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Expanding Applicability of Wireless Biosensor System for Monitoring Fish Stress Response through Abdominal Interstitial Fluid

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Fish do not change their expressions, unlike humans, making it extremely difficult to know their stress level. However, evaluating the stress response of fish in continuous aquaculture is essential because of their sensitivity to stress. A wireless biosensor system has been developed to easily visualize the stress response by monitoring the glucose level as its indicator. However, the system had a limited range of applicable fish species. The implantation site of the sensor, the interstitial fluid of the eyeball, is hardly found in saltwater fish. In this study, we focused on the implantation of the sensor in abdominal interstitial fluid to expand its range of applicable species. First, we confirmed the presence of glucose in the implantation site and confirmed that the biosensor operated normally even after immersion in the fluid. We also attempted to immerse the biosensor in the abdominal cavity of Nile tilapia (*Oreochromis niloticus*), then monitored the stress responses. Next, we implanted the biosensor in the abdominal cavity of the saltwater fish horse mackerel (*Trachurus japonicus*) and obtained some corresponding results, thus demonstrating that the biosensor can also be used in saltwater fish, expanding its application.

1. Introduction

Safe and efficient rearing methods are required in fish-rearing environments such as aquaculture farms and aquariums. For example, impaired water quality due to residual food, excreta, and overcrowding can create stressful conditions.[1–3] Long-term fish rearing in such a stressful environment can cause fish diseases.[4,5] Stress is a general term proposed by Selye that applies to a situation in which a person or an animal is subjected to a challenge that may result in a natural or symbolic danger to its integrity.[6] Such stress must be avoided as it may weaken the

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immune system of fish, leading to their death. Although chemicals are administered to prevent stress and related health problems, they may also have adverse effects on the fish.[7,8]

Some of the components released in blood, such as cortisol and glucose, may reflect the stress response. When fish are under stress, the cortisol level increases as a primary response and the glucose level increases as a secondary response.[9–12] Therefore, stress has been visualized by measuring the blood glucose level of fish. Traditionally, the colorimetric method has been used for this measurement.[13] This method requires blood sampling, which includes stimulation by syringe insertion, capture, and anesthesia. This process inevitably increases the stress of fish and becomes a stressor. In addition, the blood glucose level can only be measured at the time of blood sampling. That is, it is impossible to continuously assess the stress response.

To solve these problems, we previously developed a glucose biosensor to measure the stress response without blood sampling; glucose biosensors measure the amount of glucose by using enzymes that react specifically with glucose.[14] The sensor-calibrated and actual blood glucose levels were found to be in excellent agreement.[15] The sensor continuously measures the blood glucose level on the basis of an enzyme reaction, enabling the real-time monitoring of the stress response. It can also be used on free-swimming test fish, reducing the stress caused by handling. However, blood is unsuitable for monitoring the stress response of fish because of the decreased sensor output resulting from blood coagulation and the coalescence of blood proteins at the sensor placement site. Therefore, instead of blood, we focused on eyeball interstitial fluid (EISF), an interstitial fluid in the outer membrane of the eye, whose glucose level correlates with the blood glucose concentration.[16] This interstitial fluid also has the advantage of fewer foreign substances than blood. Biosensors have been implanted in EISF to measure stress responses.[17–19] However, most of the fish species with EISF are freshwater fish, limiting the application of the system to freshwater fish. This means that biosensors are difficult to use for saltwater fish without EISF.

On the other hand, the proportion of saltwater fish is currently higher than that of freshwater fish in the aquaculture industry, especially in Japan.[20] Developing a sensor that can be applied to saltwater fish is essential to increase the number of sites where biosensors can be used. In this study, we focused on the abdominal interstitial fluid (AISF) in the abdominal cavity as a new implantation site as an alternative to EISF to realize stress response monitoring in more fish species.

