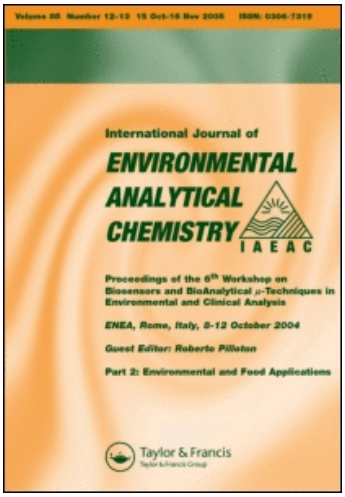

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Electrode-based immunologic assay system to monitor oocyte maturation-inducing hormone in fish

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A novel immunologic method using antigen-antibody reactions and electrochemical measurement to monitor the oocyte maturation-inducing hormone 17, 20β-dihydroxy-4-pregnen-3-one (DHP) in fish was developed. The electrode-based immunologic assay (EBIA) system was based on the competition between DHP and an acetylcholinesterase (AChE)-labelled DHP for a limited number of ant-DHP binding sites. DHP in a sample solution and acetylcholine esterase-labelled DHP were added to the anti-DHP-coated reaction vessel to promote a competitive assay. After one-hour incubation, the vessel was drained and washed five times with a washing buffer. Then, the reaction vessel was incubated with the substrate, acetylthiocholine, for a further 1.5 h. The Pt-Ir electrode was then immersed in the reaction vessel and the current generated at +650 mV at the electrode was used as the analytical signal of the EBIA system. A good correlation was observed between the output current and DHP concentration (1.95–250 pg ml^{-1}) using this method. Optimal system performance was achieved under the following conditions: temperature 26°C, ATCh concentration 10mM. One measurement could be completed within 5 min using the microelectrode and the total assay time, including sample preparation, was less than 3 h. The system was applied to monitor DHP in spawner (rainbow trout) during one month. There was a close correlation between our proposed system and the conventional method. The system can be used for a more rapid determination of the time of ovulation, compared with the conventional method.

Keywords: biosensor; immunoassay; electrode; fish; hormone; ovulation

1. Introduction

The increased demand for cultured fish as a food source will require improved productivity and egg production efficiency in fish farms. Several factors, such as the environment and variations in fish size, influence fertility and ovulation rates. Therefore, a simple, inexpensive, and field-applicable method for identifying the time of ovulation is desirable. There is a clear relationship between oocyte development and serum steroid hormones in a number of freshwater and marine teleosts [1]. Based on several studies, hormonal regulation of two important processes, oocyte growth (vitellogenesis) and final oocyte

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maturation, is generally accepted to be as follows. Vitellogenin (Vg), a yolk precursor protein, is synthesised in hepatocytes by estrogen stimulation, which is secreted from follicle cells surrounding the oocytes and excreted into the bloodstream. Vg is selectively incorporated into growing oocytes by receptor-mediated endocytosis, then proteolytically cleaved into yolk proteins and stored as yolk globules. The full-grown oocytes undergo final oocyte maturation by stimulation of C21 steroid hormone secreted from follicle cells. In salmonids (e.g. rainbow trout, sockeye salmon, amago salmon) [2–6] and cyprinids (e.g. silver carp, bitterling) [7–9], the maturation-inducing hormone has been identified as 17, 20β-dihydroxy-4-pregnen-3-one (DHP). Lou *et al.* [2] investigated changes in DHP levels in the rainbow trout *Oncorhynchus mykiss* during the breeding season. Low levels ($<20\,\mathrm{ng\,mL^{-1}}$) were maintained for several months and then the levels rapidly increased just before ovulation, indicating a clear relationship between DHP levels and ovulation and suggesting the crucial importance of monitoring DHP levels in the plasma to monitor seed production in fish.

