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# Nutritional quality of fish faeces is enhanced by highly unsaturated fatty acid-producing heterotrophic protozoa

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ABSTRACT

Highly unsaturated fatty acids such as 20:5n3 (EPA) are both hormone precursors and cell membrane components, making them important nutrients for aquatic animals. Many animals must obtain EPA from their diets because they cannot synthesize enough EPA to meet their requirements, and algae are the main source of EPA in aquatic ecosystems. In a previous study, we detected EPA in the faeces of *Danio rerio*, a freshwater fish, even though the fish consumed a green algae diet that did not contain EPA. The objective of this study was to determine why EPA was detected in fish faeces. A significant positive relationship was detected between the number of heterotrophic protozoa and the concentration of EPA in the faeces, which suggests that this EPA was of protozoan origin. In addition, another experiment showed that protozoa adhered to faeces far more than the green algal diet remnants, which indicates that protozoa preferred to swarm on faeces. Furthermore, we cultured protozoa in an EPA-free medium and fed them a bacterial diet also lacking EPA, and found that *Cyclidium* sp. synthesized EPA de novo. The results demonstrate that protozoa produce essential fatty acids and enhance the nutritional quality of animal faeces in detritus-based food webs in freshwater ecosystems.

## 1. Introduction

Highly unsaturated fatty acids (HUFA) such as 20:5n3 (EPA) and 22:6n3 (DHA) are indispensable nutrients for aquatic animals because they are hormone precursors and important components of cell membranes (Bell et al., 1986; Olsen, 1999). In the case of freshwater zooplankton, inadequate HUFA levels led to a decline in growth and survival rates (Ahlgren et al., 1990; Das et al., 2007). Animals that have Δ5 and Δ6 desaturase, an enzyme required for fatty acid synthesis, can biosynthesize EPA from its precursor, 18:3n3 (ALA) (Ahlgren et al., 1999). ALA can be synthesized from 18:1n9 with Δ12 and Δ15 desaturase (Hastings et al., 2001), but this enzyme is limited to plants and algae, and therefore animals cannot biosynthesize ALA on their own (Holman, 1986). Thus, to obtain EPA, animals must obtain EPA or ALA directly from their diet.

The main producers of EPA are algal groups such as diatoms, raphidophytes, and euglenoids (Taipale et al., 2013), whereas most green algae and cyanobacteria contain only ALA. Therefore, animals with Δ5 desaturase that assimilate green algae or cyanobacteria can produce EPA. However, higher growth rates of zooplankton was observed when EPA was obtained directly from the diet (Brett and Müller-Navarra, 1997), suggesting that this is the optimal method of procuring this nutrient.

Basen et al. (2013) detected EPA in freshwater clam pseudofaeces that originated from a cyanobacteria diet that did not contain EPA. Similarly, we previously observed EPA in the faeces of the freshwater fish *Danio rerio* that were raised exclusively on a green algae diet that did not contain EPA (20:5n3) (unpublished). Using microscopic observation, we found some heterotrophic ciliophora and flagellate on the fish faeces, even though the density of these protozoa were very low in the water column; contamination from diatom or other EPA-producing algae was not found. These results suggest that the EPA detected in the faeces could be attributed to protozoa that preferentially swarm on faeces.

A review of previous studies showed that data on the fatty acid composition of protozoa and their ability to synthesize fatty acids are lacking compared to that of algal species. However, several researches have shown that protozoa can produce EPA and DHA, although the ability to biosynthesize EPA de novo appears to be species specific. For example, Bec et al. (2010) demonstrated that *Paraphysomonas* sp. can biosynthesize EPA, but *Strombidium sulcatum* cannot (Klein Breteler et al., 2004). Further research is needed to better understand the capacity of freshwater protozoa to biosynthesize EPA.

We hypothesized that the EPA detected in *D. rerio* faeces originated

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from swarming protozoa that preferentially accumulate on faeces. Faeces are usually rich in bacteria and may attract bacterivorous protozoa. In addition, we also proposed that the protozoa can biosynthesize EPA de novo. To test these hypotheses, we conducted 2 laboratory experiments. In the first experiment, the relationship of EPA content and protozoa on the faeces of *D. rerio* was investigated to determine whether a high abundance of protozoa contribute to EPA presence in faeces. In the second experiment, the distribution of protozoa was investigated in a beaker with chlorella and chlorella originating from fish faeces to determine if protozoa show preference for fish faeces. Finally, we cultured protozoa species that preferentially swarmed on faeces with an EPA-free medium and diet to evaluate their ability to synthesize EPA.

## 2. Methods

### 2.1. Relationship between the number of protozoa and EPA in faeces

To evaluate whether of EPA in *D. rerio* faeces is generated by heterotrophic protozoa, ten individual *D. rerio* were raised in a 60 l water tank for more than 1 year under stable temperature conditions. Half of the water volume was replaced once a week. Some protozoan species occurred naturally in the water tank. Chlorella (Chlorella King, Chlorella Industry Co.), which does not contain EPA (Fujibayashi et al., 2016), was provided twice a day as the sole diet for the experimental fish.

To collect the faeces, several *D. rerio* were randomly taken and temporally (about 2 h) transferred to a 1 l beaker containing dechlorinated tap water. A pipette was used to retrieve the faeces immediately after excretion faeces were transferred to a 100 ml glass beaker. The 100 ml glass beaker was placed in the water tank capped with 1 mm mesh to prevent suspended particles in the water tank from sinking into the beaker. The faeces-providing fish were also transferred back into the initial holding tank. This procedure was repeated once a day for 6 consecutive days. On the first day of the experiment, the chlorella diet was also placed in a 100 ml glass beaker (diet) that was capped with a 1 mm mesh net and then transferred to the water tank. A total of eight, 100 ml glass beakers, containing either faeces or diet were in the tank by the seventh day (Fig. S1 in the Appendix).

