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Quantifying a trait-mediated indirect effect of an environmental stressor on population dynamics

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Shota Shibasaki[1#] & Masato Yamamichi[1,2,3,4,5,6*]

1. Center for Frontier Research, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka, 411-8540 Japan

2. Genetics Program, Graduate Institute for Advanced Studies, SOKENDAI (The Graduate University for Advanced Studies), 1111 Yata, Mishima, Shizuoka 411-8540, Japan

3. School of the Environment, The University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia

4. Department of International Health and Medical Anthropology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

5. Institute for Multidisciplinary Sciences, Yokohama National University, 79-5 Tokiwadai, Hodogaya, Yokohama, Kanagawa 240-8501, Japan

6. Department of Ecosystem Studies, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

[*]To whom correspondence: M.Y. myamamichi@nig.ac.jp

[#]Current affiliation: Faculty of Culture and Information Science, Doshisha University, Kyotanabe, Kyoto 610-0394, Japan

ORCID: SS 0000-0002-8196-0745, MY 0000-0003-2136-3399

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Abstract

Trait-mediated indirect interactions have been intensively studied to understand ecological dynamics in biological communities. On the other hand, little is known about the relative importance of trait-mediated indirect effects of environmental stressors on population dynamics. How does an environmental stressor indirectly affect the population dynamics of a focal species by altering the traits of interacting species and the strength of interspecific interactions? Here, we quantified the direct and trait-mediated indirect effects of an environmental stressor by combining rotifer-alga experiments and Bayesian parameter estimation of a dynamic model. These days, human activities salinize freshwater lakes globally, thereby increasing (decreasing) the death (birth) rates of plankton species. Salinity stress is also known to induce clump formation in certain phytoplankton species. As larger clumps work as a defense trait against gape-limited predation of zooplankton, the salinity stress can affect zooplankton not only directly but also indirectly through phytoplankton trait changes. We first show that a green alga *Chlamydomonas sphaeroides* formed larger clumps than the two model species of green algae (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*) under a moderate salinity stress (0.06M NaCl). Then, by tracking the clump size distributions of *Chlamydomonas sphaeroides*, we confirmed that small clumps are more vulnerable to predation by rotifers *Brachionus calyciflorus* as previous studies demonstrated. Finally, we co-cultured the green algae and rotifers for a week with and without salinity stress and fitted the Lotka-Volterra predator-prey model. We first estimated how salinity stress increased the mortality rate of rotifers by using *Chlorella vulgaris*, which seldom showed clump formation. Then, we estimated how salinity stress decreased the attack rate of rotifers on *Chlamydomonas sphaeroides* due to clump formation. We found that salinity stress increased the rotifer's mortality rate by 86% and decreased the attack rate on *Chlamydomonas sphaeroides* by 15%. These results indicate that salinity stress can weaken the predator-prey interaction, and thus salinization can harm freshwater zooplankton species through increasing the mortality rates and decreasing the attack rates. This will be an important step for a quantitative understanding of how environmental stressors can affect community dynamics via trait modifications.

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1 Introduction

Classic studies in ecology tended to focus on interspecific interactions mediated by population densities, but recent studies have demonstrated the importance of considering trait dynamics driven by behavioral changes, phenotypic plasticity, or rapid evolution (Bolker et al. 2003, Miner et al. 2005, Bolnick et al. 2011, Hendry 2017). Particularly, trait-mediated indirect interactions (TMII) have been intensively studied to understand community dynamics (Werner and Peacor 2003, Schmitz et al. 2004, Utsumi et al. 2010, Ohgushi et al. 2012, Golubski et al. 2016). For example, trophic cascades were traditionally investigated through the perspective of density-mediated indirect interactions: carnivores reduce the population density of herbivores, which increases plant biomass (Hairston et al. 1960). Studies on TMII, in contrast, emphasized the importance of behavioral change of intermediary species with the foraging-predation risk trade-off: carnivores may change the behavior of herbivores ("landscape of fear"), which can further increase or even decrease local plant biomass (Schmitz et al. 2004).