Several methods for measuring steroid hormone levels, including DHP, have been reported, such as liquid chromatography-mass spectrometry, radioimmunoassay (RIA), and enzyme-linked immunosorbent assays (ELISA). Inoue *et al.* [10] developed analytical techniques for accurate quantification of bisphenol (a stabilising or antioxidant for plastics) in human semen samples using liquid chromatography–mass spectrometry with electrospray ionisation. The method allowed for sensitive detection of BPA with a detection limit of $0.5\,\mathrm{ng\,mL^{-1}}$. Although the method is highly sensitive and specific, but require time-consuming pre-treatment steps that do not allow for rapid processing of samples. Moreover, these instruments are complex and expensive, and are not easy to use for on-site measurement in aquaculture. Recently, for fishery sciences, RIA and ELISA were used to determine DHP levels. Rinchard *et al.* [11] determined DHP levels in plasma of the lake whitefish *Coregonus clupeaformis* by RIA. Radioactivity was counted in a toluene-based liquid scintillation cocktail on a Beckman LS3801 beta counter. In female ovulating lake whitefish, the levels of DHP varied widely from 0.2 to $13\,\mathrm{ng\,mL^{-1}}$. Asahina *et al.* [12] developed an ELISA technique using 3-carboxymethyle oxime of DHP conjugated horseradish peroxidase as a label and a homologous antiserum raised in rabbits. A calibration curve was obtained in the range of 30–3840 $\mathrm{pg\,mL^{-1}}$. The sensitivity and precision of their proposed ELISA method was almost the same as those of ordinary steroid RIA. Because of the requirement for radioisotopic labels, however, RIA is also associated with problems of radioisotope disposal and operation safety. Although a commercial ELISA kit is popular for determining DHP levels in the aquaculture field, the technique requires an expensive optical sensing element and is not convenient for use in outdoor areas, such as a fish farm, due to the sizes of the devices. Thus, the development of a new simple assay system as an alternative to the conventional methods is needed, especially for DHP measurement in fish farms.

Many biosensors have been developed and are widely used for determining levels of hormones and other important substances. We recently developed several biosensors and bioassay systems for use in fisheries and environmental research fields [13–19]. In particular, we have focused on the development of specialised biosensors for fish. For example, a wireless biosensor system consisting of a needle-type micro-electrode, glucose oxidase, and a wireless potentiostat, was developed to monitor blood glucose levels in fish. Glucose levels in fish blood can be monitored in free-swimming fish for 3 days using this new system. The sensor-calibrated glucose levels and actual blood glucose levels were in good agreement (0.18–144 $\mathrm{mg\,dL^{-1}}$) [17]. For the rapid detection of fish disease-causing

bacteria such as *Flavobacterium psychrophilum*, we have also developed new sensing methods using immunomagnetic separation coupled with flow cytometry [18,19]. In such work, carbonyl iron powder was used as magnetic beads for immunomagnetic separation [18]. Bacterial concentration was determined using this method, which correlate with 10–10^8 colony-forming units per milliliter obtained using a conventional colony-counting method [20]. Using immunomagnetic separation, one assay can be completed within 60 s and the total assay time, including sample preparation, is less than 2 h [18].

We have developed various bioassay systems that are specialised for use in the fishery environment. Our aim is to establish a wireless monitoring system for DHP under free-swimming conditions for fish as well [17]. As a first step, it is necessary to establish a new electrochemical system in which a biochemical reaction such as an antigen-antibody reaction is transformed into an electronic signal. As described above, although ELISA is popular for determining DHP levels in the aquaculture field, the method requires an optical sensing device. Because the device cannot be attached to fish due to its sizes and configuration, it is difficult to perform wireless monitoring under free-swimming conditions. Here we describe the development and use of an electrode-based immunological assay system (EBI assay system) utilising antigen-antibody reactions and electrochemical measurement to detect the oocyte maturation-inducing hormone DHP.

2. Experimental

2.1 *Reagent*

Maturation-inducing steroid (salmonid) enzyme immunoassay (EIA) antiserum (anti-DHP polyclonal antibody), and Maturation-inducing steroid (salmonid) EIA acetylcholinesterase tracer (AChE-labelled DHP), and DHP standard solution (25 ng mL^{-1}) were purchased from Cayman Chemical (Ann Arbor, MI, USA). The specificity of the anti-DHP polyclonal antibody was as follows; 17, 20β-dihydroxy progesterone: 100%, 17, 20α-dihydroxy progesterone: 0.04%, 20β-hydroxy progesterone: 0.1%, allopregnanolone: $<0.01\%$. SigmaUltra ethylenediaminetetraacetic acid tetrasodium salt hydrate, (tetrasodium EDTA), acetylthiocholine (ATCh; acetylthiocholine chloride, minimum 99% TLC), and bovine serum albumin (FractionV, approx. 99%; BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween 20 was obtained from Kanto Chem. Co. (Tokyo, Japan). All other reagents were of analytical grade. Various concentration of DHP standard solutions (1.95–250 pg mL^{-1}) and ATCh solution (2–20 mM) were prepared by diluting buffer consisting of 0.01% NaN_3, 2.34% NaCl, 0.037% EDTA, 0.1% BSA in 0.1 M phosphate buffer (pH 7.4).

