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Discrimination of fish egg quality and viability by Raman spectroscopy

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ABSTRACT

Egg or embryo quality is typically assessed by viewing egg morphology and by observing the cleavage rate of the embryo. Assessment of egg or embryo quality based on bio-molecular information might lead to improved outcomes for aquaculture or in vitro fertilization (IVF) treatments.

To assess egg quality, we performed Raman spectroscopy on fish eggs from the Japanese medaka (*Oryzias latipes*). Good classification results from the yolk were obtained using principal component analysis (PCA) and linear discrimination analysis (LDA). The results show that the supply of oil energy starts immediately after fertilization and embryogenesis is initiated. The presence or absence of fertilization can be evaluated by fatty acid Raman bands with 95.7% accuracy. The Raman results show that the key factors that indicate the viability of fish eggs are amino acid production and carotenoid pigment deposition. The LDA algorithm based on the Raman bands of these substances indicates whether the development is normal or abnormal with 80.3% accuracy. These results show that Raman spectroscopy can be a powerful tool for non-invasive assessment and real time monitoring of fish egg quality and viability.

1. INTRODUCTION

A sexual reproductive body can be produced from a fertilized ovum. Once the ovum is fertilized by the sperm, it undergoes cell division, differentiates into several cells, and forms a complete body. The fertilized ovum has totipotency and is in the unique position to be able to create a new generation with biodiversity. Although cloning technology and induced pluripotent stem (iPS) cells has made it possible for humans to create a new body or various types of organs, we have not succeeded in creating the origin of life, i.e., a fertilized ovum itself. The fertilized ovum contains all the genetic information required to develop a complete organism. However, not all fertilized ova are viable. Some of them develop abnormally and ontogenesis stops. Various studies have mentioned that the survival rates of eggs or embryos are closely correlated with egg quality. Quality is typically estimated by observing blastomeric morphology.

In aquaculture, the ova, i.e., the fish eggs that have a high survival rate for fertilization, eyeing, hatching, and first feeding, are regarded as high-quality eggs [1]. There are several methods currently in use to measure marine fish egg quality. Fish eggs from some marine species such as the Sea Bass (*Dicentrarchus labrax* L.) are characterized by observing whether the eggs float or sink in seawater; floating eggs are shown to have good quality and sinking eggs have low quality [2, 3]. However, this criterion for the judgment of egg quality is not always applied to other marine fish species, such as the Atlantic halibut *Hippoglossus hippoglossus*. For this species, egg quality is assessed by observing the blastomeric morphology in terms of size, shape, and timing of cleavage [4, 5]. Atlantic halibut is a very important commercial fish, but

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the hatching rates are often less than 1% in aquaculture [6]. In the case of salmon, where the method is quite well established, the hatching rate is about 50% [1]. To improve the hatching rates, it is necessary to improve the method used to inspect and select viable fish eggs and monitor the culturing conditions.

Visual inspection is also a conventional method that is used to assess human embryos after in vitro fertilization (IVF). Edwards suggested that implantation and pregnancy rates after IVF showed a close relationship with embryo cleavage rates and morphological features [7]. Some previous studies introduced a grading method for the human ovum based on cleavage [8-11]. The authors of these previous studies indicated that blastomeric morphology was a predictive factor for embryonic quality and viability. The method is, however, based on observation and potentially prone to observe bias. This method is currently used in fertility therapy.

The accurate estimation of egg or embryo quality is very important for aquaculture and for IVF treatments. We hypothesized that it may be possible to improve the reliability of fish egg inspections as well as IVF success, by using Raman spectroscopy. In the present study, we demonstrate the potential of Raman spectroscopy as a tool to evaluate freshwater fish egg hatching rate.

2. MATERIAL AND METHODS

2.1 Fish and Eggs

Male and female wild type Japanese medaka (*Oryzias latipes*) were purchased from an aquarium shop and cultivated in a cistern. The species lives in fresh water and grows to a length of about 4 cm. The fish are easily cultivated and are often used as a laboratory model to test compounds for carcinogenicity [12, 13]. The natural breeding season of the fish is from May to August in Japan [14, 15]; however, they deposit eggs anytime when the water temperature is above 25 ℃. The eggs hatch within two weeks if incubated at room temperature. Because the egg of *Oryzias latipes* is transparent, it produces little fluorescence in Raman measurement, a property that is beneficial in our study.