On the other hand, little is known about the relative importance of direct and trait-mediated indirect effects of environmental stressors on population dynamics. Human activities are currently affecting various species negatively, and researchers tend to focus on the loss of biodiversity thorough the direct effects of environmental stressors on death or birth rates. But if some species change their traits in response to anthropogenic disturbances, it can alter the demography of other interacting species (i.e., the trait-mediated indirect effects) (Abrams 1995, Werner and Peacor 2003, Peacor et al. 2020). For example, human activities can invoke fear in some species and reduce their foraging behaviors (Smith et al. 2024), which can increase their prey's performance (Leighton et al. 2010, Mehlhoop et al. 2022). While an increasing number of studies proposed the potential importance of trait-mediated indirect effects, it was in general difficult to quantify the relative contributions of direct and indirect pathways of environmental stressor to focal species' population dynamics.

Here we use a rotifer-alga experimental system (Fig. 1) for quantifying trait-mediated indirect effects. The rotifer-alga system has been used in various studies to understand species coexistence (Rothhaupt 1988), predator-prey population cycles (Fussmann et al. 2000, Verschoor et al. 2004, Scheuerl and Stelzer 2019, Blasius et al. 2020), and eco-evolutionary dynamics (Fussmann et al. 2003, Yoshida et al. 2003, Yoshida et al. 2007, Becks et al. 2010, Becks et al. 2012, Hiltunen et al. 2014, Kasada et al. 2014, Declerck et al. 2015, Haafke et al. 2016, Hermann and Becks 2022). Particularly, many studies focused on eco-evolutionary dynamics driven by rapid

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evolution of algal defense against rotifer predation (reviewed in Yamamichi 2020). In the freshwater unicellular green alga *Chlamydomonas reinhardtii*, the defense trait is palmelloid clump formation, which is too big to be eaten by gape-limited predators including rotifers (Lurling and Beekman 2006, Sathe and Durand 2016, Réveillon and Becks 2024), and such multicellularity is heritable and can evolve in laboratory experiments (Becks et al. 2010, Herron et al. 2019, Bernardes et al. 2021). Interestingly, predation is not a sole driver of clump formation: clump formation in green algae is also induced by environmental stressors (Cheloni and Slaveykova 2021, de Carpentier et al. 2022, Cornwallis et al. 2023) including salinity stress (Khona et al. 2016, Farkas et al. 2023). Human activities salinized freshwater systems globally through, for example, roadway de-icing (Dugan et al. 2017, Kaushal et al. 2021, Cunillera-Montcusí et al. 2022), impeding population growth of freshwater phyto- and zooplankton species (Sarma et al. 2006, Venâncio et al. 2019). Therefore, human activities may not only directly increase death rates of zooplankton species, but also indirectly harm them by inducing palmelloid clump formation of green algae.

Here, to understand trait-mediated indirect effects of salinity stress, we conducted laboratory experiments using four freshwater plankton species: a rotifer (*Brachionus calyciflorus*) as a predator and three green algae (*Chlamydomonas sphaeroides*, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris*) as prey. *Chlamydomonas sphaeroides* is not a model organism, but phylogenetically close to *Chlamydomonas reinhardtii* (Yumoto et al. 2013) and was used as a food of *B. calyciflorus* in previous experiments (Rothhaupt 1988). We first show that *Chlamydomonas sphaeroides* formed remarkably larger clumps than the other two algal species under a moderate salinity stress (0.06M NaCl) by phenotypic plasticity. Then, we show that large clumps of *Chlamydomonas sphaeroides* were less likely to be consumed by rotifer by comparing the clump size distributions before and after exposure to predation under salinity stress. Finally, we show that the rotifer's attack rate on the green alga decreased by 15% under salinity stress by Bayesian parameter estimation of the Lotka-Volterra model based on rotifer-alga co-culture time-series data with and without salinity stress. These results indicate that salinity-induced clump formation can weaken predator-prey interactions in freshwater ecosystem, highlighting the importance of understanding trait-mediated indirect effects of environmental stressors to understand community dynamics in changing environments.