2.2 *Fish*

Rainbow trout *Oncorhynchus mykiss* were reared in a concrete-lined pond ($2 \times 1 \times 1$ m) supplied with running well water at the Oizumi Station (Yamanashi, Japan), Tokyo University of Marine Science and Technology. Water temperature fluctuated between 10.5 and 13.5°C throughout the year. Fish were fed with commercial trout food. For blood sampling, fish were anesthetised with 2-phenoxyethanol (2 μg mL). A heparinised syringe fitted with a 20G needle was then inserted into the caudal vein of the fish to collect 1.0 to 2.0 mL of whole blood, which was transferred to a test tube. Each blood sample was

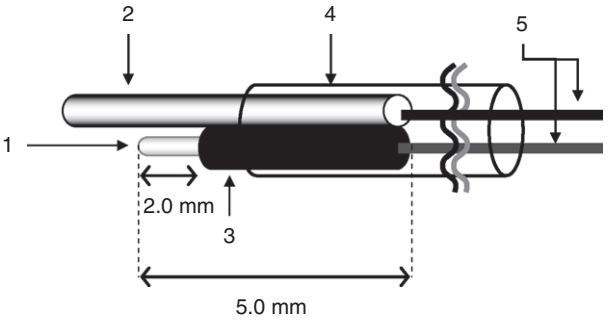

Figure 1. Schematic diagram of the microelectrode. 1. Platinum-iridium (Pt-Ir) wire working electrode. 2. Silver/silver chloride (Ag/AgCl) reference/counter electrode. 3. Teflon-coated Pt-Ir wire. 4. Heat-shrink tubing. 5. Lead wire.

centrifuged at 3000 rpm for 15 min to prepare a plasma sample. Plasma samples were separated from the resulting pellet and stored at −30°C until assayed.

2.3 *Microelectrode preparation*

Figure 1 shows a schematic diagram of microelectrode. A two-electrode electrochemical system with a platinum-iridium (Pt-Ir) wire working electrode and a silver/silver chloride (Ag/AgCl) reference/counter electrode was used. The working electrode was made using a 5 mm length of Teflon-coated Pt-Ir wire (diameter: 0.178 mm, Nirako Co., Tokyo, Japan). The Teflon was stripped at one end to expose 2.0 mm length of the Pt-Ir wire as the sensing surface. The other side of the Teflon-coated Pt-Ir wire was wound with lead wire, and sealed with heat shrink tubing and epoxy resin. The reference electrode/counter electrode preparation is described below. Ag/AgCl paste (BAS, Tokyo, Japan) was applied to the lead wire and dried. The tip of the wire was sealed with heat shrink tubing and epoxy resin to construct the working electrode. Two electrodes were then positioned together by heat shrink tubing (inner diameter: approximately 3 mm).

2.4 *Preparation of immobilised antibody*

Anti-DHP (50 μL; antibody) was added to several reaction vessels (volume: 350 μL) pre-coated with Mouse Anti-Rabbit IgG (Cayman Chemical, USA). The vessels were sealed and incubated for 18 h at 4°C. After removal of the unbound antibody, the vessels were washed five times with 200 μl washing buffer (0.1 M phosphate (pH 7.4) buffer and 0.05 vol% Tween-20). The reaction vessels were used for immune and electrochemical reactions in the EBIA system according to the procedures described below.

2.5 *EBIA system procedure*

Initially, a Pt-Ir electrode was immersed in 20 mL of 0.1 M phosphate buffer (pH 7.4) at 26°C. A potential of +650 mV (versus Ag|AgCl) was applied to the electrode to obtain a stable background current. Meanwhile, free DHP in a sample solution (50 μL) and

acetylcholine esterase-labelled DHP (50 μL) were added to the anti-DHP-coated reaction vessel to promote a competitive assay. After a 1 h incubation at 26°C, the vessel was drained and washed five times with a washing buffer. Then, the reaction vessel was incubated with 250 μL of 10 mM of the substrate, acetylthiocholine, at 26°C for a further 1.5 h. The Pt-Ir electrode was then immersed in the reaction vessel and the current generated at +650 mV at the electrode was used as the analytical signal of the EBIA system. A conventional amperometry technique was used for the final detection 5 min after the output current had stabilised.