AISF is medically classified as an interstitial fluid similarly to EISF.[21] It is responsible for the supply of nutrients and the transport of excreta, and its glucose level is also expected to correlate with the blood glucose level. Furthermore, in humans, the glucose level in ascites fluid, synonymous with AISF, correlates with the blood glucose level.[22] Therefore, stress response monitoring in AISF is expected to be feasible and to promote stress research in various fish species, including saltwater fish.

2. Materials and Methods

2.1 Reagents

Glucose oxidase (GOx, from *Aspergillus niger*; E.C. 1.1.3.4, type VII-S; 147,000 units g^{-1}) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Nafion®

dispersion solution (DE521 CS type, 5 wt%), glutaraldehyde (grade I, 25% aqueous solution), glucose CII-Test, 2-phenoxy ethanol, sodium chloride, ammonia solution (25%), and heparin sodium were purchased from Wako Pure Chemical Industries (Osaka, Japan). All the other reagents used for this research were of commercial or laboratory grade.

2.2 Test fish

For the tested freshwater fish, we used Nile tilapia (*Oreochromis niloticus*) cultured at Tokyo University of Marine Science and Technology, following the Guide for the Care and Use of Laboratory Animals of Tokyo University of Marine Science and Technology. The aquarium was equipped with an upper filtration tank and constantly aerated with an air pump. The room temperature was 25.0 °C and the lighting hours were 9:00–18:00.

For the tested saltwater fish, we used horse mackerel (*Trachurus japonicus*) fished in Tateyama Bay and bred at the Tateyama station of Tokyo University of Marine Science and Technology. The fish were reared in an aerated environment with seawater pumped from offshore stations. The water temperature was 25.5 °C.

2.3 Biosensor preparation

The biosensors were prepared using a 10-mm-long Teflon-coated platinum–iridium (Pt–Ir) wire (diameter: 0.178 mm) as the working electrode and Ag/AgCl paste (BAS, Tokyo, Japan) as the counter/reference electrode. The Teflon was stripped from one end to expose 1.0 mm of the Pt–Ir wire. A copper wire was coiled around the Teflon-coated surface. The Ag/AgCl paste was applied to the area where the copper wire was twisted. The sensor tip was dipped in a 5% Nafion® dispersion solution and air-dried for 10 min. GOx (5,650 units mL^{-1}) and BSA (6.0 mg) were mixed with 0.25 mL phosphate buffer (PB, 0.1 M, pH 7.8) in the enzyme solution. The Nafion®-coated electrode was dipped in the enzyme solution and air-dried for 10 min. This procedure was carried out three times. The sensor was placed in an enclosed space and maintained at 35 °C, and 0.05 mL glutaraldehyde (25%) was added to induce cross-linking between the glucose oxidase and BSA for 6 h. The sensor was placed in the PB and stored in a refrigerator at 4 °C. Before use, a standard glucose solution (5,000 mg dL^{-1}) was added dropwise to confirm the sensor response to glucose. The sensor output current was measured when the glucose level in the measurement solution was increased in a stepwise manner.

2.4 Collection of EISF and AISF

An injection needle (23 G) was inserted into the infraorbital membrane of the eye of Nile tilapia, and approximately 0.3 mL EISF was collected. The sample was placed in an Eppendorf tube and stored in a refrigerator (4 °C). Similarly, an injection needle (23 G) was inserted into the abdominal cavity of Nile tilapia, and approximately 0.3 mL AISF was collected. The sample was placed in an Eppendorf tube and stored in a refrigerator (4 °C).

2.5 Collection of plasma

An appropriate amount of heparin sodium solution (3000 units mL^{-1}) was placed in a syringe fitted with a needle. The needle was inserted from the posterior edge of the anal fin of Nile tilapia toward the vertebrae, and approximately 0.8 mL blood was collected. The blood was placed in an Eppendorf tube and centrifuged (800 × g, 10 min, 4 °C). Then, the supernatant plasma sample was dispensed and stored in a refrigerator (4 °C).

