*Research article*

# Educational Material Research on the Color of Crayfish for Conversion to Edible Resources

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**Abstract** The crayfish (*Procambarus clarkii*) is an invasive alien species consumed as a food source worldwide. However, there is an extremely low demand for it as a food resource in Japan, and it is typically targeted for extermination. One reason for the lack of progress in considering crayfish a sustainable food resource is limited education on the issue during secondary education programs. It is well known that astaxanthin is the pigment responsible for the body color of common red crayfish and crustaceans such as shrimp and crabs. At the same time, while there are ample study materials on plant pigments in the science education curriculum in junior high and high schools in Japan, there are no such materials to our knowledge that focus on animal pigments. Therefore, this research sought to develop teaching materials to deepen understanding of color as a food component of crayfish through an experiment involving extracting and separating their pigments. Using pigments extracted from red crayfish exoskeletons, we developed experimental teaching materials to observe astaxanthin by employing thin-layer chromatography and high-performance liquid chromatography. In addition, an experiment was conducted to observe the effect of different foods on crayfish body color. For the experiment, food was developed to make crayfish bodies white (decolorized) and orange. When the decolorized crayfish were fed norbixin or bixin, which are components of the orange annatto pigment, norbixin tended to accumulate as orange in the exoskeleton more than bixin based on the apparent body color change, color extraction from images, and thin-layer chromatography. These findings are expected to be useful for

science and nutrition education, supporting the development of the students' awareness and understanding of crayfish as a food resource.

**Keywords** education, teaching material, *Procambarus clarkii,* pigment, food resource, SDGs

## INTRODUCTION

Crayfish (*Procambarus clarkii*), which live in rivers, ponds, rice paddies, swamps, and other bodies of water in the city, are a globally invasive alien species native to the United States (Gheradi, 2013). Although the damage they cause to ecosystems is regarded as a problem, crayfish are used as a food resource in the United States, China, and European countries (Hara, 2021). Furthermore, in the future, their excrement and shells are expected to be used as agricultural resources such as fertilizers, as raw materials for textiles, and as medical resources (Ifuku et al., 2004; Yihun et al., 2016).

Crayfish were introduced to Japan in 1927 as food for bullfrogs. Since then, the species' high fertility rate and omnivorous nature have led to substantial damage to local ecosystems and agriculture. Thus, crayfish have become a well-known representative of alien species in Japan. As part of environmental protection activities, crayfish are routinely exterminated and culled, but they are not actively used. One reason for this is the lack of opportunities for students to learn about the usefulness of crawfish as a resource in elementary and secondary education. In elementary education, crayfish are familiar observation materials because of their ease of care and management; however, in secondary education, they are only treated as an invasive alien species and representatives of crustaceans. Therefore, we thought that, in order to promote the recognition of crayfish as a food resource, experimental materials focusing on the useful food components contained in crayfish might be necessary. We focused on carotenoid pigments, which are a factor in crayfish body color that children are likely to be interested in and have functional properties such as antioxidant effects and nutrients. In Japanese secondary education, carotenoid pigments are derived only from photosynthetic organisms, and their functions are rarely discussed. Among carotenoid pigments, astaxanthin, which is found in the wild crayfish, salmon, and crustaceans (shrimp and crab) that are familiar to the Japanese people, is utilized in a variety of products because of its high functionality. Although the usefulness of astaxanthin is known commercially, it has not yet been used in Japanese secondary education. Therefore, the development of experimental teaching materials focusing on astaxanthin using crayfish will provide new science experiment teaching materials focusing on animal pigments and will enable students to learn about the usefulness of crayfish.

Astaxanthin is abundant in the exoskeletons of crayfish. The exoskeleton can easily extract pigments, and the use of molting shells obtained each time the crayfish grows is useful as a type of teaching material for collecting samples without killing the organism. Astaxanthin in the exoskeleton of crayfish exists in free and ester forms. However, the form of astaxanthin in exoskeletons derived from molted shells is unknown. Therefore, in this study, we will examine the separation conditions using thin-layer chromatography (TLC) and high-performance liquid chromatography (e-HPLC), which are treated in secondary education, as teaching materials for observing the ontogeny of astaxanthin in molting shells.