2.6 *Conventional method for determination of DHP*

The conventional measurement of DHP was performed using an ELISA kit according to the manufacturer's instructions (Cayman Chemical, USA). This test kit is based on the principles of a competitive binding assay. The ELISA 96-Well microplate was coated with mouse monoclonal anti-rabbit antibodies. First, DHP in the plasma samples (50 μL) or DHP standard solutions (50 μL) were added to wells of a microplate. Then, a specific quantity of AChE-labelled DHP (50 μL) and the anti-DHP antibody (50 μL) were added to each well. 'Non-labelled' and 'labelled' DHP competed for a limited number of binding sites with anti-DHP antibodies overnight at 4°C. After incubation (ca. 12 h), unbound components were washed away by wash buffer comprising 0.1 M phosphate (pH 7.4) buffer and 0.05 vol% (v/v) Tween-20. Ellman's Reagent (200 μL), containing the substrate for the esterase enzyme, was added to each well and developed for approximately 90 min. The activity of bound AChE-labelled DHP was measured by reading absorbance at a wavelength of 405 nm using a plate reader.

3. Results and discussion

3.1 *Typical response curve of the EBIA system*

The EBIA system was prepared to monitor DHP in fish based on antigen-antibody reactions and electrochemical measurement. The principle of the system was based on the competition between unlabelled DHP and an acetylcholinesterase (AChE)-labelled DHP for a limited number of anti-DHP binding sites. Because the concentration of AChE-labelled DHP was held constant while the concentration of DHP was varied, the amount of AChE-labelled DHP that binds to the anti-DHP was inversely proportional to the concentration of DHP. After antigen-antibody binding, acetylthiocholine (ATCh) was added, and the ATCh was hydrolysed by the AChE to produce thiocholine, as follows:

$$\mathrm{ATCh} + \mathrm{H_2O} \xrightarrow{\mathrm{AChE}} \text{thiocholine} + \text{acetic acid} \quad (1)$$

Thiocholine was then detected by an electrocatalytic oxidation reaction at the sensing surface of the Pt-Ir working electrode, producing an electric output current, and regenerating O_2:

$$\text{thiocholine} \longrightarrow \text{dithio-bicholine} + 2\mathrm{H^+} + 2\mathrm{e^-} \quad [19]$$

Therefore, since the amount of AChE-labelled DHP bound to the vessel was inversely proportional to the amount of DHP present in the vessel, DHP concentration could be measured from the amount of thiocholine detected by the electrode.

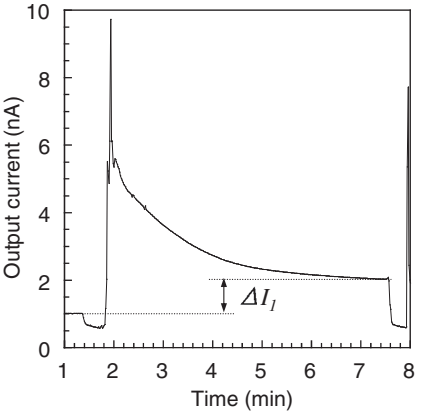

Figure 2. Typical response curves of EBIA system. The ATCh concentration, volume of ATCh, and temperature were 10 mM, 250 µl, and 26°C, respectively.

Initially, based on Li *et al.* [21], we used cyclic voltammetry (data not shown) to determine that +650 mV was the optimal potential for oxidation of ATCh at a Pt-Ir electrode. Figure 2 shows a typical response curve of the EBIA system for the DHP standard solution (10 pg mL^{-1}). When a potential of +650 mV was applied to the microelectrode immersed in 10 mM ATCh, the output current transiently increased from baseline, then gradually decreased and stabilised after 5 min. This was attributed to the thiocholine in the solution being hydrolysed by AChE, and the increase in thiocholine was measured at the microelectrode as an oxidation current. The response decreased with an increase in the DHP concentration. When a different concentration of DHP standard solution (1.95–250 pg mL^{-1}) was measured, the sensor response curve was similar within 5 min. In subsequent experiments, the current difference (ΔI) after 5 min between the base current obtained at a microelectrode in a buffer solution and the current obtained in a sample solution was used as an analytical signal in the EBIA system.