2.6 Effects of AISF immersion on sensors *in vivo*

We held Nile tilapia so that the abdominal surface faced upward, and we removed the scales. The descaled area was widened using an indwelling animal needle (14 G) and a gimlet. After widening, a sensor with a waterproof wireless transmitter was implanted in the fish and fixed with Aron Alpha®. After implantation, the test fish was transferred to an isolation tank. It was then anesthetized again, and the sensor was removed. Before and after the sensor implantation, its glucose characteristics were examined by the dropwise addition of a standard glucose solution into PB. Calibration curves for each time were prepared for comparison before and after *in vivo* implantation.

2.7 Stress monitoring system

A simplified diagram of the experimental system is shown in Fig. 1. The wireless stress response monitoring system consisted of a receiver, a personal computer (PC), a USB receiver (ToCoStick, Monowireless Corporation, Kanagawa, Japan), and a wireless transmitter (23 × 40 mm^2). The waterproof wireless transmitter was connected to the sensor. A voltage (+650 mV) set on the PC was applied via a wireless transmitter, and the measured output current was received by the USB receiver and displayed on the screen of the PC in real time.

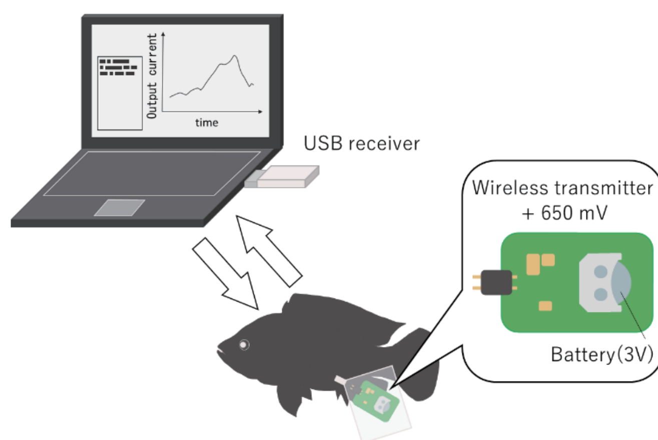

Fig. 1. (Color online) Experimental setup using a wireless biosensor system.

2.8 Implantation in EISF and AISF

An injection needle (23 G) was inserted into the EISF behind the eye of the test fish, and a hole was made in the EISF. The sensor was inserted through the hole and fixed with bioadhesives. For the implantation in AISF, we held the test fish so that the abdominal surface faced upward and removed the scales. The descaled area was widened with an indwelling animal needle (14 G) and a gimlet. After widening, a waterproof sensor with a wireless transmitter was implanted in the fish and fixed with bioadhesives.

2.9 Stress response monitoring of freshwater and saltwater fish

An experimental tank (450 × 300 × 300 mm^3) containing 25 L of rearing water was prepared and aerated. Test fish (Nile tilapia, *Oreochromis niloticus*) was selected from the rearing tanks and transferred to the prepared experimental tank for acclimation. The test fish was fasted for at least 12 h before the start of stress response monitoring to suppress the increase in glucose level caused by feeding. Next, the sensor was implanted in the abdominal cavity of the fish using the method mentioned above. After monitoring for 15 min, we started to force swimming by stimulating the caudal fin using a stick with a length of approximately 30 cm. After 3 min of forced swimming, the stress response was continuously monitored until the output current returned to the prestress load base value.

For the saltwater fish, an experimental tank (1400 × 600 × 490 mm^3) containing 200 L of seawater was prepared and aerated. Test fish (horse mackerel, *Trachurus japonicus*) were acclimatized to the experimental tank. The test fish was fasted for at least 12 h before the start of stress response monitoring to suppress the increase in glucose level caused by feeding. Next, the sensor was implanted in the abdominal cavity of the fish using the method mentioned above. Stress response monitoring was started after the sensor output current was confirmed to be stable. After monitoring for 15 min, we started to force swimming by stimulating the caudal fin using a stick. After 3 min of forced swimming, the stress response was continuously monitored until the output current returned to the prestress load base value.