Previously, we produced crayfish that changed their body color to blue or pink by feeding astaxanthin to genetically fixed white crayfish (Takeda, 2021). We also found that feeding another carotenoid pigment, annatto pigment, to white crayfish turned their bodies orange. However, the food pigment annatto contains bixin and norbixin as components, and the difference in body color due to these components is unknown. Wild crayfish are familiar for use as general teaching material, but since they retain their original colors, they must be decolored. Therefore, in this study, as teaching material to explore the factors involved in rearing, we prepared decolorized wild crayfish diets and conducted feeding tests of diets containing mainly bixin and norbixin. Then, the experiment will compare the body color change for each color value and study the conditions to observe the substances that cause these body colors by TLC.

## OBJECTIVE

This research explored the development of teaching materials focusing on pigments, which are both body color factors and nutrients in crayfish, with the aim of utilizing them in science and food education to highlight their usefulness as food resources.

## METHODOLOGY

### Experiment Focusing on Astaxanthin Contained in Crayfish Exoskeleton and Separation of Astaxanthin using TLC

We experimented to explore astaxanthin in crayfish exoskeletons as a body-color factor. Acetone containing 0.01% astaxanthin (Fujifilm Wako) and krill were used as controls. It has been reported that krill have astaxanthin and esters of astaxanthin (monoesters, diesters) (Takaichi et al., 2003). Since the eyes of krill contain substantial amounts of these carotenoids (Maoka et al., 1985), krill eyes were used in the experiment.

The sample for the experiment included the exoskeletons of living crayfish and molted exoskeletons. For pigment extraction, the abdominal segment of each exoskeleton was shredded to about 1 mm, and 100 µl of acetone was added to 0.01 g of the material. They were mixed lightly, allowed to stand for 15 minutes, and centrifuged for 20 seconds. Each sample was spotted on a TLC plate (TLC silica gel 60 $F_{254}$ [4 × 8 cm], Sigma-Aldrich), and then the TLC was placed in the solvent (petroleum ether: acetone = 7:3) in the deployment tank to separate the pigments.

### Isolation of Astaxanthin Using e-HPLC

"Kotori" (Uniflows, Japan) is an HPLC developed for educational purposes. It is smaller and cheaper than the general HPLC. Thus, it is used in high schools in Japan. An e-HPLC was used to detect astaxanthin contained in the molted exoskeletons of crayfish. As a control, 0.001% astaxanthin (Fujifilm Wako) was dissolved in acetone, and 100 µl of acetone was added to 0.01 g of the abdominal segment as a sample. A Cadenza CD-C18 column (Φ6 × 50 mm) (manufactured by Intact Co., Ltd.) was equilibrated with 50% methanol: THF (1:1) eluent at 600 µL/min at room temperature (25°C). Each sample of 2.7 µL was injected into the column; then, the peak of astaxanthin was detected at 405 nm.

### Creation of Bait for Decolorization and Feeding Tests

Material containing no carotenoid pigments was used to create decolorization bait. Rice powder was used as a carbohydrate, soybean, pork bone, and fish powders were used as protein, and sardine oil was used as a lipid. Rice powder, soybean powder, pork bone powder, fish powder, and sardine oil were mixed at 2:3:2:2.8:0.2, and an appropriate amount of water was added. The ingredients were then molded into a round shape, steamed for 20 minutes, and then solidified and used as feed. Four crayfish aged about 8 weeks old were selected for a feeding test in which any changes to their body color after consuming the decolorization bait were observed. The crayfish were fed 0.05 g of bait once a day in the morning for 8 weeks. In addition, it was confirmed that there was no bait left over from the previous day, and any molted exoskeletons were removed, the water was changed daily before feeding. As a control experiment, commercially available crayfish bait (Kyorin) was used under the same conditions.

