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EVALUATION OF DIETARY MICROALGAE IN THE CULTURE OF *ACARTIA STEUERI* (COPEPODA, CALANOIDA)

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Abstract: Adult females of the calanoid copepod *Acartia steueri* were individually reared for eight days with different microalgal diets to determine the optimal microalgal diet for their survival and reproduction. Five mono-microalgal diets (*Thalassiosira weissflogii, Rhodomonas salina, Tetraselmis suecica, Phaeodactylum tricornutum*, and *Isochrysis galbana*) and one mixed microalgal diet (*Tetraselmis suecica* + *Thalassiosira weissflogii*) were tested. The survival rate of the females, egg production rates and hatching success were measured every day for eight days. *A. steueri* fed with *R. salina, P. tricornutum*, and *I. galbana* had lower rates of egg production over time and eventually egg production ceased. This phenomenon indicates that these microalgae are unfavourable as dietary substitutes for the adults of *A. steueri*. The cumulative number of eggs produced for eight days was 24.8 ± 4.2 eggs female^{-1} when fed with *T. suecica*, which was the highest value among the mono-microalgal diet treatments. The viable egg production was maximised with a mixed diet of *T. suecica* and *T. weissflogii*, and therefore the mixed diet can be considered a suitable diet for *A. steueri* adults.

Keywords: Copepod, cultivation, egg production, hatching success, microalgae**.**
Abbreviations: Filtered seawater (FSW), egg production rate (EPR).

Introduction

Copepods are natural prey items for most marine fish larvae, accounting for nearly 80% of their stomach contents (Tanaka *et al*., 1987; Blaxter *et al*., 1998; Okazaki *et al*., 2019). In aquaculture and ornamental fish industries, copepods are considered the preferred live feed for marine fish larvae over the commonly used organisms such as *Artemia* and rotifers (Støttrup, 2003; Rasdi & Qin, 2016; Radhakrishnan *et al*., 2020). Marine fish larvae fed with copepods, instead of *Artemia* and rotifers, had better survival rates (Wilcox *et al*., 2006), better pigmentation (Næss *et al*., 1995; Busch *et al*., 2011), and better growth (Støttrup & Norsker, 1997; Øie *et al*., 2017). Despite the obvious advantages of using copepods as a live feed, their use is still limited owing to low productivity and cost efficiency when cultured intensively.

Copepods from the genus *Acartia* are pelagic, can be found in coastal waters worldwide (Hansen *et al*., 2016), and are good candidates for a live feed because their body size, swimming behaviour, and biochemical composition are suitable for many marine fish larvae which have small mouth gapes (Rajkumar & Vasagam, 2006; Wilcox *et al*., 2006). In addition, *Acartia* species produce dormant eggs (quiescent arrested embryogenesis) which can be stored and hatched to start new cultures or feed fish larvae (Drillet *et al*., 2006; Drillet *et al*., 2008; Hansen *et al*., 2016). *Acartia steueri* Smirnov, 1936 is widely distributed in the coastal waters of the western Pacific Ocean from South Kuril Bay, Russia, to Kabira Bay, Okinawa Island (Kos, 1958; Nishida, 1985), Japan, and is an essential food source for the larvae of commercially important fishes in

their natural habitats (Tanaka *et al*., 1987). Recently, this species was used in a trial culture on a laboratory scale because its mortality and fecundity were independent of the effect of crowding even at a density of 2000 inds. L^{-1} (Takayama *et al*., 2020; 2021).

Diet critically affects the egg production rate, hatching success, survival rate, growth rate, and population growth of copepods. One of the underlying difficulties in the intensive cultivation of copepods is the varied dietary requirements between species (Buttino *et al*., 2009; Alajmi & Zeng, 2015; Takayama *et al*., 2022). Generally, calanoid copepods feed on live microalgae. Feeding copepods with multiple microalgal species can provide for a more balanced diet for the copepods and improve several aspects of copepod cultivation. Previous studies demonstrated that cultivation with mixed microalgal diets improved the egg production of copepods compared with those fed with mono diets (Li *et al*., 2008; Alajmi & Zeng, 2015). For example, mixed microalgal diets used in the culture of *Acartia sinjiensis, Bestiolina similis*, and *Parvocalanus crassirostris* enhanced their overall performance compared to monoalgal diets (Milione & Zeng, 2007; Camus *et al*., 2009; Camus & Zeng, 2010; Ohs *et al*., 2010; Alajmi & Zeng, 2015). Thus, in the present study, *A. steueri* was fed five different mono-microalgal diets and one mixed diet for eight days, and the effects of these diets on the survival, egg production rate, and hatching success of the copepod were studied.