When we performed our experiment, we followed the fundamental guidelines for proper conduct of animal experiment and related activities in academic research institutions under the jurisdiction of Ministry of Education, Culture, Sports, Science and Technology in Japan.

2.2 Cleavage Stages of the Fish Egg

The egg size is about 1.5mm in diameter. In the unfertilized egg, oil droplets are scattered at random in the cytoplasm (Stage 0). The egg contains enough energy to support embryonic development until the first feeding [16]. The fertilized egg progresses into the cleavage stages. Images of the stages of normal development during the first 8 h after the fertilization are shown

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in Figure 1. Once the egg is fertilized, fat droplets distributed through the egg gradually begin to coalesce. About 30 min after the fertilization, an unequal distribution of the droplets can be observed (Stage I). In the fish egg, the unequal cleavage takes place at its animal pole, which looks like a bulge on the yolk. About 1 h after the fertilization, oil droplets fuse together into bigger droplets and settle at the vegetal pole (Stage II). The cytoplasm then begins to gather near the animal pole, forming a blastodisc, after which the cell goes into cleavage, generally dividing into an 8-celled stage within about 2.5 h after fertilization. Thus, the blastodisc is transformed into an embryonic body. The cell cleavage continues and the blastodisc is divided into 32 cells within 4 h after fertilization (Stage III). The cell size becomes smaller due to additional cleavages. The egg at 8 h after fertilization is referred to as the morula or blastula stage (Stage IV). More precise and detail classification of developmental stages of *Oryzias latipes* is found in reference [15].

Raman spectra were obtained from three sites in the egg: the vegetal pole (oil droplets), the animal pole (cytoplasm), and the equatorial area (the center of the yolk). The laser irradiation did not appear to influence the eggs. It was confirmed that all eggs that showed a correct cleavage hatched without any after-effects, even with repeated Raman measurements.

Typically, abnormal fertilized eggs were observed within 8 h of incubation (Type A). It was generally observed that fat droplets were not assembled correctly in the vegetal pole and the eggs were not viable.

Frozen storage techniques for fish eggs have not yet been established because of the rich oil components contained in fish egg. To examine some of the factors that change with the freeze-thaw cycle, frozen-thawed eggs (Type B) were used. Fertilized eggs at the stage of 2–8 cells were instantly frozen with liquid nitrogen, stored at -80 ℃ for 1–4 weeks, and thawed with water at room temperature. After the treatment, these eggs did not restart the cleavage, as was expected.

2.3 Raman Measurements

The Raman system consists of a 785 nm diode laser (Toptica Photonics, Germany), a single polychromatic Raman spectrometer (F 4.2, focal length 320 nm, 750 nm blazed 600 l/mm grating; Photon Design Co. Ltd. Japan), and a Peltier-cooled charge coupled device detector (CCD; DU420-BRDD, ANDOR Technology Co. Ltd., Northern Ireland) with objective lens (Mitutoyo M11002805A, 20×/0.40, focal length 20 mm). The excitation power is typically 60 mW at the sample point and the exposure time is 60 s (30 s × 2).

The measured spectra are treated with Savitzky–Golay smoothing and the background noise is subtracted by 5th-order polynomial fitting. The spectral intensity is normalized with a band at 1003cm^{-1} due to phenylalanine or at 1450cm^{-1} due to the CH deformation depending on the

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target molecules to be analyzed. Principal component analysis (PCA) and linear discrimination analysis (LDA) were employed for processing the data [17, 18]. These calculations were performed with chemometrics software (Unscrambler: CAMO, USA).

3. RESULTS AND DISCUSSIONS

Raman spectroscopy was used to study the molecular compositional changes in the yolks of fish eggs. Averaged Raman spectra of the normal fertilized eggs were compared at the equatorial area ((a); n = 122), animal pole ((b); n = 58), and vegetal pole ((c); n = 99). A subtracted spectrum ((b)-(a), (d) in Fig.2) was also compared. Bands at 853, 938, and 1003 cm^{-1} were assigned to tyrosine, proline, and phenylalanine [19, 20]. It appears that the band due to glycogen also overlaps with the band at 938 cm^{-1}. Bands at 1265, 1447 and 1658 cm^{-1} include contributions of amide III, CH-bending, and amide I modes of proteins, and of =C-H bending, CH deformation, and C=C stretching modes of lipids. The spectrum of the vegetal pole has a strong contribution from lipids. A band at 1745 cm^{-1} is assignable to the C=O stretching mode of ester groups. Bands at 1656 and 1265 cm^{-1} are assigned to C=C stretching and =C-H bending modes, reflecting the fact that the fat droplet near the vegetal pole includes a relatively high concentration of unsaturated lipids. The subtracted spectrum (d) is similar to that of glycogen, which suggests the localization of glycogen at the animal poles.