Pigment-containing bait was then created using the decolorization bait before it underwent steaming. The bait and pigment were mixed at a ratio of 9:1, and about 0.5 ml of water per 1 g of bait was added to assess the degree of viscosity. The pigments used were norbixin (Fujifilm Wako) and bixin (Fujifilm Wako); they are components of annatto pigment (Scooter, 2009) used for food coloring. The ingredients were then molded into a round shape, steamed for 20 minutes, then solidified. This pigment-containing bait was then used in a feeding test of crayfish decolored using decolorization bait. Any changes to the crayfish's body color were observed. Four crayfish were fed

norbixin-containing bait, and four were fed bixin-containing bait. A piece of pigment-containing bait was fed at a rate of 0.03 g per 0.1 g of crayfish weight in the morning once a day for 9 days. In addition, it was confirmed that there was no bait left over from the previous day and any molted exoskeletons were removed; the water was changed daily before feeding.

### Color Extraction from an Image of Body Color Change

Pictures were taken of the subject crayfish every day under the same conditions. The red, green, and blue (RGB) values of the sixth abdominal segment were measured using a color selection software called Spoitkun. The resulting data were expressed as the average value ± standard deviation.

### Confirmation of Color Change Due to Boiling

It was important to confirm how the color would change after boiling as this is how crayfish are prepared as food. Therefore, we compared the color of the first pectoral leg of wild crayfish and crayfish colored using norbixin before and after boiling for 15 min.

### Detection of Substances Causing Body Color Using TLC

Acetone containing 0.01% norbixin (Fujifilm Wako) and 0.01% bixin (Fujifilm Wako) was used as a control, and the tail fins of crayfish were used as a sample. For each 0.01 g of tail fin mass, 50 µl of acetone was added. The solution was lightly mixed and then centrifuged for 20 seconds to extract the pigment. After pigment extraction, each sample was quickly spotted on a TLC plate (TLC Silica gel 60 RP-18 $F_{254}$s [5×7.5 cm], Sigma-Aldrich). Then, the TLC was placed in the solvent (water: acetone 1:9) in the deployment tank to separate the pigments.

## RESULTS AND DISCUSSION

### Experiment Focusing on Astaxanthin Contained in Crayfish Exoskeleton and Separation of Astaxanthin using TLC

Astaxanthin esters were mainly present in the abdominal segment of the crayfish (lane 3) as well as the krill (lane 2), and astaxanthin was also detected (Fig. 1A). Astaxanthin was observed as a major pigment in the abdominal segment of the molted exoskeleton (lane 4) (Fig. 1A). Although it has been reported that astaxanthin esters and astaxanthin are the main pigments in the exoskeleton of wild crayfish (Nakagawa et al., 1974), no research was found describing the predominance of freeform astaxanthin in molted exoskeletons. This experiment revealed that there is a difference in the presence of astaxanthin in crayfish exoskeletons before and after molting.

Since crustacean molting is a lifelong phenomenon, molted exoskeletons can be continuously collected as samples for experiments. In addition, since the pigment contained is mainly astaxanthin in the free form, it is expected that it will be easy for children to understand. Using molted exoskeletons for experiments to observe pigments contained in the exoskeletons of crayfish is considered suitable.

### Isolation of Astaxanthin Using e-HPLC

As with the control freeform astaxanthin, elution peaks were also confirmed in the molted exoskeleton at 200 s–230 s (Fig. 1B). Freeform astaxanthin was confirmed to be the main pigment contained in the molted exoskeleton using not only TLC but also e-HPLC.

"Kotori" can be used to confirm astaxanthin in a short time. Thus, it is possible to perform multiple analyses even within a typical 50-minute class period. The device and column are very small compared to general HPLC, and, since it is very portable, it is considered suitable for conducting advanced experiments aimed at separating astaxanthin in various educational settings.