3.2 *Effects of analytical conditions on the output current*

The response of the EBIA system based on an enzyme reaction is influenced by analytical conditions such as substrate (ATCh) concentration, and reaction temperature. For this reason, it is necessary to establish optimal assay conditions to enhance the sensitivity of the system. The effects of these parameters on the response of the EBIA system were investigated. To confirm the influence of the enzymatic reaction, ultrapure water without DHP was used as sample. Figure 3 shows the effect of the concentration of ATCh on the output current. Various concentration of ATCh samples (2–20 mM) were prepared by diluting with 0.1 M phosphate buffer (pH 7.4). The output current increased with an increase in the ATCh concentration, but became less variable at ATCh concentrations above 10 mM. In this experiment, we investigated the saturating concentration of ATCh, because the principle of the system was based on the competition between DHP and an AChE-labelled DHP for a limited number of anti-DHP binding sites. Thus, an ATCh concentration of 10 mM provided a stable signal and was used for the enzyme reaction. Figure 4 shows the effect of reaction temperature on the output current. The output

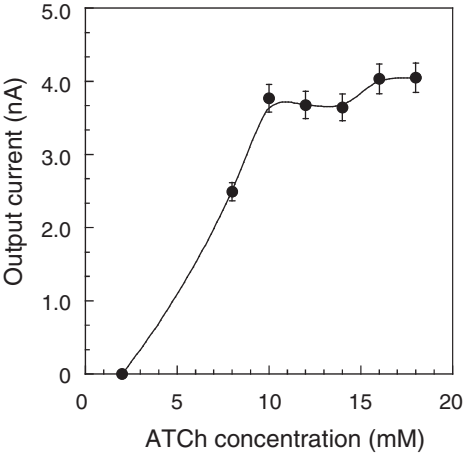

Figure 3. Effect of ATCh concentration on output current. The experimental conditions were the same as those described for Figure 2, except for ATCh concentration. The measurements were run in quadruplicate for one sample.

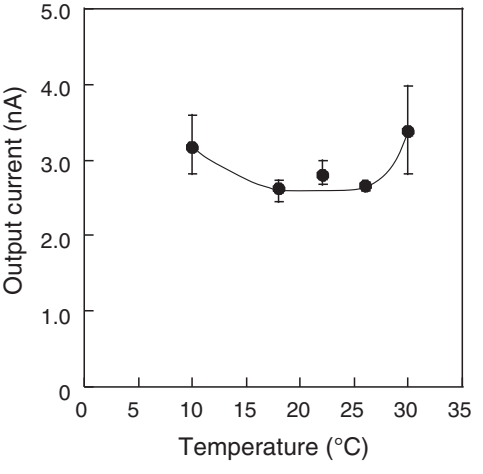

Figure 4. Effect of incubation temperature on output current. The experimental conditions were the same as those described for Figure 2, except for temperature. The measurements were run in quadruplicate for one sample.

response of the system was observed for temperatures ranging from 10 to 30°C. At 10 and 30°C, however, the response was unstable as indicated by the large error bars in the figure. The output current was stable for temperatures ranging from 18 to 26°C. Therefore, for subsequent experiments, the reaction was performed at 26°C, which had the least variable response. Under the same analytical conditions, the effect of ATCh concentration, temperature, and stability on the output current was very slight. The disparity in the sensor output was due to differences in the properties of the microelectrode used for each experiment. The optimum conditions for each sensor, however, could be determined.

3.3 *Microelectrode stability*

Microelectrode stability of the EBIA system is shown in Figure 5. The measurements were performed by immersing the microelectrode in one reaction vessel containing two components (i) AChE-labelled DHP anti-DHP (antibody) binding compounds and (ii) ATCh solution. The electrode was evaluated by calculating the relative standard deviation (RSD%) in the output current. After 60 measurements, the RSD% was 8.14%. The low RSD values show that the electrode can be used to determine AChE-labelled DHP with satisfactory precision. Thus, the micro-electrode exhibits good reproducibility for continuous measurement of 60 times with stable and accurate results.