2 Materials and Methods

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2.1 Strain Information

The unicellular green algae, *Chlorella vulgaris* (NIES-227), *Chlamydomonas reinhardtii* (NIES-2236), and *Chlamydomonas sphaeroides* (NIES-2242), were obtained from the National Institute for Environmental Studies (NIES), Japan. The green algae were maintained in the C medium (Ichimura 1971) at 23□ with the light cycle 14:10. The details of the culture conditions of the green algae were identical to our previous study (Shibasaki and Yamamichi 2024).

The facultative parthenogenetic rotifer *Brachionus calyciflorus* (*elevetus*) (*sensu* Otake et al. 2025) was isolated from a water sample in Kanazawa Castle Park, Ishikawa prefecture, Japan, obtained in November 2023 by Dr. Yurie Otake at Kyoto University. The rotifer has been maintained in C medium with *Chlorella vulgaris* as prey under 25□ with the light cycle 14:10. The culture of the rotifer has been transferred into a fresh C medium with a weekly dilution rate ca. 10 % while adding 1 ml culture of *Chlorella vulgaris*.

Before adding the rotifers to experimental flasks, the stock culture of rotifers was filtered twice with a 40 μm cell strainer, left for rotifers to starve for four hours in the dark at 25□, and then filtered and washed again so that *Chlorella vulgaris* did not contaminate the experimental flasks. This protocol follows a previous study with a similar experimental system (Hermann and Becks 2022).

2.2 Comparing the clump sizes

We first compared the clump sizes of the three green algae in the following four conditions with three replicates in each: (i) control (C medium), (ii) with predators (adding rotifers at initial density ca. one female individual/ml), (iii) with salt (0.06M NaCl C medium), and (iv) with predators and salt (adding rotifers at initial density ca. one female individual/ml to 0.06M NaCl C medium). We grew the green algae (and rotifers, if applicable) in 50 ml of the media in a 300 ml Erlenmeyer flask for seven days at 25□ with the light cycle 14:10 (light intensity 145 ± 5 μmol $m^{-2}s^{-1}$) while shaking (60 rpm). After the cultivation, we collected 1 ml of culture from each flask and added 10 μl of Lugol's solution (20 g of potassium iodide and 10 g of iodine mixed in 200 ml of distilled water) to fix the green algae. We measured the sizes (i.e., the number of cells per clump) of 300 clumps per sample under microscopy.

2.3 Evaluating salinity-induced clump formation as a defense trait

Next, we examined whether large clumps of *Chlamydomonas sphaeroides* induced by the salinity stress reduced the rotifer predation. We first grew *Chlamydomonas*

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sphaeroides in 50 ml of 0.06M NaCl C medium for seven days in six 300 ml Erlenmeyer flasks so that the green algae formed clumps. Then, we sampled the 5 ml of culture from each flask and fixed them using 50 µl of Lugol's solution. We measured the sizes of 300 clumps in each. 5 ml of rotifer cultures (in 0.06M NaCl C medium) were added to three of the six flasks (i.e., test cases) so that the initial density of rotifers was 0.1 individual/ml, while we added 5 ml of fresh 0.06M NaCl C medium to the rest of the three flasks (controls). The six cultures were grown for six hours under light (light intensity 145 ± 5 µmol $m^{-2}s^{-1}$) while shaking (60 rpm) at 25□. We then sampled the 5 ml of culture from each flask, fixed them with Lugol's solution, and measured the clump sizes as explained above.