On the seventh day of the experiment, all 100 ml glass beakers that contained fish faeces or diet were retrieved from the water tank. Faeces or diet were transferred to 15 ml glass tubes with a pipet to which dechlorinated water was added to adjust to a total volume of 7 ml. These glass tubes were ultrasonicated with 30 s and homogenized with a vortex mixer. Next, 0.3 ml of subsample was examined using an optical microscope to identify and enumerate the number of protozoa. Counting was conducted three times for each glass tube. Faeces or diet that remained in the glass tube were centrifuged and retrieved, and then freeze-dried and weighed. Freeze-dried faeces and diet were analyzed for fatty acid composition (see the “Fatty acid analysis” section below). The relationship between EPA content and the number of protozoa was then examined. Absence of other algal species was confirmed by microscopic observation.

### 2.2. Protozoa preference test

To determine whether protozoa selectively swarm on faeces, 500 ml of dechlorinated tap water and 300 ml of BLEY medium that contained 1000 cell $ml^{-1}$ of *Cyclidium* sp. were added to a 1 l glass beaker. Fifteen 1.5 ml glass vials were then placed in the beaker. Five of the vials contained faeces of *D. rerio* that were collected in the same manner as described above. Another five vials contained chlorella. The final 5 vials were used as a control (Fig. S2 in the Appendix). After 3 days, the number of *Cyclidium* in each glass vial was counted with an optical microscope.

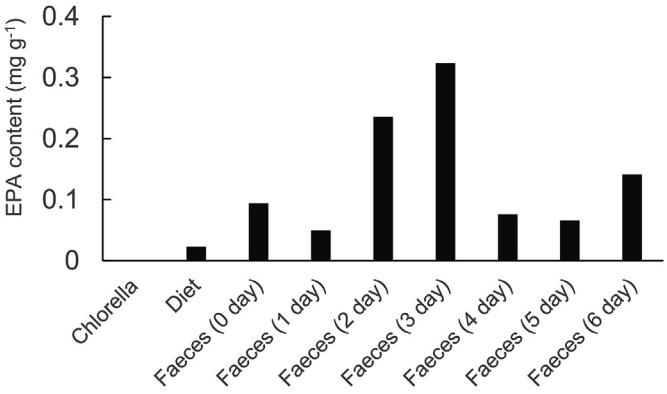


**Fig. 1.** Eicosapentanoic acid (EPA) content in fish faeces, chlorella and diet (chlorella).

### 2.3. Protozoan cultures

To analyze fatty acids in the protozoan cells, ciliate *Cyclidium* sp. and *Glaucoma* sp., which were associated with the EPA content of *D. rerio* faeces (see result) were cultured. These strains were isolated from an activating sludge and have stocked in BLEY medium subculture. The BLEY medium was made by mixing 0.5 g/l of boiled young barley leaf powder and 1.5 g/l of boiled egg yolk. Because BLEY medium contains ALA, parts of protozoa in BLEY medium was transferred to an inorganic modified Taub medium (Tanaka et al., 1994; Taub and Dollar, 1964) that contained no ALA. After a 7-day cultivation, part of this mixed culture were transferred to the inorganic modified Taub medium again. This procedure was repeated five times. In this way, the initial BLEY medium was diluted 100,000 times, and its effect on the fatty acid composition of the protozoa was considered negligible.

The prey bacterium, *Alcaligenes faecalis* NBRC13111, was obtained from the Biological Resource Center, National Institute of Technology and Evaluation, Japan. The stock culture of *A. faecalis* was inoculated into a sterile TG medium (Taub medium added to 10 g/l of glutamate) in an Erlenmeyer flask and precultivated at 30 °C for about 96 h. The centrifuged bacterium was rinsed and resuspended in the Taub medium, to which the protozoan solution was added.

### 2.4. Fatty acid analysis

The fatty acid composition of the freeze-dried and filtered (Whatman, GF/D) protozoa was analyzed following a slightly modified version of the one-step method developed by Abdulkadir and Tsuchiya (2008). Briefly, the super surface layer of the glass filter that contained the protozoa was scraped, and this sample was placed in a 50 ml glass tube with 4 ml of hexane and 1 ml of internal standard (1 mg of tricosanoic acid with 20 ml of hexane). The glass tube was then placed in a 100 °C water bath for 2 h. After cooling to room temperature, 1 ml of hexane and 2 ml of ultrapure water were added. The glass tube was shaken vigorously and then centrifuged for 3 min at 2500 rpm. The upper layer of hexane, which contained the fatty acid methyl esters (FAME), was poured into a 1.5 ml GC vial.

One microliter of the FAME solution was injected into a gas chromatograph (GC-2014, Shimadzu) equipped with a capillary column (Select FAME, 100 m × 0.25 mm i.d., Agilent Technologies). The column temperature was programmed from 150 °C (with a 5 min hold) to 230 °C (4 °C/min) and held for 10 min. The temperature was then increased to 250 °C at 4 °C/min and held for 10 min. Helium gas was used as a carrier. The temperatures of the injection and FID detector were 270 °C and 280 °C, respectively.

Fatty acids in the samples were identified by comparison with the retention times of commercial authentic standard mixtures (Supelco Inc. Supelco 37 component FAME Mix, BAME Mix, PUFA3, Me. 14-