The concentration of glycogen in the blastodisc increases relative to the center of the yolk. This was confirmed using Raman spectra, which showed that the averaged concentration of glycogen in the yolk during Stages I–IV was lower than that in Stage 0. The blastodisc in the animal pole is composed of active cytoplasm, and it is collected in the animal pole by the oscillation caused by fertilization [21]. Therefore, our result can be interpreted as showing that the cytoplasm is rich in glycogen and the materials in the cytoplasm are collected from all around the egg yolk after fertilization. The present result indicates that the transfer and variation of the material within the egg is successfully detected using Raman spectroscopy.

Small positive bands at 723, 782 and 814 cm^{-1} in the subtracted spectrum were assigned to DNA and RNA. They show that the concentrations of DNA and RNA are higher in the animal pole than in the equatorial area (yolk). Because ontogenesis generally reduces the entropy in the system, the localization of the molecules would be expected to change after fertilization. Thus, the molecular composition in different parts of the egg before fertilization would be obviously different from that after fertilization.

Subtraction spectra for the equatorial areas of various eggs at Stage 0 are shown in Figure 3. It is expected that conditions will be different before and after fertilization. Figure 3 shows that unsaturated lipids are high in Stage I, and then decreased during Stage II. Figure 4(a) shows a plot of the PCA score obtained for Stages 0 and I. It is clear that these two egg types can be

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discriminated using PC1. The feature observed in the PC1 loading plot seems to have a strong contribution from fatty acids.

The LDA diagnostic model used these bands as discrimination factors and the results were validated for the two types of eggs by the leave-one-data-site-out, cross-validation method. The validation results indicate that the LDA model has a 95.7% (22/23) reliability, as shown in Figure 4(b). This suggests that the supply of energy starts immediately after fertilization and that embryogenesis will be initiated. This is consistent with the fact that lipids in fish egg are the major source of metabolic energy throughout embryonic development [22, 23].

Amino acid production is active in Stage III, and carotenoid accumulation can be seen in Stage IV of normal development. In contrast, a decrease of amino acid and deposition of carotenoid take place in abnormally developed eggs like Type A and B.

PCA analysis was performed on the data sets, including all development stages and abnormal types, to discriminate the abnormal eggs from normal eggs. The result shows that two types (normal and abnormal developments) are well discriminated by PC1 as shown in Figure 5(a). Positive bands in the PC1 loading plot (Fig. 5(b)) can be assigned to some amino acids and the negative bands correspond to carotenoid. The results suggest that the key factors to discriminate the normal development in early stages are protein and carotenoid. It is expected that amino acids are actively generated in a normal developing egg and that pigment deposition takes place in irregular eggs. Furthermore, it is also clear from Figure 3 and 5(b) that biosynthesis of amino acids becomes most active in Stage III and is less active afterward. By using Raman spectroscopy, it is possible to monitor the alteration of the bio-substances that occurs hourly.

Taking into account all the above results, the key factors to discriminate the viable normal egg from the abnormal ones are amino acid and carotenoid contents. The LDA model is obtained by using amino acid and carotenoid Raman bands extracted by PCA. Sixty-one samples were used to make the LDA model and the reliability of the model was validated. Forty-nine samples were correctly predicted and 12 were misjudged (Fig. 6), with an accuracy of 80.3%. The present results confirm that detection of the amino acid and carotenoid production are important for egg classification. Raman spectroscopy is a powerful tool for non-invasive assessment of fish egg quality and viability.

In the animal pole, a part of the egg swells out and a blastodisc is formed in Stage II. The PCA was applied to the data sets of the spectra measured at the animal pole in Stage II, III, and IV. The data indicate that amino acid production is most active and the concentration of glycogen is high in stage III and less active in the other stages. The tendency is for the active material production to occur in the yolk.