2.4 Predator-prey dynamics and parameter estimation

To investigate how the salinity stress alters the predator-prey dynamics, we grew the rotifers and green algae with and without salinity stress (C medium with 0.06M NaCl and 0M NaCl, respectively) for seven days. The plankton species were grown in 50 ml of the growth medium in a 300 ml-Erlenmeyer flask at 25□ with the light cycle 14:10 (light intensity145 ± 5 µmol $m^{-2}s^{-1}$) while shaking (60 rpm). We estimated the population densities of green algae and rotifers daily by sampling 1 ml of culture from each flask. Because it was difficult to estimate the algal densities with clumps using an automated cell counter Countess II FL (Thermo Fisher Scientific Inc.), we centrifuged the sampled cultures (4□, 2120 x g, 10 min), removed the supernatant, added 1 ml of C medium to each, and left them for two hours at 25□ under lights. This protocol resolved many clumps because the clump formation in this system was caused by phenotypic plasticity (Khona et al. 2016; see also Fig. S11). After two hours, we fixed the samples by adding 10 µl of Lugol's solution to each. For each sample, we measured the densities of the green algae three times by Countess II FL (using 30 µl culture) and counted female rotifers under microscopy.

To distinguish the direct (i.e., increasing the mortality rate) and indirect (i.e., decreasing the attack rate due to the salinity-induced clump formation of prey) impacts of salinity stress on the rotifer's population dynamics, we fitted our experimental data to the following Lotka-Volterra predator-prey model with prey logistic growth and predator's linear (Holling type I) functional response:

$$\frac{dX_i}{dt} = X_i\left[r_{ij}\left(1-\frac{X_i}{K_{ij}}\right) - a_{ij}Y\right],$$

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$$\frac{dY}{dt} = Y\left(c_{ij}a_{ij}X_i - m_j\right) \quad (1)$$

where X_i is the $\log_{10}$ density of the green algae i, Y is the $\log_{10}$ density of female rotifers, r_{ij} is the intrinsic growth rate of the green algae i in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress, K_{ij} is the carrying capacity of the green algae i in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress, a_{ij} is the rotifer's attack rate on green algae i in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress, c_{ij} is the conversion rate of the green algae i to the rotifer's density the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress, and m_j is the mortality rate of the rotifers in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress.

The parameter values were estimated using Bayesian statistics (see Supporting Information for technical details). First, we estimated posterior distributions of the intrinsic growth rates r_{ij} and the carrying capacities K_{ij} of *Chlorella vulgaris* (i = 1) and *Chlamydomonas sphaeroides* (i = 2), respectively, in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress from nine-day mono-culture experiments (Supporting Information S1.1 and Figs. S1-S4). Next, we estimated the mortality rates of rotifers m_j in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress based on the seven-day co-culture experiments with *Chlorella vulgaris* (Supporting Information S1.2 and Figs. S5-S7). We assumed that the negative impact of salinity on rotifers come through the mortality rate only because *Chlorella vulgaris* seldom formed clumps in the presence of the 0.06M NaCl salinity stress (see Results). Indeed, the difference in the estimated attack rates a_{1j} with and without salinity stress was negligibly small (at most 10%) and 95% credible intervals (CIs) were overlapping with zero (Fig. S7: note that we assumed c_{1j} = 1 for convergence of posterior distributions). Finally, we estimated the attack rates on *Chlamydomonas sphaeroides* a_{2j} in the absence (j = 1) or presence (j = 2) of the 0.06M NaCl salinity stress along with the conversion efficiency c_{2j} (Supporting Information S1.3 and Figs. S8-S9) utilizing that the estimated mortality rates m_j based on the co-culture experiments with *Chlorella vulgaris*.

2.5 Experimental evolution of clumping formation

We also cultivated *Chlamydomonas sphaeroides* under salinity stress for 91 days to see whether larger clumps can evolve or not. Previous studies showed that palmelloid clump formation of *Chlamydomonas reinhardtii* can evolve rapidly in laboratory