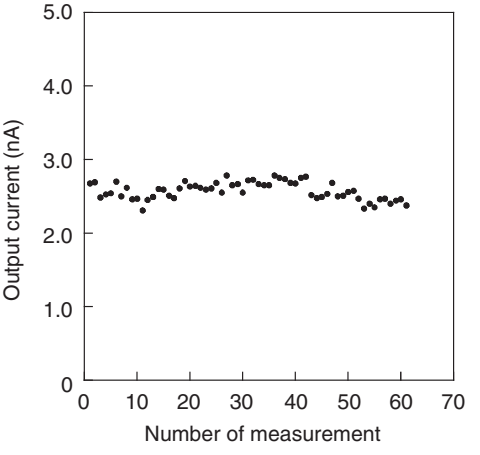

Figure 5. Stability of the microelectrode of the EBIA system. The experimental conditions were the same as those described for Figure 2.

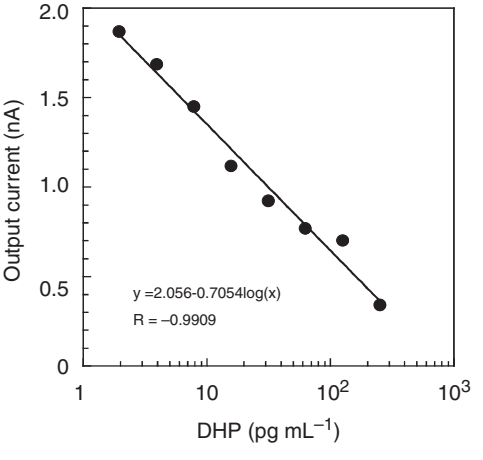

Figure 6. Calibration curve of the EBIA system for DHP. The experimental conditions were the same as those described for Figure 2. The measurements were run in triplicate for one sample.

3.4 *DHP calibration curve of EBIA system*

We investigated the relationship between the EBIA system output current and the concentration of the DHP standard solution (Figure 6). When samples with various concentrations of DHP standard solution were measured by the system (3 times per sample), a rapid and stable response was attained. The output current decreased with increasing concentration of DHP. A linear calibration plot was obtained in the range of 1.95 to 250 pg mL^{-1} (correlation coefficient: -0.9909). In the aquaculture field, since it was important to be able to monitor rapid increases in DHP (50–300 ng mL^{-1}) in fish [2], the measurement range of 1.95 to 250 pg mL^{-1} could be sufficient for monitoring of DHP in fish. The obtained correlation coefficient of -0.9909 is statistically significant since it produced a t value of 18.0 that is much greater than the critical t value of 2.45 at a 95% confidence level [22]. The fitted function was expressed as $Y = a + b\log(X)$ ($a = 2.056 \pm 0.145$, $b = 0.7054 \pm 0.0958$ under the 95% confidence limits), where Y and X stand for the output current (nA) and the DHP concentration (pg mL^{-1}). On the proposed