In the vegetal pole, the oil components were compared for normal and abnormal eggs, but no differences were detected. Many researchers have examined the relationship between egg

quality and dietary fatty acid [1, 23] and have indicated that the oil composition and viability of the egg have a close relationship. The discrepancy between their findings and our results may come from the fact that the fishes were fed the same meal in our research, which was commercially available, and the nutrition conditions were the same.

4. SUMMARY

In our study, fish egg quality and viability were assessed based on biomolecular information obtained by Raman spectroscopy. This method is different from the traditional, empirical, and phenomenological method based on blastomere morphology. Laser irradiation was performed for three areas of fish eggs: the vegetal pole, animal pole, and equatorial area. Good classification results from yolk were obtained by PCA and LDA. The results show that the supply of oil energy starts just after fertilization and embryogenesis will be initiated. The presence or absence of fertilization can be assessed by fatty acid Raman bands with 95.7% accuracy.

The concentration of glycogen in the animal pole is higher than that in the center of the yolk. We also confirmed that the concentration of glycogen in the yolk after fertilization was lower than that of Stage 0. This result indicates that glycogen rich cytoplasm is collected from all around the egg yolk after fertilization. The present results show that the transfer and variation of the material within the egg is successfully detected by Raman spectroscopy. Furthermore, it makes clear that key factors for egg classification are amino acid and carotenoid production. Biosynthesis of amino acids becomes the most active in stage III, in the animal pole and center of the yolk, and it is less active thereafter. Raman spectroscopy can be used to monitor the alteration of bio-substances occurring hourly. An LDA model based on these material Raman bands validates the egg viability with 80.3% accuracy. The results provide new insights that Raman spectroscopy is useful to evaluate the fish egg quality and viability.

All radiated eggs with normal development hatched even after repeated Raman measurement, and no recognizable influences were noted. The result shows the safety of laser irradiation.

Assessing fish egg quality is very important, not only from the aspect of aquaculture but also from the aspect of reproduction of wild fish and preservation of endangered species. Raman spectroscopy provides a non-invasive assessment method and real-time monitoring of fish egg development.

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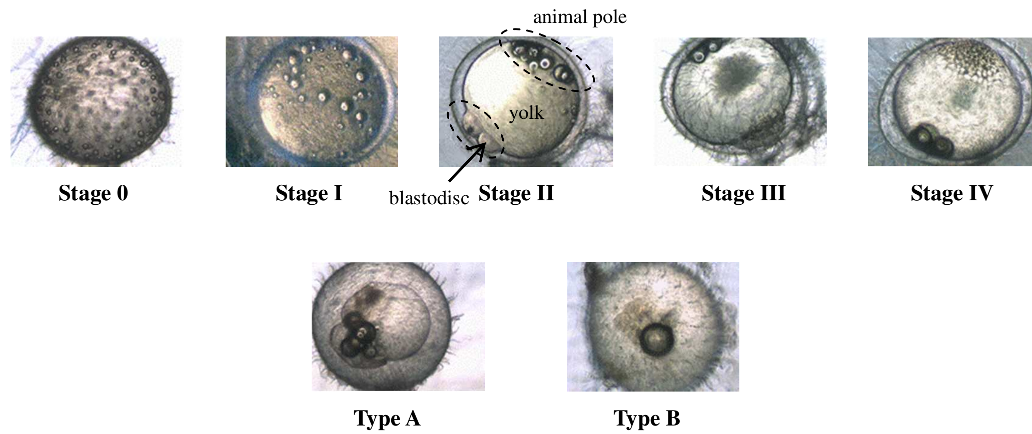

Figure 1: Images defining normal developmental stages and abnormal egg types.