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experiments (Becks et al. 2010). Although clump formation of *Chlamydomonas sphaeroides* is phenotypic plasticity, we expected that we might be able to observe genetic assimilation, where clumps originally formed in response to salinity stress later become genetically fixed via selection (Pigliucci et al. 2006). Such evolved strains would allow us to investigate whether larger clumps were more unlikely to be consumed by rotifers or not in the absence of salinity stress. We grew the ten lines of the green algae in 20 ml of C medium in 200 ml Erlenmeyer flasks at 25□ with the light cycle 14:10 (light intensity 145 ± 5 μmol $m^{-2}s^{-1}$) while shaking (120 rpm). Within the ten lines, five lines were grown under salinity stress (0.06M NaCl) while the rest five were without salt (0M NaCl). The initial algal density was approximately 1×10^5 cells/ml. Every week, we collected 200 μl of culture medium from each flask, centrifuged the sampled cultures (4□, 2120 x g, 10 min), removed the supernatant, and added each sample to 20 ml of fresh C medium with or without NaCl. Note that we accidentally sampled 1000 μl, instead of 200 μl, of cultures and transferred into fresh media on day 21.

We weekly measured the algae densities and mean diameter of particles (i.e., either single cells or multi-cellular clumps) by an automatic cell counter analyzer, CASY TT (Innovatis). In addition, we also examined whether *Chlamydomonas sphaeroides* under salinity stress lost the phenotypic plasticity. We collected 5 ml of culture medium from each line under salinity stress, centrifuged the sampled cultures, removed the supernatant, and grew in 2 ml of fresh C medium with and without NaCl, respectively, for six hours. After six hours, we measured the mean diameter of the particles by CASY TT. If the green algae lost phenotypic plasticity, the mean diameter without salinity stress was expected to be as high as with salinity stress. In contrast, the six-hour culture under salinity stress would not affect the mean diameters. Note that we do not have a part of the data on the mean diameter on days 42 and 91, because we could not use CASY TT due to its trouble on December 31st, 2024, and February 18th, 2025, respectively.

2.6 Statistical analyses

All statistical analyses were performed in R version 4.3.1 (R Core Team 2024). The effects of salt and rotifers on clump sizes of the green algae were analyzed by the non-parametric two-way ANOVA with the aligned rank transformation in ARTool library version 0.11.1 (Kay et al. 2025). The linear mixed model was implemented by lmerTest library version 3.1.3 (Kuznetsova et al. 2017). The parameter values in the

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predator-prey model were estimated by RStan library version 2.32.6 (Stan Development Team 2025).

3 Results

3.1 *Chlamydomonas sphaeroides* generates large clumps under salt

We first compared the clump sizes of the three freshwater green algae, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Chlamydomonas sphaeroides*, in the presence of either or both of two stressors: salt and predators (rotifers). Although all four planktons in this study are freshwater species, they can survive in the C medium containing 0.06M NaCl (see Figs. S1-S9 as examples) (Shibasaki and Yamamichi 2024). Fig. 2 shows that the median clump sizes of the three green algae were ones in the control cases, confirming that they were unicellular algae. While *Chlorella vulgaris* remained almost unicellular in the presence of salt and rotifers, two *Chlamydomonas* species increased their clump sizes in the presence of salt (0.06M NaCl). The median clump size of *Chlamydomonas reinhardtii* increased from one to two due to the salinity stress, but their typical clump sizes were less than four cells per clump. *Chlamydomonas sphaeroides* increased the median clump size from one to eight or more cells under salinity stress, and we even observed more than 30 cells existed in a single clump (see the bottom panels of Fig. 2). The statistical analysis also suggested that the salinity had a large impact on the clump sizes of *Chlamydomonas sphaeroides* (Table 1). Because the green algae immediately dissolved the clumps when the salinity stress was removed (Fig. S11), the palmelloid clump formation in response to salinity stress seems phenotypic plasticity, not evolution.

3.2 Larger clumps impeded the predation by the rotifers

Next, we examined whether large clumps of *Chlamydomonas sphaeroides* under salinity stress could work as a defense trait against gape-limited predation by rotifers. We addressed this question by comparing the clump size distribution before and after exposure to rotifer predation for six hours. Fig. 3 shows how the distributions of clump sizes changed with (Fig. 3A) and without (Fig. 3B) rotifers. In the absence of rotifers, the median clump sizes decreased or seldom changed in six hours (Fig. 3B). On the other hand, the median clump sizes clearly increased in the presence of rotifers (Fig. 3A). The violin plots also show that the density of small clumps (four or less cells per clump) decreased with predators. The linear mixed model also suggested that the clump sizes, on average, decreased in six hours but increased in the presence of the rotifers

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(Table 2). These results suggest that rotifers consumed small clumps but did not eat large clumps.