Materials and Methods

Microalgal Culture

The present study used five microalgal species, namely, *Thalassiosira weissflogii, Rhodomonas salina, Tetraselmis suecica, Phaeodactylum tricornutum*, and *Isochrysis galbana*, which are commonly used in marine hatcheries throughout the world and are relatively easy to maintain (Table 1). Batch cultures of the microalgae were grown on f/2 medium (Guillard, 1975) in 50 mL conical flasks at 20°C in an incubator (FLI-301N, EYELA), under a photoperiod of 12:12 h light: dark cycle and a light intensity of 120 µmol m^{-2} s^{-1}. Cell sizes of the microalgae were measured under a light microscope (OPTIPHOT-2, Nikon Co., Ltd.) with an ocular micrometre. Cells were counted using a haemocytometer (Hirschmann, Eberstadt) under a light microscope to determine the cell densities in the cultures. The microalgae fed to the copepods were harvested during the mid– to late logarithmic phase.

Collection and Preculture of Experimental Specimens

Field sampling was conducted at Manazuru Port (35°09'49"N, 139°10'33"E; maximum depth 6 m), located in the north-western part of Sagami Bay, central Japan (Figure 1). Manazuru Port is a typical temperate embayment area where environmental factors change abruptly as a result of irregular inflows of fresh water and tidewater as well as complex topography and

Table 1: Microalgae used as diets for the copepods in the present study

Microalgae	Classification	Cell size (µm)
Tetraselmis suecica	Chlorophyte	7.0 ± 1.1
Rhodomonas salina	Cryptophyte	10.3 ± 2.7
Thalassiosira weissflogii	Diatom	17.1 ± 3.8
Phaeodactylum tricornutum	Diatom	15.0 ± 1.3
Isochrysis galbana	Haptophyte	4.5 ± 1.1
Mixed diet (*T. suecica* & *T. weissflogii*)	–	–

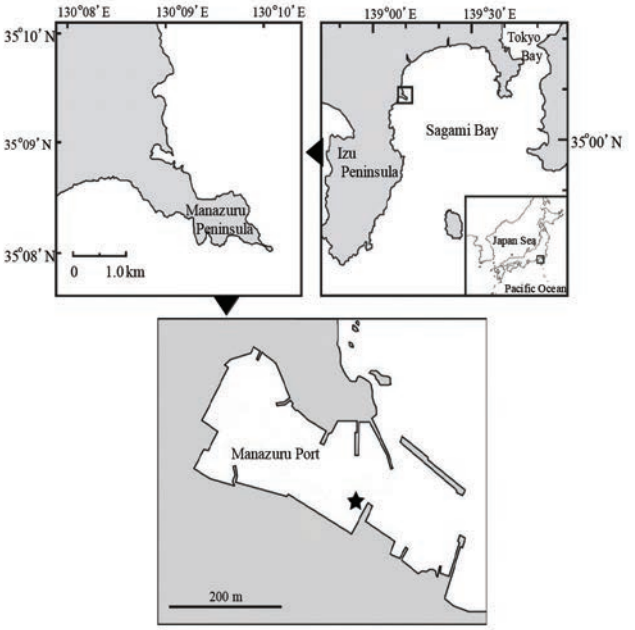

Figure 1: Map of the study area. The location of the sampling site is denoted by the closed star

seasonal changes (e.g. Satoh *et al*., 2000; Toda *et al*., 2000; Tsuchiya *et al*., 2013; Takayama *et al*., 2018).

Zooplankton samples were collected using a plankton net (mesh size 180 µm, diameter 30 cm, length 100 cm) towed gently and obliquely from the sea bottom to the surface in May and June 2019. At the same time, surface seawater was collected with a bucket and was used to culture the collected copepods. After sampling, the zooplankton samples were immediately transferred (within 30 minutes) to a field laboratory. Adult females of *A. steueri* were sorted from the collected samples under a dissecting microscope (WILD M10, Leica Co., Ltd.) based on morphological characteristics described by Ueda (1997). Four bottles containing 25 females each were filled with 250 mL ambient seawater pre-screened through a 65-µm mesh. The females were acclimatised at 19°C (near-ambient temperature of the surface seawater), fed with a sufficient supply (500 µg C L^{-1}) of a mixed microalgal diet consisting of *T. weissflogii, R. salina, T. suecica*, *P. tricornutum,* and *I. galbana* at 1:1:1:1:1 carbon ratio, and kept inside an incubator (Biotron, NK system) with a photoperiod of 12:12 h light: dark cycle for 24 hours to negate the effects of the prior food environment in the field. After acclimatisation, healthy female copepods were selected from the stock culture and used in all the experiments described below.