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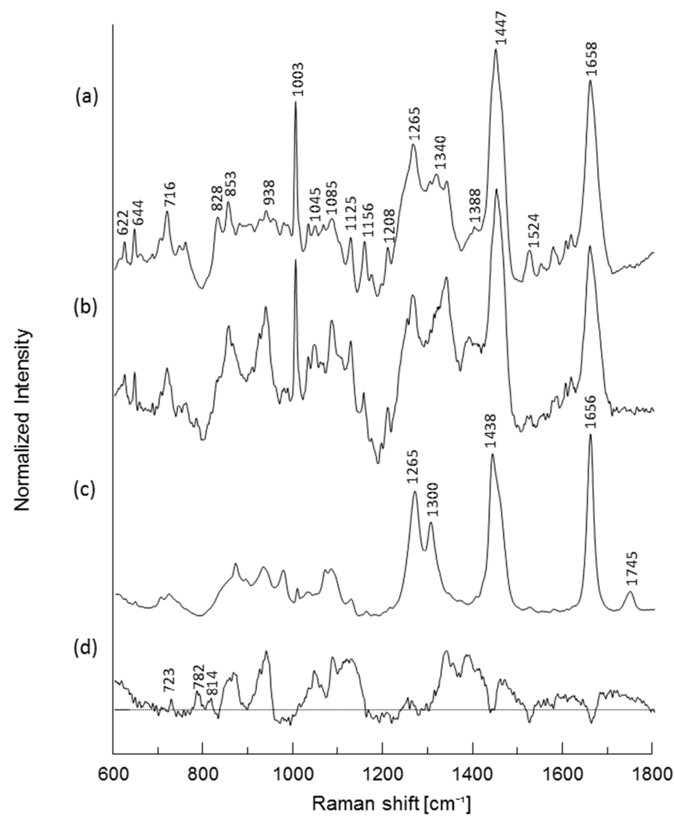

Figure 2: Averaged Raman spectra from (a) the equatorial area (n = 122), (b) animal pole (n = 58), (c) vegetal pole (n = 99), and (d) the subtracted spectrum calculated as (b) - (a).

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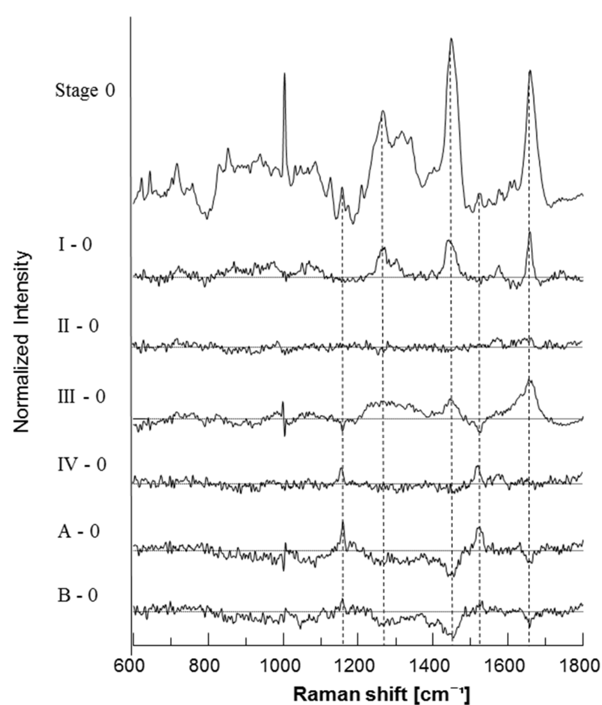

Figure 3: Subtraction spectra for the equatorial areas of various eggs calculated based in Stage 0.

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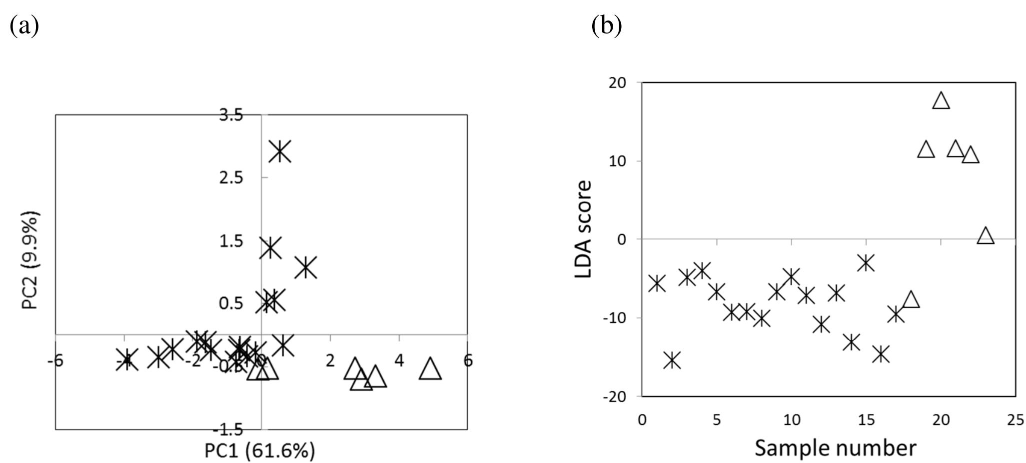

Figure 4: (a) Score plot of principal component analysis (PCA). ∗ is Stage 0 and ∆ is Stage I. (b) Result of linear discrimination analysis (LDA) classification with the leave-one-site-out cross-validation method. The LDA algorithm showed 95.7% (22/23) accuracy.