We also examined how salinity stress changed predator-prey dynamics by conducting co-culture experiments of the green algae and rotifers for seven days. While the green algae *Chlamydomonas sphaeroides* densities did not change significantly depending on salinity stress (Fig. 4A), we observed a remarkable decrease in rotifer's population density in the presence of salinity stress (Fig. 4B). To disentangle the relative importance of direct and trait-mediated indirect impacts of salinity stress, we fitted the experimental data to the Lotka-Volterra predator-prey model (Eq. 1) by performing the Bayesian estimation (see Supporting Information and Figs. S1-S9 for more details). Fig. 4C shows that the attack rate on *Chlamydomonas sphaeroides* was 15% (95% CI [−0.01, 0.37]) larger in the absence of salinity stress (a_{21}) than in the presence of salinity stress (a_{22}). Although the 95% CI included zero (the vertical dashed line in Fig. 4C), the posterior distribution indicated that the attack rate without salinity stress was larger than that with salinity stress in 93%. The mortality rate was, on the other hand, 86% (95% CI [−0.98, −0.71]) larger in the presence of salinity stress (m_2) than without salinity stress (m_1). Taken together, the salinity-induced clump formation was likely to decrease the growth rate of the rotifers by decreasing the attack rate, but this impact was less intensive than the increase in the rotifer's mortality rate.

3.3 The salinity stress did not lead to the evolution of larger clumps or loss of phenotypic plasticity.

We also examined whether the clump size evolved or not when *Chlamydomonas sphaeroides* were grown under salinity for 91 days. Fig. S10 shows that, while all five lines without salinity stress (i.e., controls; orange in Fig. S10A) grew constantly during the experiment, the lines under salinity stress (blue in Fig. S10A) gradually decreased the growth rates from day 7 with some variation: one line (Salt-1) maintained a moderate population density (approximately 1-2×10^5 cells/ml) during the experiment, whereas another (Salt-4) continued to decrease its density from day 70 and went extinct (its density was <100 cells/ml) at the end of the experiment. Fig. S10B shows the dynamics of mean diameters of the particles (single cells and multicellular clumps) during the experiment. Without salinity stress, the mean diameter remained around 10 μm, suggesting that the green algae were unicellular. On the other hand, the mean diameter under salinity stress was about 20 μm. This indicates that, if a single cell size did not change, a single clump would be composed of 2^3 = 8 cells on average, which is

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consistent with our observation under microscopy (Figs. 1-2). However, there was no evidence that clump size evolved to be larger under salinity stress.

We also investigated whether *Chlamydomonas sphaeroides* lost phenotypic plasticity (i.e., forming clumps without salt) during the experiment by transferring the green algae into fresh C media with and without NaCl for six hours. Fig. S11 shows the mean diameter returned to approximately 10 μm without salinity stress, while the mean diameter remained 20 μm with the 0.06M NaCl salinity stress. These results indicate that the green algae dissolved the clumps when the salinity stress was removed, and the phenotypic plasticity was kept during the 91-day experiment.

4 Discussion

While human activities can directly threaten organisms by reducing their habitats or harvesting, these impacts can also propagate communities by altering species interactions: i.e., trait-mediated indirect effects (Abrams 1995, Werner and Peacor 2003). Despite its potential importance, the relative importance of direct and indirect effects was unclear. Here, we used a rotifer-alga system to quantify a trait-mediated indirect effect in freshwater communities. We found that the 0.06M NaCl salinity stress induced the algal clump formation (Fig. 2), and it weakened the interspecific interaction (predation) between the green algae and rotifers (Figs. 3-4). In freshwater systems, salinization is a severe problem caused by human activities (Dugan et al. 2017, Kaushal et al. 2021, Cunillera-Montcusí et al. 2022). Our results demonstrate the importance of a quantitative understanding of how environmental stressors can affect community dynamics via trait modifications.