Survival Rate, Egg Production and Hatching Success

Adult females of *A. steueri* were cultured with different diet treatments to examine the effects of the microalgal diets on their survival rate, egg production rate (EPR), and hatching success. The present study consisted of two experiments. In the first experiment, copepods collected in May 2019 were tested with five mono-algal diets (*T. weissflogii, R. salina, T. suecica*, *P. tricornutum*, and *I. galbana*) and starved. In the second experiment, copepods collected in June 2019 were tested with one mono-algal diet of *T. suecica*, one mixed diet (*T. suecica* + *T. weissflogii* at 1:1 carbon ratio), and one batch was starved.

In both experiments, individual adult females were placed into separate culture chambers (diameter 40 mm, height 80 mm), which were placed inside beakers containing 50 mL seawater filtered with a 0.22 µm membrane (Merck Millipore) (Figure 2). The culture chamber retaining the adult females has a 180-µm nylon mesh placed 5 mm above the bottom to prevent cannibalism (Onoue *et al*., 2004; Takayama *et al*., 2019) while allowing passage of the eggs. The copepods were fed daily at 300 µg C L^{-1} (15 µg C ind. $^{-1}$) and maintained under a photoperiod of 12:12 hour light: dark cycle at 19°C in an incubator for eight days, with fifteen replicates. During the culture period, agitation was conducted twice a day by gentle pipetting to disperse the microalgal diets in the water. Survival rate (%) was calculated from the total number of individuals in each treatment and the number of individuals that died. The seawater in each chamber was replaced daily with freshly filtered seawater (FSW). The discharged seawater was passed through a 35-µm nylon mesh to collect the spawned eggs (70 µm diameter). Collected eggs were counted under a dissecting microscope, and the egg production rate (EPR) was calculated as:

$$\text{EPR (eggs female}^{-1}\text{ day}^{-1}) = n/N/d$$

where n is the number of eggs counted; N is the number of females; and d is the incubation period (days).

After counting, the eggs were incubated in a petri dish containing FSW at 19°C in an incubator (CN-25C, Mitsubishi) in the dark for 48 hours (Uye, 1980) to measure the hatching success. After incubation, all individuals (hatched nauplii and unhatched eggs) were fixed in a 5% buffered formalin–seawater solution. Hatching success (%) was calculated from the number of nauplii and the total number of eggs. Viable fecundity was estimated by multiplying fecundity with hatching success.

Statistical Analyses

Data was verified to have met the parametric test assumption. Differences in egg production between the different diet treatments were analysed using a one-way analysis of variance (ANOVA). Tukey–Kramer *post hoc* test was performed when ANOVA showed a significant difference at $p < 0.05$.

Ethics Statement

All copepod samples were collected following the national legislation in Japan, and all necessary permits were obtained before conducting the research.

Results

First Experiment (Comparison among Five Mono-microalgal Diet Conditions)

In the first experiment comparing the mono-diets, survival rates of the adult females of *A. steueri* fed with *T. suecica* and *T. weissflogii* at day 8 were 86.7% and 53.3%, respectively (Figure 3a). Other microalgal diets (*R. salina, P. tricornutum,* and *I. galbana*) showed similar temporal variations with the starvation condition in survival rate and were less than 15% at day 8.

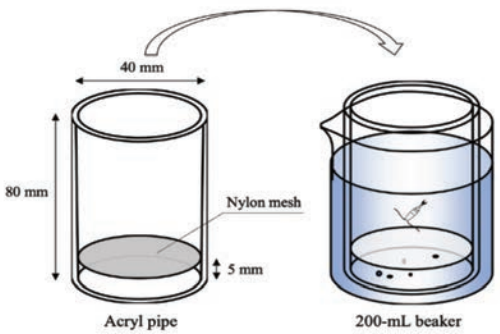

Figure 2: The culture chamber was used for egg production experiments in the present study