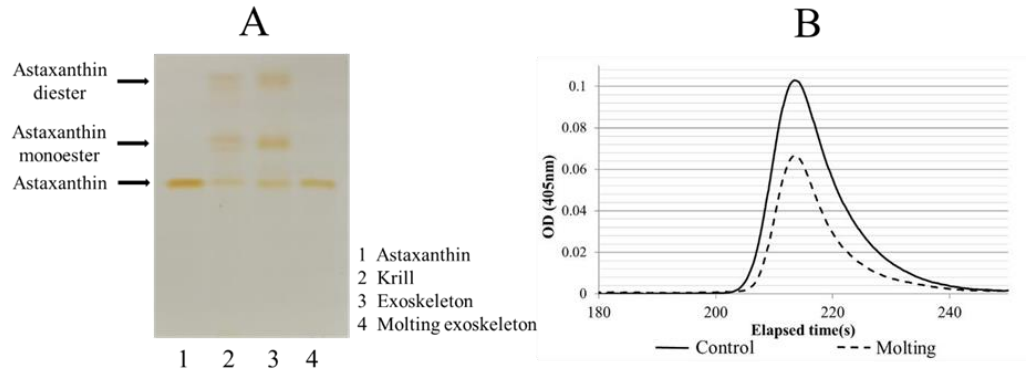


**Fig. 1 Isolation of astaxanthin in wild crayfish exoskeletons A (TLC), B (e-HPLC)**

## Creation of Bait for Decolorization and Feeding Tests

In the feeding test using TL2-3 cm crayfish for 8 weeks, the bodies of the crayfish fed with the commercial bait turned brown (Fig. 2A-a) while the bodies of the crayfish fed with decolorized bait turned white (Fig. 2A-b). Body color changes to blue and white with each molt are known to occur by feeding horse mackerel with a low amount of carotenoid pigment. However, horse mackerel bait is likely to cause contamination and odor in the breeding water. The decolorized bait created in this experiment can also be used as an alternative bait, which is considered to have the advantage of ease of breeding.

In the feeding test with pigment-containing bait, the bodies of all four crayfish consuming the norbixin-containing bait (Fig. 2B-abc) rapidly changed to orange while those consuming the bixin-containing bait gradually changed to yellow, although there were individual differences (Fig. 2Bdef). In our previous research, genetically fixed white crayfish fed with a diet containing annatto pigment (TCI, Japan) diet turned orange. However, in that research, it was unclear whether it was norbixin or bixin that was related to the orange body color. In this study, decolored wild crayfish turned dark orange when fed with norbixin (Fig. 2B-c) and yellow when fed with bixin (Fig. 2B-f). Thus, it was apparent that norbixin is the main factor contributing to orange body color. Fig. 2 shows the change over time in the crayfish with the most colored body on day 9.

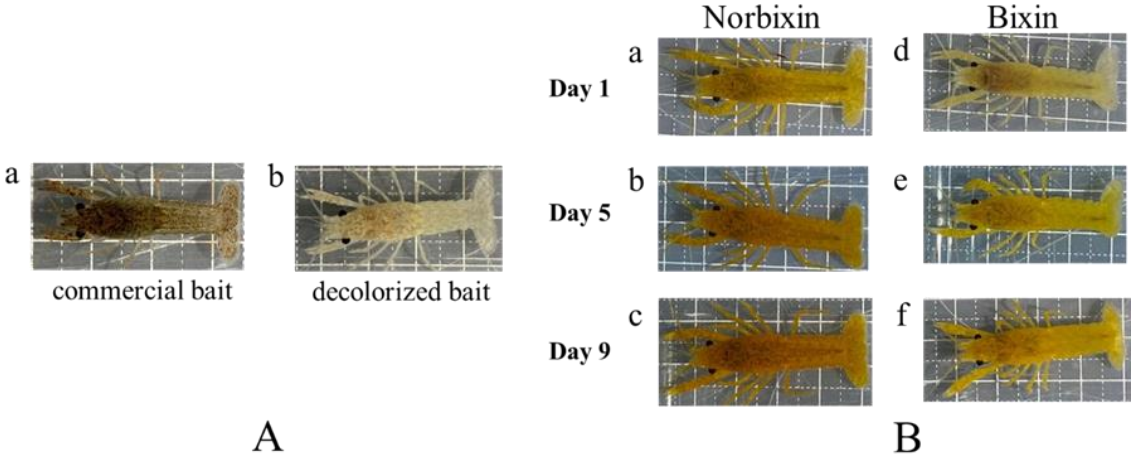


**Fig. 2 Body color change by feeding test A: commercial bait (Left) and decolorized bait (Right) B: Norbixin (Left) and Bixin (Right)**

