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Starvation induced changes in nucleic acids and protein contents of crustacean zooplankton

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Abstract

We compared changes in nucleic acids (DNA and RNA) and protein (PRO) contents of crustacean zooplankton (*Calanus sinicus* and *Artemia* sp.) to starving exposure. We measured a reduction in the individual DNA, RNA and PRO of meta-naupliar stage of *Artemia* sp. following 2-days of starvation, while such response was not clear for starved copepodite stage 5 of *C. sinicus*. We measured a significantly reduced RNA:DNA ratio for the starved individuals of both species after 2 days, whereas there was no significant difference in the RNA:PRO ratios of fed and starved individuals of both species. Although DNA, RNA and PRO of individuals were slightly reduced for *C. sinicus* following prolonged starvation (6-days), the declines of RNA:DNA and RNA:PRO ratios were disappeared due to the relative decrease of DNA and PRO to RNA. These results demonstrate significant negative responses of RNA:DNA ratio to starving exposure and highlight these biochemical measurements as useful proxies of physiological responses to food limited conditions for crustacean zooplankton. However, we also note that RNA:DNA ratio is representative of short-term feeding history (i.e., short starvation) for crustacean zooplankton.

Introduction

Zooplankton population dynamics are largely determined by rates of development, growth, and mortality [1]. The amount of information on *in situ* rates of zooplankton development and growth has been increased over the last century because of the widespread use of serial sampling and incubation techniques [2]. On the other hand, estimates of mortality rates are very difficult to measure in the field. Cohort analysis with frequent sampling [3] can be used but diffusion and advection of water masses can limit our ability to track the same populations over time [4]. Moreover, predator exclusion can limit the value of incubation-based estimates of mortality [4]. Thus, we have no routinely applicable method for measuring natural mortality rates for pelagic zooplankton.

Over the last four decades, zooplankton ecologists have explored the potential utility of a variety of biochemical techniques for measuring physiological processes [5, 6, 7]. Variation in nucleic acids content represents a biochemical measurement which has been proposed as a proxy of feeding history and/or starvation for zooplankton [8, 9, 10]. While starvation is considered to increase their mortality risks due to the reduction of their swimming activity [11], we still don't know how starvation can be identified for natural zooplankton populations and/or communities in food-diluted environments. Although biochemical approaches have been used to study physiological responses of *Acartia* spp. to starvation [12, 13], we have little information on their use for other crustacean zooplankton.

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The aim of the present study was to investigate the changes in individual content of DNA, RNA and protein (PRO) and their ratios of differently crustacean zooplankton (*Calanus sinicus* and *Artemia* sp.) in response to starvation. We also compared the changes in DNA, RNA and PRO of *C. sinicus* between the different durations of starving exposure.

Materials and Methods

To compare changes in DNA, RNA and PRO of crustacean zooplankton, we selected meta-naupliar stage of *Artemia* sp. (hereafter *Artemia* sp.) and copepodite stage 5 of *Calanus sinicus* (hereafter *C. sinicus*). *Artemia* sp. resting eggs (Tetra Japan Co., Ltd.) were hatched (28°C) and development monitored to meta-naupliar stage in a 5 L polyethylene tank containing 0.2 μm filtered seawater. Animals were fed a mixture of *Isochrysis* sp., *Pavlova sp., Thalassiosira weissflogii* and *Tetraselmis* sp. (Shellfish Diet 1800TM: Instant Algae Co.) using a flow-through system [14]. *C. sinicus* were collected from 100 m to sea surface in the northern Kagoshima Bay using a North Pacific Standard net [15] (diameter 45 cm, mesh size 0.335 mm, vertically towing speed 0.5 m sec^{-1}) equipped with a 3 L cod end (0.335-mm mesh on it). On deck, healthy specimens of *C. sinicus* were quickly identified to copepodite stage 5 under a dissecting microscope and 100 individuals were kept in a 500 mL polycarbonate bottle containing the 0.2-μm filtered seawater.

Specimens of *Artemia* sp. and *C. sinicus* were incubated for 2 days in a 5 L polyethylene tank containing 0.2 μm filtered seawater with and without phytoplankton. The tank was maintained at ambient temperature (16°C) on a 12L:12D cycle in an incubator (EYELA MTI-201B) and the water was gently aerated. We also carried out an additional, longer (6 day) starvation experiment for *C. sinicus*. Food concentrations in the "fed" treatment (i.e., tank with phytoplankton) were kept at a constant 3.5×10^8 cells L^{-1} (food-saturated) using the flow-through system. Specimens for biochemical analyses were collected from treatment tanks, sorted into 2 mL vials, and stored at -80°C at the start and end of experiments,.