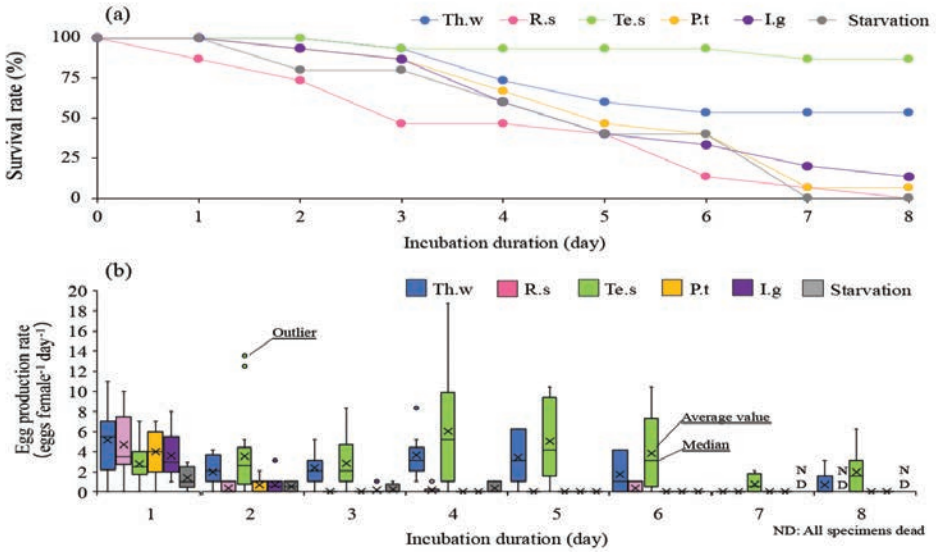

Figure 3: Temporal variations in (a) survival rate and (b) egg production rate of female *Acartia steueri* cultured with different microalgal diets for 8 days, in the first experiment conducted in May 2019. Th.w: *Thalassiosira weissflogii*, R.s: *Rhodomonas salina*, Te.s: *Tetraselmis suecica*, P.t: *Phaeodactylum tricornutum*, I.g: *Isochrysis galbana*. N.D. in the figure indicates that the egg production rate could not be measured because all specimens died

The egg production rate (EPR) ranged from 0.0 ± 0.0 to 5.8 ± 5.3 eggs female^{-1} day^{-1} (Figure 3b). In the *R. salina, P. tricornutum,* and *I. galbana* treatments, EPR decreased after day 1, and egg production eventually ceased. The cumulative number of eggs produced over eight days (i.e. individual fecundity) differed significantly ($p < 0.05$) among the different microalgal diet treatments, with a significantly greater number of eggs produced when the copepods were fed with *T. suecica* (24.5 ± 17.7 eggs female^{-1}) compared with the *R. salina, P. tricornutum, I. galbana* treatments, and those left in the batch that were starved (one-way ANOVA, Tukey–Kramer, $p < 0.05$) (Figure 4).

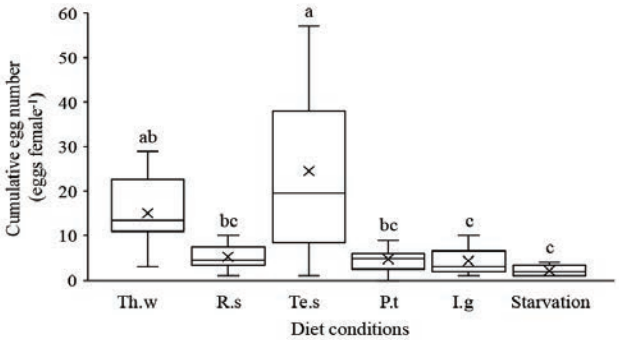

Figure 4: Individual fecundity of *Acartia steueri* cultured with different microalgal diets for 8 days in the first experiment conducted in May 2019. The error bar shows the standard deviations (n=15). Different letters on the top of the bars indicate a significant difference in each experimental condition (one-way ANOVA, Tukey-Kramer, p<0.05). Th.w: *Thalassiosira weissflogii*, R.s: *Rhodomonas salina*, Te.s: *Tetraselmis suecica*, P.t: *Phaeodactylum tricornutum*, I.g: *Isochrysis galbana*

The hatching successes in the *T. weissflogii* and *T. suecica* treatments were 92.6% and 94.4%, respectively (Table 2). 14.0 and 23.1 viable eggs female^{-1} were estimated under the mono-diet condition of *T. weissflogii* and *T. suecica*, respectively.