3. Results and Discussion

3.1 Comparison of glucose levels in each sample

The glucose levels in AISF, EISF, and plasma samples are shown in Table 1. First, glucose was present in the AISF of Nile tilapia. This may be because the peritoneum is lined with

Table 1
Glucose level of each sample.

Sample	Glucose level (mg dL^{-1})
AISF	35.8
Plasma	32.6
EISF	17.6

intercostal arteries and veins, and the visceral organs of the abdominal cavity are lined with the celiac integumentary artery, which also supplies glucose to the AISF.

There were some differences among the glucose levels of the samples. The glucose level was highest in AISF, followed by plasma and EISF. This difference may be due to differences in glucose transport mechanisms at the site of each body fluid.

3.2 Stress response monitoring of freshwater fish

Figure 2 shows the monitored stress response of Nile tilapia with the sensor in AISF. The shaded area indicates that the fish were under stress loading. The output current increased after the start of stress loading and gradually decreased after the end of stress loading to the value before stress loading. This suggests that the glucose level in AISF increases in response to stress loading and then recovers, indicating that AISF is an appropriate target for the glucose biosensor. On the other hand, Fig. 2 shows that the response decreases before the end of stress loading. This is because the fish consumed glucose because of the swimming exercise, and the rate of glucose consumption exceeded the rate of glucose production associated with stress loading, resulting in a decrease in total glucose content.

In addition, the implanted sensor did not become detached in this experiment, and stable measurement was possible. Since the biosensor is expected to be used for a long time, our experiment confirmed that the abdominal cavity is an appropriate location for implantation, because firm implantation and stable measurement are required for glucose monitoring.

3.3 Characterization of glucose biosensors in AISF

To see if there is any impact of *in vivo* measurements on the ability to measure, the sensors were characterized before and after immersion in AISF. The measurement method was described

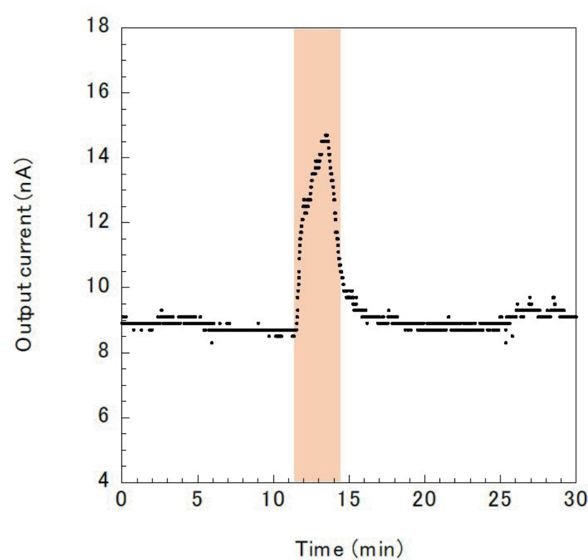

Fig. 2. (Color online) Monitored stress response of Nile tilapia (AISF, forced swimming).

in Sect. 2.3, and the obtained results are shown in Fig. 3. The solid line shows the graph before immersion, and the dotted line shows that after immersion. Both lines show that the output current of the sensors increases with the glucose level. There was a strong correlation between the glucose level and the increase in output current, and this correlation was similar before and after immersion. These results confirm that the sensor can measure changes in glucose level even after immersion.

This suggests that the glucose biosensor can measure changes in glucose level over time without being significantly affected by immersion in the abdominal puncture or by substances contained in AISF.

3.4 Stress response monitoring of saltwater fish

The glucose biosensor retained its good sensing characteristics after the experiment with Nile tilapia, a freshwater fish. A subsequent experiment was conducted with horse mackerel, a saltwater fish, under the same conditions. The monitored stress response of the horse mackerel is shown in Fig. 4. The output current increased after the start of stress loading and decreased after the end of stress loading. This may be because the fish tended to rapidly run away immediately after the start of forced swimming during the stress response measurement. However, they soon became exhausted, and their swimming slowed. This can be attributed to stress loading on the fish due to the forced swimming. In saltwater fish, glucose concentrations increase after forced exercise,[23] as also indicated by the experiment in this study. Our results suggest that the sensor in AISF can measure stress responses in freshwater and saltwater fish.