Previous studies investigated the clump formation of *Chlamydomonas reinhardtii* as a defense trait against predation (Lurling and Beekman 2006, Becks et al. 2010, Becks et al. 2012, Sathe and Durand 2016, Bernardes et al. 2021). However, green algae form multicellular clumps under other kinds of stress (Cheloni and Slaveykova 2021, de Carpentier et al. 2022, Cornwallis et al. 2023) including salinity stress (Khona et al. 2016, Farkas et al. 2023). Our study shows that *Chlamydomonas sphaeroides* was sensitive to the salinity stress and formed larger clumps than the two model green algae, *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, under 0.06M NaCl (Fig. 2). The salinity-induced clump formation was caused by phenotypic plasticity because the green algae dissolved the clumps and returned unicellular when the salinity was removed from the media (Fig. S11). The green algae formed clumps without predators (Figs. 2 and S10B), and thus its effect on the predator-prey interaction

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is a by-product of adaptation to salinity stress. Nevertheless, the salinity-induced clump formation worked as a defense trait against predation by rotifers as shown in previous studies (Becks et al. 2010, Réveillon and Becks 2024): our experiments showed that the rotifers were unlikely to consume large clumps (Fig. 3) and their attack rate on *Chlamydomonas sphaeroides* decreased under salinity (Fig. 4C). Our results, therefore, indicate that salinity stress weakened the predator-prey interaction by inducing the algae clump formation.

We found that the trait-mediated indirect effect of salinity stress (decreasing the attack rate on *Chlamydomonas sphaeroides* a_{2j} by 15%) was less intensive than the direct effect (increasing the rotifer's mortality rate a_{2j} by 86%) (Fig. 4). However, when we extrapolate the results, it can have a significant effect: if we assume that the attack and mortality rates change linearly in response to salinity (the black solid lines in Figs. S12A-B), the rotifer populations are expected to go extinct at ca. 0.2M NaCl (the solid curve in Fig. S12C) based on the Lotka-Volterra predator-prey model (Eq. 1). On the other hand, if we assume that the attack rate does not change in response to salinity (the black dashed line in Fig. S12B), rotifer extinction occurs at ca. 0.35M NaCl (the dashed curve in Fig. S12C). Thus, clump formation may greatly promote rotifer extinction. This is in stark contrast to the idea of indirect evolutionary rescue, where prey defense evolution prevents predator extinction (Yamamichi and Miner 2015, Cortez and Yamamichi 2019, Hermann and Becks 2022). Hermann and Becks (2022) showed that increasing salinity reduces the rotifer density through the direct effect, and the reduced predation pressure selects for less defended *Chlamydomonas reinhardtii* due to the defense-growth trade-off (Becks et al. 2010, Réveillon and Becks 2024). Because of the reduced frequency of defended algae, in turn, rotifer populations may be rescued from extinction. But in our system, clump formation is induced by salinity stress, and it increases the extinction risk of rotifers (Fig. S12). It will be interesting to integrate these two opposite consequences of clump formation for better understanding how clump formation under salinity stress can pleiotropically affect salt tolerance and predator-prey dynamics.

