood lactate concentration, which is an indicator of fatigue; and blood cholesterol concentration, which is an indicator of disease resistance, can also be measured by various biosensors developed in our laboratory (Hibi et al., 2012; Takase et al., 2014). Through these measurements, the relationship between the stress response and these parameters can be elucidated. In this paper, we attempt that if the environment was changed into an unbearable point, fish will feel stress and reflect the stress from the variation of glucose concentration shows by our sensor system instead explore the stressors. However, the stress induced glucose concentration variations in this study are measured based on single stress source. In real situation, the stress is the complex reaction of various environmental conditions. It is very difficult to distinguish all the stressors from each other only by observe the trajectory of glucose concentration at this stage. In next step, we want to build a diary on breeding fish which breed in a synthetic composite environment and importing more physiological indicators into the measurement like cortisol and cholesterol, *etc*. We may understand which stressor will be the main factor caused the stress *e.g.* if we find a high level in glucose and low level in cholesterol, we ought to consider whether the fish were infected by bacteria and the infection of bacteria would be the stressors which experience by fish. The transmission radius of sensor in this paper is 10 m in air and attenuation will arise in water especially in seawater (Endo et al., 2010). To solve these problems, we are trying to devise a receiver which setting above the water or near the feeding site for getting the data from the sensor implanted on fish. Then, the computer gets the data from the receiver which the transmission will be carried out in air. We are also plan to develop some new transmission systems such as optics or ultrasound device which hold a better transmission capacity in water which can bring a better transmission radius and stability. On the basis of this knowledge, improvements to the aquatic environment and farming technology can be implemented to enhance fish-friendly fisheries.

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