## Color Extraction from an Image of Body Color Change

When crayfish color is expressed as a mixture of Red (R), Green (G), and Blue (B) in 16 bits (256 steps) using color extraction of images taken over time, the R value did not change significantly for either control, bixin, or norbixin. In terms of G values, it was confirmed that the feeding period

of bixin was almost constant while norbixin was gradually declining. In addition, the B value was almost zero in one day for norbixin while, for bixin, it gradually decreased (Fig. 3).

In Japan industry standard color names, yellow is R ≒ G > B (255, 212, 0), and orange is R > G > B (243, 152, 0). In addition, both orange and yellow become darker orange and yellow as the B value decreases to 0. Therefore, orange and yellow can be compared from the relative ratio of R and G. This result supports the visual color shown in Fig. 2B. Efforts to quantify changes in the color of the eyes seen using color extraction software will lead to activities to grasp observation events more scientifically and can be used for not only science but also STEAM education.

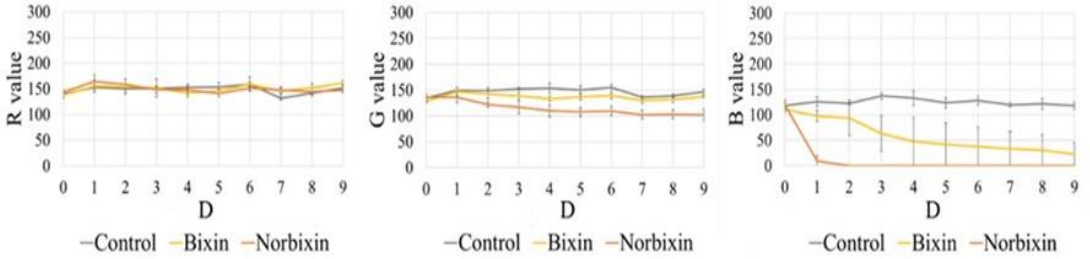


**Fig. 3 Changes in RGB values of crayfish exoskeletons over time using pigment-containing baits**

## Confirmation of Color Change Due to Boiling

Just as many crustaceans turn red when boiled, wild crayfish are red black before boiling (Fig. 4A) but turn red when boiled (Fig. 4B). Interestingly, the first pectoral leg of a crayfish, which had turned orange with norbixin (Fig. 4C), remained orange (Fig. 4D) when boiled. Orange has the same effect on food palatability as red (Birren, 1963). Although boiled crayfish as food have typically come only in red, the above finding can be used to develop new colors of crayfish for food products. It is also hoped that crayfish will be used as a teaching material for the development of crayfish as a food source in Japan.