RNA and DNA were measured following the microplate fluorescent assay (MFA) developed by Wagner *et al.* [16]. Each specimen was thawed and then homogenized (in 0.2 mL extraction buffer with 1% sarcosyl) by vigorous shaking with 5 zirconia beads. Samples were homogenized for 20 minutes at room temperature on a vortex mixer equipped with a multiple-vial head. The samples were then diluted with Tris buffer to reduce the sarcosyl concentration to 0.1%, and were then shaken for an additional 40 minutes. The samples were then centrifuged (15000×g) for 10 min at 4°C to separate insoluble brine shrimp/copepod remains. In each run, 100 μL aliquots of sample supernatant, 8 replicates of DNA (calf-thymus, Sigma-Aldrich D4522) ranging from 0.15 to 10.0 μg mL^{-1}, and 8 replicates of RNA (bakers-yeast, Sigma-Aldrich R7125) ranging from 0.15 to 5 μg mL^{-1}; were transferred to 96-well microplates (Nunc). The zero concentrations of standard solutions were treated as reagent blanks (i.e., containing all chemicals but no brine shrimp/copepod homogenate). Exactly 0.1 mL of 0.1% SYBER Green I (Sigma-Aldrich S9430) was added to each well, and the plates were then kept at room temperature for 20 minutes. Reaction fluorescence was then scanned (First scan) using a microplate reader (Perkin-Elmer, ARVO MX1420) with 530 nm (excitation) and 590 nm (emission) filters. RNase solution (10 μL) was added to each well. The microplate was then kept at room temperature for 15 minutes, after which, the SYBER Green fluorescence was scanned (Second scan). The RNA concentrations (μg mL^{-1}) were calculated as the differences between the first (DNA+RNA) and second (DNA) scans. Absolute concentrations were calculated using the standard curve of RNA versus the fluorescence established from the first scan of the same plate. DNA concentrations (μg mL^{-1}) were computed from the second scan and the standard curve of DNA versus fluorescence established at the second scan (RNase treated) of the same plate.

Protein content was determined from a sub-sample of brine shrimp/copepod homogenate according to the protein-binding method of Bradford [17]. Aliquots (1000 μL) of homogenate supernatant were transferred to a glass tube containing 1000 μL of the Bradford Regent (Sigma-Aldrich B6919) and kept at room temperature for 20 minutes. The absorbance of the dye color was measured at 595 nm using a spectorophotometer (Hitachi, U1800). Protein concentrations (μg mL^{-1}) were computed from a standard curve of protein standard (bovine serum albumin: Sigma B2518) concentration versus absorption.

Results

DNA and PRO were not significantly different between fed and starved *Calanus sinicus* after incubation for 2 days (Welch's t-test, $p>0.05$) (Fig. 1). Although we measured a slight reduction in RNA after 2-days starvation, the difference

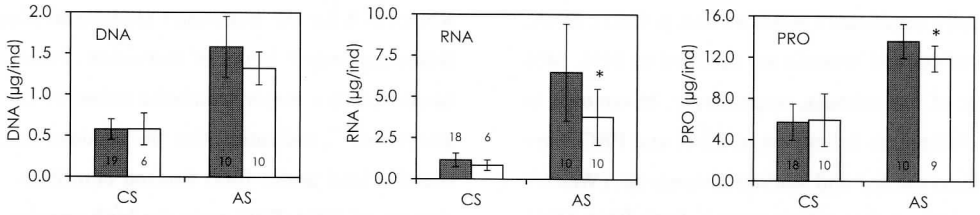

Figure 1. Comparison of individual DNA, RNA, and PRO content for *Calanus sinicus* (CS) copepodite stage 5 of and *Artemia* sp. (AS) metanauplii. Animals were either fed (grey bars) or starved (white bars) for 2 days. Significant differences between treatments at $p<0.05$ (Welch's t-test indicated by an asterisk, *. Error bars represent standard error. Numbers in each panel show replicates.

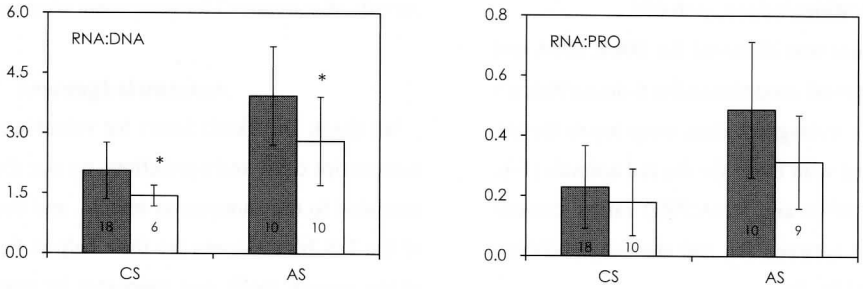

Figure 2. Comparison of RNA:DNA and RNA:PRO ratios for *Calanus sinicus* (CS) copepodite stage 5 and *Artemia* sp. (AS) metanauplii. Animals were either fed (grey bars) or starved (white bars) for 2 days. Significant differences between treatments at $p<0.05$ (Welch's t-test indicated by an asterisk, *. Error bars represent standard error. Numbers in each panel show replicates.