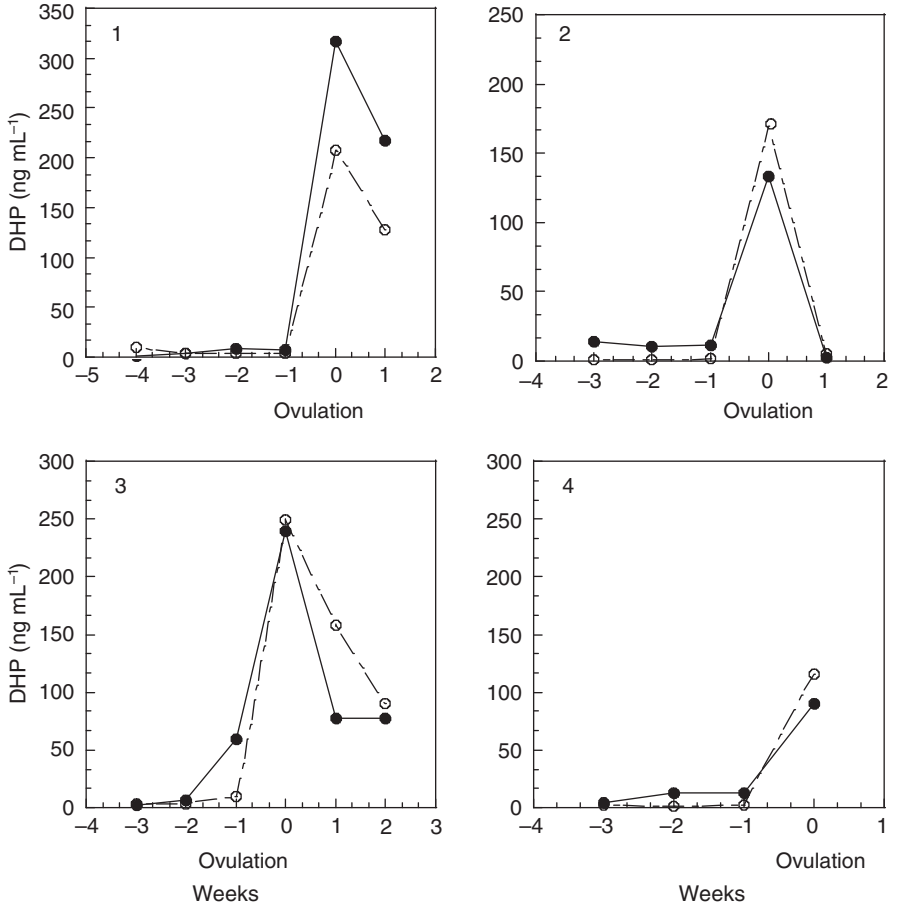

Figure 7. Monitoring of the DHP concentration in spawning strain of rainbow trout. Time course of DHP concentration in fish (sample Nos. 1–4), filled circles represent the EBIA data, open circles represent the ELISA data.

EBIA system, one measurement could be completed within 5 min using the microelectrode. Moreover, the total assay time, including sample preparation, was less than 3 h, which was substantially less than the 12 h required for the conventional ELISA method.

3.5 *Measurement of DHP in fish plasma sample by the EBIA system*

To evaluate the potential use of the EBIA system for actual samples, the EBIA system was applied to determine the DHP concentration in female rainbow trout during the breeding season. Ovulation was checked once per week from October to November 2008. Figure 7 shows the time course of the DHP concentration in spawning strains of four rainbow trout determined by the EBIA system and the conventional ELISA method. The ovulation point was indicted at "0" on the abscissa. The four fish were reared at about 11°C for 3 to 5 weeks in an outdoor tank. In the EBIA method, all plasma samples were diluted 1000-fold in buffer and the DHP levels were estimated from the calibration plot shown in Figure 6. As shown in Figure 7, DHP levels measured by the ELISA method showed a drastic increase at 1 week before ovulation. The value determined by the EBIA system also increased at this time. DHP levels determined by the EBIA system paralleled those measured by the conventional ELISA method. The results monitored by our proposed method agreed with those obtained using the ELISA method.

Thus, the proposed method could establish an electrode-based immunologic assay system for monitoring DHP. Our findings could contribute to the development of an *in vitro* wireless DHP monitoring system for fish. Currently, however, the method is somewhat problematic due, for example, to the competition between unlabelled DHP and AChE-labelled DHP for a limited number of anti-DHP binding sites. Future improvements to address these problems may result in the development of a suitable system for fish.

4. Conclusions

We developed the EBIA system to monitor DHP, an oocyte maturation-inducing hormone, in fish based on antigen-antibody reactions and electrochemical measurement. The microelectrode was prepared from Pt-Ir wire (working electrode) and Ag/AgCl paste (reference/counter electrode). When the microelectrode was immersed in sample solution containing DHP, the electrode produced an output current. The output current generated corresponded to the oxidation current of thiocholine to dithiobicholine. The response of the EBIA system decreased with an increase in the DHP concentration because of the competition between DHP and an AChE-labelled DHP for a limited number of anti-DHP binding sites. The optimum assay conditions were as follows; ATCh concentration: 10 mM, reaction temperature: 26°C. The micro-electrode has excellent reproducibility for 60 consecutive measurements. A correlation was observed between the output current and DHP concentration (1.95–250 pg mL^{-1}) using the EBIA system. The EBIA system was applied to determine DHP levels in female rainbow trout during the breeding season. DHP concentrations determined by the EBIA system were consistent with those measured by conventional ELISA method in the range of 2.5 to 320 ng mL^{-1}. One measurement could be completed within 5 min and the total assay time, including sample preparation, was less than 3 h. Our proposed method using the electrode-based immunologic technique is applicable for the rapid and simple determination of DHP in fish.

Acknowledgements

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