Second Experiment (Comparison between a Mono-microalgal Diet and a Mixed Diet)

In the second experiment, copepods were tested with a mono-diet treatment of *T. suecica*, which showed maximum individual fecundity in the first experiment, and a mixed diet (*T. suecica* + *T. weissflogii*). In terms of female survival rates, both conditions showed similar temporal variations with 60% at day 8 (Figure 5a).

Egg production in both conditions was consistent throughout the incubation period except for the mono-diet treatment on day 2, and EPR varied from 0.0—3.8 ± 4.6 eggs female^{-1} day^{-1} (Figure 5b). The cumulative egg number over eight days in the mixed diet treatment was

Table 2: Hatching success and cumulative number of viable eggs over 8 days (viable-fecundity) of *Acartia steueri* raised under different food conditions in the first and second experiments. N.D. indicates that hatching success could not be measured due to insufficient sample numbers. Viable fecundity was estimated by multiplying fecundity with hatching success.

	Food condition	Hatching success (%)	Viable-fecundity (viable eggs female^{-1})
1st exp.	*Thalassiosira weissflogii*	92.6	14.0
	Rhodomonas salina	N.D.	-
	Tetraselmis suecica	94.4	23.1
	Phaeodactylum tricornutum	N.D.	-
	Isochrysis galbana	N.D.	-
	Starvation condition	N.D.	-
2nd exp.	*Tetraselmis suecica*	89.5	5.5
	Mix diet (*T. suecica* & *T. wissflogii*)	98.1	13.5
	Starvation condition	N.D.	-

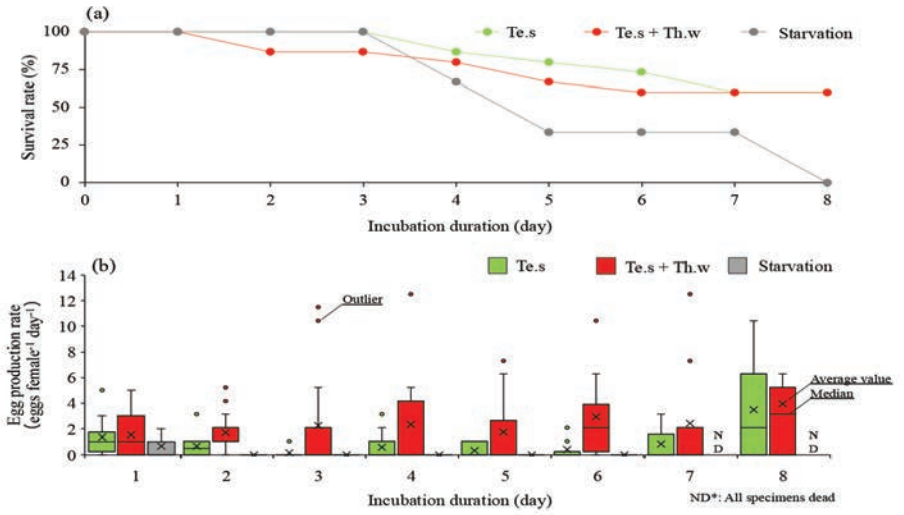

Figure 5: Temporal variations in (a) survival rate and (b) egg production rate of female *Acartia steueri* cultured with different microalgal diets for 8 days during the second experiment conducted in June 2019. Te.s: *Tetraselmis suecica*, Te.s + Th.w: mixed diet of *Tetraselmis suecica* and *Thalassiosira weissflogii*. N.D. in the figure indicates that the egg production rate could not be measured because all specimens died

significantly higher than that of the batch that was left to starve. (one-way ANOVA, Tukey–Kramer, $p < 0.05$), but no significant difference was found between the mono-diet treatment (6.1 ± 5.6 eggs female^{-1}) and the mixed diet treatment (13.7 ± 13.2 eggs female^{-1}) (one-way ANOVA, Tukey–Kramer, $p > 0.05$) (Figure 6).

The hatching successes in the mono-diet treatment of *T. suecica* and the mixed-diet treatment (*T. suecica* + *T. weissflogii*) were 89.5% and 98.1%, respectively (Table 2). Viable fecundity of 5.5 and 13.5 viable eggs female^{-1} were estimated in the mono-diet and mixed diet treatments, respectively.