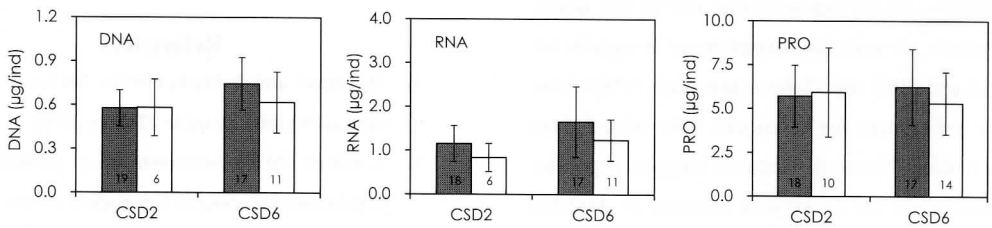

Figure 3. Comparison of individual DNA, RNA and PRO content for fed (gray bars) and starved (white bars) copepodite stage 5 of *Calanus sinicus* (CS) following 2 days (D2) and 6 days (D6). Significant differences between treatments at $p<0.05$ (Welch's t-test indicated by an asterisk, *. Error bars represent standard error. Numbers in each panel show replicates.

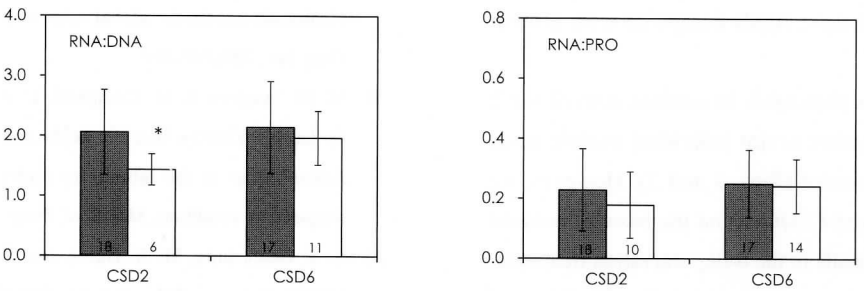

Figure 4. Comparison of RNA:DNA and RNA:PRO ratios for fed (gray bars) and starved (white bars) *Calanus sinicus* (CS) copepodite stage 5 following 2 days (D2) and 6 days (D6). Significant differences between treatments at $p<0.05$ (Welch's t-test indicated by an asterisk, *. Error bars represent standard error. Numbers in each panel show replicates.

was not statistically significant (Welch's t-test, $p>0.05$). DNA, RNA and PRO of starved *Artemia* sp. declined to 84%, 58% and 88% of that of fed animals, respectively. In contrast to *C. sinicus*, the differences in individual RNA and PRO were significant between the fed and starved *Artemia* sp. (Welch's t-test, $p<0.05$). After 2 days of starvation, both RNA:DNA and RNA:PRO ratios for *C. sinicus* were reduced relative to fed animals, however, this difference was only significant for RNA:DNA (Fig. 2; Welch's t-test, $p<0.05$). *Artemia* sp. also showed a significant difference in RNA:DNA ratio between fed and starved animals (Welch's t-test, $p<0.05$).

No significant difference was observed for DNA, RNA and PRO between fed and starved conditions after 6 days (Welch's t-test, $p>0.05$), while the averaged values were lower for the starved animals compared with those for the fed animals (Fig. 3). The resultant RNA:DNA and RNA:PRO ratios showed no significant difference between fed and starved conditions (Welch's t-test, $p>0.05$) (Fig. 4).

Discussion

An earlier study found that DNA of *Acartia tonsa* reduced in response to starvation [13]. They concluded that the reduction under starving exposure was due to cell death. In the present study, *Artemia* sp. experienced a significant decrease in RNA and PRO after 2-days starvation, while these responses were not evident for *C. sinicus* after both 2-days and 6-days starvation. These differences suggest a greater tolerance to starvation for *C. sinicus* relative to *Artemia* sp. Some of the improved tolerance to starvation may be resulted from the reserved lipids because copepodite stage 5 of *C. sinicus* eventually accumulates lipids in preparation for dormancy and eventual gonad maturation [18]. Although such accumulation was no evident for *Artemia* spp., *Artemia franciscana* consumed more lipids compared with protein under starving [19].

RNA:DNA ratio was detectable in animals starved for 2 days, regardless of whether or not individual nucleic acids or protein content decreased (Figs. 1 and 2). However, we could not identify starved *C. sinicus* on the basis of reduced RNA:DNA ratio for animals in the 6-day starvation treatment. Our results with *C. sinicus* are consistent with those of Speekmann et al. [13], who found that both DNA and RNA decreased in a relative manner, making difficult to identify starved animals (*Acartia tonsa*) on the basis of ratios. In contrast, Vrede et al. [20] measured significant reductions of RNA:DNA for the freshwater cladoceran (*Daphnia galeata*), following only 5 hours of starvation. Protein has been also identified as a major metabolic substrate for *Artemia* under starvation [19], indicating that the accumulated protein is also metabolized under food limited condition. No significant decline of RNA:PRO ratio for both species might be more decrease of protein relative to RNA under starvation. These results suggest that RNA:PRO ratio is not suitable for identifying starvation and variation of RNA:DNA ratio is only representative of very recent feeding history (i.e., short-term periods of starvation) for crustacean zooplankton.

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