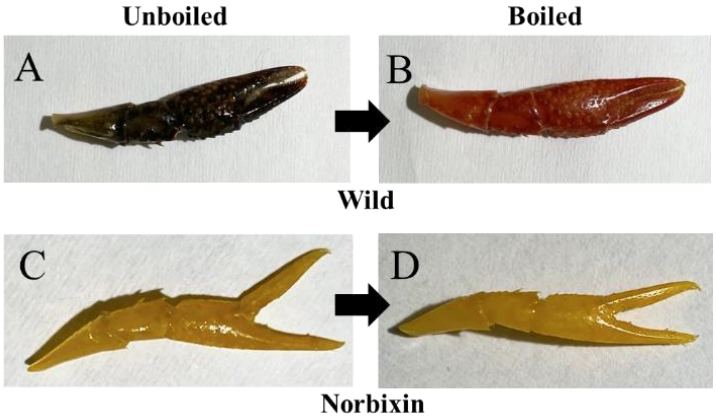


**Fig. 4 Change in color of the first pectoral leg before and after boiling**

## Detection of Causative Substances of Body Color using TLC

TLC revealed bands at the same position in norbixin (lane 1) and the tail fans colored by norbixin (lane 2) and bixin (lane 3) and the tail fans colored by bixin (lane 4) (Fig. 5). This confirmed that the pigments contained in the bait were accumulated in the body. In this experiment, a tail fan derived from a living body was used, but the same result was obtained for the molted exoskeleton (data not shown).

By conducting this experiment in parallel with the feeding test, it is possible to use it as multiscale experimental teaching material to detect changes in body color and substances that cause body color.

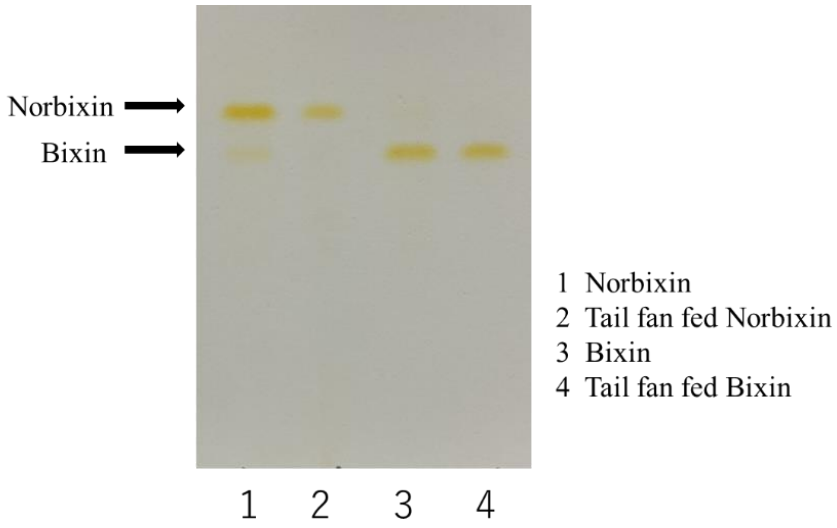


**Fig. 5 Separation of norbixin and bixin in TLC**

## CONCLUSION

Astaxanthin is contained in red crustaceans and fish. It is a factor in body color as well as an important substance with antioxidant effects. The exoskeleton of the crayfish contains a large amount of astaxanthin and is also useful as a teaching material because crayfish is treated in educational courses and is a familiar creature. In addition, the development of experimental materials focusing on astaxanthin using crayfish will be a new scientific experimental material focusing on animal pigments and will enable students to learn about the usefulness of crayfish.

In this study, astaxanthin contained in crayfish exoskeletons was isolated using TLC and e-HPLC. TLC is an experimental technique used in secondary education for the separation of pigments derived from photosynthetic organisms. The e-HPLC is an instrument developed for educational purposes and used in secondary schools specializing in science education. In this study, we have clarified the experimental conditions under which astaxanthin can be detected in more detail and in a shorter time by using e-HPLC as well as TLC; these experimental methods can be implemented in school education. Interestingly, we found that the presence of astaxanthin in the exoskeleton of wild crayfish in living tissue differed from that derived from molting.

For wild crayfish, we developed a decolorized bait that regulated the carotenoid pigments contained in the material and succeeded in whitening it. In addition, by feeding bait containing norbixin and bixin, which are components of annatto pigment for food coloring, it was revealed that body color turned dark orange with norbixin and yellow with bixin. Furthermore, it was clarified that norbixin turns a dark orange color in a shorter period of time than bixin from changes in appearance, TLC, and color extraction.

The findings and experimental methods of this research can be implemented in schools and used as new experimental materials focusing on animal pigments. Since these are teaching materials that focus on color, it is expected they will be used in science and food education that will lead to the edible use of crayfish.

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