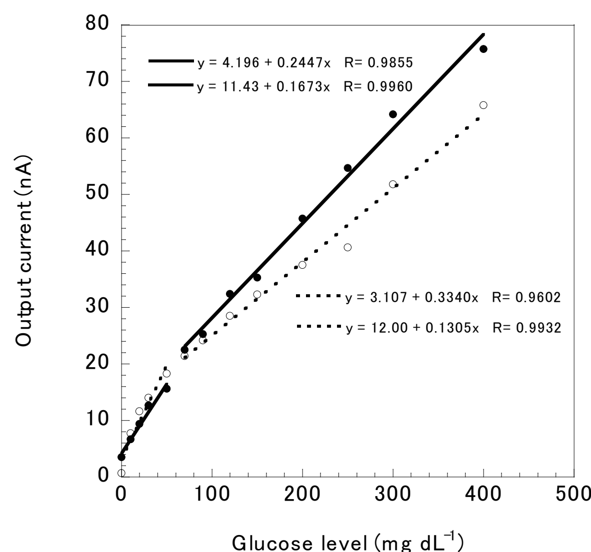

Fig. 3. Results of characterization before and after immersion under *in vivo* conditions.

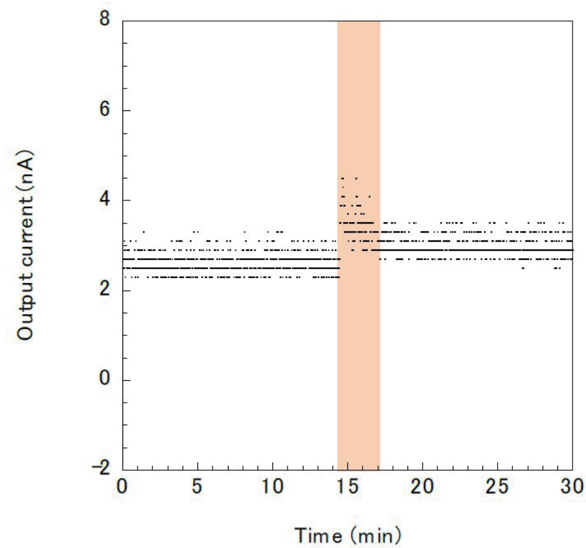

Fig. 4. (Color online) Monitored stress response of horse mackerel (AISF, forced swimming).

4. Conclusions

We experimentally investigated the possibility of using AISF as a new sensor implantation site for the real-time stress response monitoring of freshwater and saltwater fish. The findings of this study are described as follows.

First, the glucose level in AISF was measured; glucose was present in AISF, and its level was higher than those in plasma and EISF. On this basis, we suggested that the glucose level could also be measured in the AISF, EISF, and plasma.

Next, to confirm that a glucose biosensor can be used to measure glucose in AISF, a sensor was implanted in the abdominal cavity of Nile tilapia, a freshwater fish. The output current increased after stress loading, suggesting that the measurement is possible even when the sensor is placed in AISF.

We also investigated the effect of immersion in AISF on the sensor. To confirm the sensor response to glucose, its output current was measured when the glucose level in the measurement solution was increased in a stepwise manner by the dropwise addition of a standard glucose solution. Then, a calibration curve showing the increase in output current with the glucose level was drawn. A strong correlation was observed between the glucose level and the increase in output current.

Finally, to investigate the possibility of using a sensor implanted in the abdominal cavity to measure stress responses in saltwater fish without EISF, a sensor was implanted in the abdominal cavity of horse mackerel. The sensor response increased during stress loading, then decreased after stress loading, suggesting that the sensor implanted in the abdominal cavity measures the stress response to the stressor. The sensor will be subjected to further experimentation towards realizing its full-scale application in saltwater fish in the future.

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