Discussion

In the first experiment to determine a favourable diet for adults of *A. steueri* from five mono-microalgal diets, the copepods fed with *T. suecica* showed the highest cumulative egg number (i.e. individual fecundity), followed by those fed with *T. weissflogii*. In contrast, the treatments of other microalgal diets (*R. salina, P. tricornutum,* and *I. galbana*) showed similar values to the starvation condition in survival rate, egg production rate (EPR), and individual fecundity. Therefore, these results suggest that these diets are unsuitable for adult *A. steueri*. One difficulty with the mass cultivation of copepods is the varied dietary requirements of individual species. *I. galbana* is known as a DHA-rich alga and favourable diet of *Acartia southwelli, Acartia tonsa, Apocyclops royi, Centropages typicus* and *Pseudodiaptomus annandalei* (Drillet *et al*., 2011; Zhang *et al*., 2013). It implies that *A. steueri* may have a different nutritional requirement for reproduction to compare with these species. Previous studies demonstrated that cultivation with mixed diets improved egg production compared to copepods on mono-diets (Li *et al*., 2008; Alajmi & Zeng, 2015). Thus, in the second experiment, we compared the effects of a mono-diet of *T. suecica*, chosen as a representative of the mono-diets, and a mixed-diet of *T. suecica* + *T. weissflogii*, which are both effective microalgal diets for female *A. steueri*. The number of viable eggs produced by a female fed with the mixed diet increased by 2.5 times compared with that of a female on the mono-diet treatment (Table 2). Hence, the mixed diet of *T. suecica* + *T. weissflogii* can be considered a suitable diet for adults of *A. steueri* among the microalgal species in the present study, which are commonly available and relatively easy to culture. The use of mixed diets may be effective for copepods such as *A. steueri* that require a specific diet and show relatively low fecundity under mono-diet conditions.

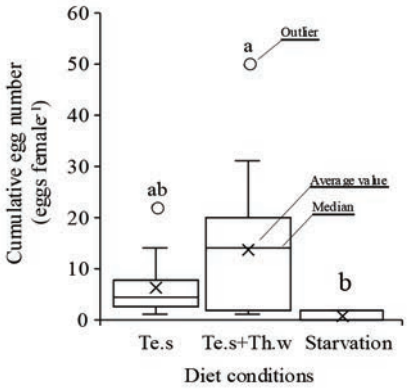

Figure 6: Individual fecundity of *Acartia steueri was* raised under different microalgal diets for 8 days in the second experiment conducted in June 2019. The error bar shows the standard deviation (n=15). Different letters on the top of the bars indicate a significant difference in each experimental condition (one-way ANOVA, Tukey-Kramer, p<0.05). Te.s: *Tetraselmis suecica*, Te.s + Th.w: Mixed diet of *Tetraselmis suecica* and *Thalassiosira weissflogii*

Previous research suggested that mono-microalgal diets may be limited in elemental ratios (Urabe & Watanabe, 1992), highly unsaturated fatty acids (Müller-Navarra *et al*., 2000), sterols (Klein Breteler *et al*., 1999) or amino acids (Kleppel *et al*., 1998) and thus cannot sustain a high EPR in copepods. In the second experiment of the present study, when a mixed diet of *T. suecica* + *T. weissflogii* was fed to *A. steueri*, the copepods' fecundity was higher than when fed *T. suecica* alone. *T. suecica* is well known to be rich in amino acids but with poor fatty acid content (Zhang *et al*., 2013). In contrast, *T. weissflogii* is rich in polyunsaturated fatty acid, which is an important nutrient for egg production in calanoid copepods (Jónasdóttir & Kiørboe, 1996; Payne & Rippingale, 2000; Kikuchi, 2020). The further improvement in egg production when a mixed diet was used might be due to the increased quantity and quality of fatty acids by the addition of *T. weissflogii* to *T. suecica*.

The *T. suecica* treatment in the first and second experiments of the present study showed different values in fecundity even under the same incubation conditions such as temperature, salinity, and food conditions. The timing of the collection of the copepods used in the two experiments from the field was different and might have caused the differences in fecundity. It was also suggested that the EPR of copepods decreases with age in the adult stage (Mauchline, 1998). Previous research demonstrated remarkable energy accumulation ability and starvation tolerance of *A. steueri*, and reported that EPR measured in the laboratory is influenced by the food environment in the field when the copepods were collected (Hirahara & Toda, 2018; Hirahara *et al*., 2018). The different values between the two experiments in the present study might have been caused by differences in the age of the population collected and the food environments in the field experienced by the respective specimens.