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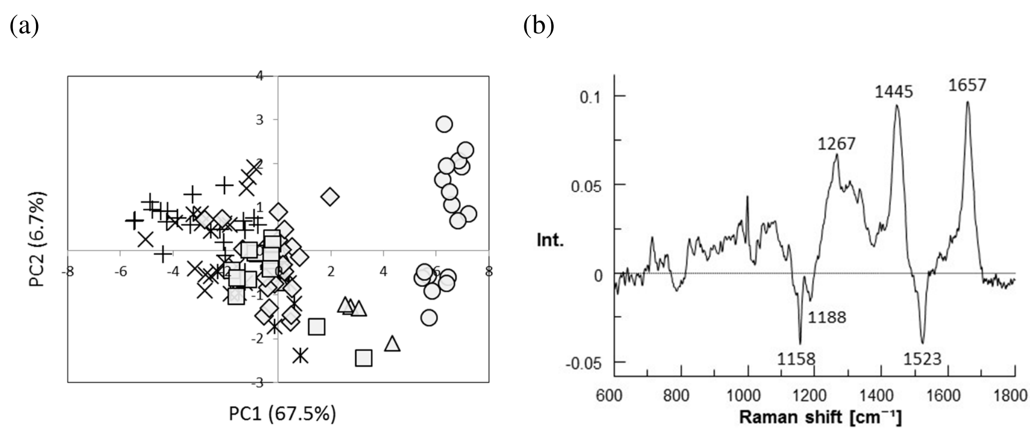

Figure 5: (a) Score plot of principal component analysis (PCA) . ∗ expresses stage 0, △ stage I, ◇ stage II, ○ stage III, □ stage IV, +type A, and × type B. (b) Loading plot of PC1. Positive bands can be assigned to the amino acid and negative bands corresponding to carotenoids.

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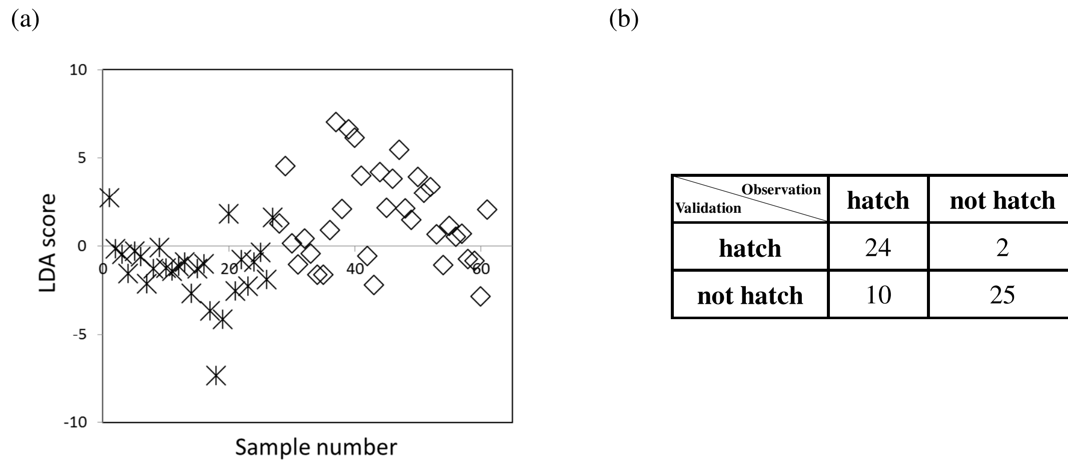

Validation \ Observation	hatch	not hatch
hatch	24	2
not hatch	10	25

Figure 6: (a) Score plot of linear discrimination analysis (LDA) validation. ✱ expresses an egg with abnormal development and non-activated egg (Stage 0). ◇ is a viable egg activated by fertilization and showing normal development. Sixty-one samples were used to create a LDA model, and remaining 61 samples were validated. (b) The validation result. Among the 61 sample data, those for 49 samples were correctly predicted, whereas 12 were misjudged. LDA algorithm showed discrimination of the egg types with 80.3% accuracy.

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