Copepods were sub-cultured in the laboratory and the EPR of the copepods was measured within three days after reaching the adult stage to evaluate the performance of the mixed diet of *T. suecica* + *T. weissflogii*. The EPR of the sub-cultured copepod fed with the mixed diet was 10.8 ± 4.6 eggs female^{-1} day^{-1} (N=7). Reported EPR values in *A. steueri* were summarized in Table 3, and mixed diets used in the present study showed comparable values with the maximum EPR of this species. The maximum EPR of this copepod as observed in the field in Sagami Bay, Japan was 23.5 ± 5.5 eggs female^{-1} day^{-1} (Onoue, 2006). Copepods fed the mixed diet of *T. suecica* + *T. weissflogii* in the present study achieved 46% of the maximum EPR in *A. steueri*. EPR in calanoid species incubated in the laboratory generally never exceed the levels recorded in field populations (Pond *et al*., 1996; Carotenuto *et al*., 2006). This may be due to the availability of mixed diets at sea which could have supplemented specific essential nutrients or antiproliferative compounds (Turner *et al*., 2001; Ianora *et al*., 2003; Buttino *et al*., 2009). *Acartia* copepods ingest not only microalgae but also protozoa such as dinoflagellates and ciliates (Mauchline, 1988; Besiktepe & Dam, 2020). The population of *A. steueri* in Okkirai Bay, Japan is known to utilise dinoflagellates and oligotrich ciliates as primary food sources during their dominant season in the bay (Yamada *et al*., 2020). Further studies using several diets from different taxonomic groups might be needed to understand which nutrient is the limiting factor for EPR in *A. steueri*.

Table 3: Comparison of reported egg production rates of the calanoid copepod *Acartia steueri*

Study Type	Food	Food Conc. (µg C L^{-1})	Sampling Location	EPR (Eggs $Female^{-1}$ Day^{-1})	Hatching Success (%)	Ref.
Field investigation	*in situ*	-	Ilkwang Bay	3.9~10.1		Jung *et al.* 2004
	in situ	-	Sagami Bay	1.3~11.9		Onoue *et al.* 2004
	in situ	-	Sagami Bay	0.05~23.7		Onoue 2006
	in situ	-	Sagami Bay	0~7.7		Kikuchi 2020
	in situ	-	Okirai Bay	1.3~15.0		Yamada *et al.* 2020
Lab experiment using collected wild animal	*Thalassiosira weissflogii*	150	Sagami Bay	~6.8	-	Kikuchi 2020
	Chaetoceros gracilis	150	Sagami Bay	~2.3	-	Kikuchi 2020
	Dunaliella tertiolecta	150	Sagami Bay	~3.3	-	Kikuchi 2020
	Thalassiosira weissflogii	1500	Sagami Bay	8.2~18.8	77.4	Takayama *et al*. 2020
	Mix (*Thalassiosira weissflogii* & *Tetraselmis suecica*)	800	Sagami Bay	0~7.7	58.3-100	Takayama *et al.* 2021
	Thalassiosira weissflogii	300	Sagami Bay	0~5.0	93	This study
	Tetraselmis suecica in 1st experiment	300	Sagami Bay	0~5.8	94	This study
	Tetraselmis suecica in 2nd experiment	300	Sagami Bay	0.1~2.0	90.0	This study
	Mix (*Thalassiosira weissflogii* & *Tetraselmis suecica*)	300	Sagami Bay	1.6~3.8	98.0	This study
Lab experiment using sub-cultured animal	Mix (*Thalassiosira weissflogii* & *Tetraselmis suecica*)	300	Isolated from Sagami Bay	10.8 ± 4.6	100	This study

Conclusion

Our results showed clear evidence that microalgal diets dramatically impacted the survival and egg production of *A. steueri*. Among the mono-microalgal diet treatments, the highest cumulative number of eggs produced for eight days was 24.8 ± 4.2 eggs $female^{-1}$ when fed with *T. suecica*. The viable egg production was maximised for batches that were on a mixed diet of *T. suecica* and *T. weissflogii*, and this mixed diet can be considered a suitable diet for *A. steueri* adults. This study also illustrated that the reproduction of *A. steueri* was significantly improved when fed with the mixed diet of *T. suecica* + *T. weissflogii* compared with specimens on the mono-algal diet.

Acknowledgements

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