Reducing the attack rate in response to salinity stress can potentially cause other complex dynamics: the hydra effect (Abrams 2009). When we assume that the the attack rate is more sensitive to salinity (the gray line in Fig. S12B), the rotifer density can increase in response to the increasing salinity stress (Fig. S12D). This is because, despite the direct effect increasing the mortality rate, salinity stress decreases the attack rate, thereby increasing the population densities of algae and rotifers. The hydra effect has often been investigated in the context of rapid evolution and eco-evolutionary

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411 dynamics (Cortez and Yamamichi 2019), but it will be important to explore its
412 possibility with phenotypic plasticity as well.
413 Expanding our experimental system may provide deeper insights into how
414 human salinization activities disturb freshwater communities. For example, we will be
415 able to combine two or more stressors (e.g., pH, temperature) to experimentally show
416 how realistic environmental changes alter ecological dynamics (Kawaguchi and
417 Yamamichi 2025). Although we focused on interactions between two species, we can
418 introduce another green alga that may be less sensitive to salinity stress (e.g., *Chlorella*
419 *vulgaris*) to the rotifer-*Chlamydomonas* system to investigate how salt changes the
420 outcome of exploitative and apparent competition between the two algae species.
421 Another possibility is introducing a zooplankton species that consumes (e.g.,
422 *Asplanchna brightwelli*) (Verschoor et al. 2004) or competes with (e.g., *B. rubens*)
423 (Rothhaupt 1988) *B. calyciflorus*. Such expansion would allow us to investigate how
424 salinization would change the stability and structure of complex food webs. Shibasaki
425 and Terui (2024) recently showed that species richness and three-species subgraph in a
426 food web (i.e., motifs: Milo et al. 2002) alter food chain length, which represents the
427 vertical dimension of biodiversity (Wang and Brose 2018). Because salinity stress
428 would reduce the number of freshwater species by decreasing their growth rates and
429 simplify the food web by the trait-mediated indirect effects, salinization may shorten
430 food chain length in freshwater systems. Future studies can test this hypothesis by
431 extending our experimental system.
432 In conclusion, the present study demonstrates that rotifer-alga system is useful
433 for investigating trait-mediated interaction effects caused by environmental stressors.
434 We focused on the salinization of freshwater systems and analyzed how salinity stress
435 changes the predator-prey interaction between green algae *Chlamydomonas sphaeroides*
436 and rotifers. In addition to the direct effect of salinity stress increasing the rotifer
437 mortality rate, salinity stress induced the multicellular clump formation of the green
438 algae, which worked as a by-product defense trait against gape-limited predation by
439 rotifers. Therefore, the salinity impeded the growth of rotifers both directly and
440 indirectly. Future study could investigate how freshwater salinization alters food webs
441 by extending this experimental system.
442

443 Acknowledgment

444 The three strains of the green algae *Chlorella vulgaris* (NIES-227), *Chlamydomonas*
445 *reinhardtii* (NIES-2236), and *Chlamydomonas sphaeroides* (NIES-2242), were obtained

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Author contribution

S.S. and M. Y. conceived the project, S.S. performed the experiment, conducted the statistical analyses, and wrote the initial draft. M.Y. supervised the project and acquired the funding. All authors reviewed and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data availability

The experimental codes and programming cades will be deposited to a public repository upon acceptance.

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Tables

Table 1. Non-parametric two-way ANOVA and its effect size (partial η^2). Df is the degree of freedom.

Species	Stressor	Df	Df.res	*F* value	*p* value	partial η^2
Chlamydomonas sphaeroides	Salt	1	1196	1626.542	0	0.576
	Predator	1	1196	65.765	0	0.052
	Slat*Predator	1	1196	21.373	0	0.018
Chlamydomonas reinhardtii	Salt	1	1196	241.7099	0	0.168
	Predator	1	1196	14.1246	0	0.012
	Slat*Predator	1	1196	3.3138	0.069	0.003
Chlorella vulgaris	Salt	1	1196	7.1755	0.008	0.006
	Predator	1	1196	20.3152	0	0.017
	Slat*Predator	1	1196	4.5742	0.033	0.004

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658 **Table 2**. Fixed effect in the linear mixed model on the clump sizes after predation. S.E.
659 is standard error and Df is the degree of freedom.

Variable	Estimate	S.E.	Df	*t* value	*p* value
Intercept	2.054	0.081	5.157	25.307	0
6 hours	−0.169	0.056	3592	−3.015	0.003
Predators	−0.27	0.115	5.157	−2.354	0.064
6 hours with predators	0.343	0.115	5.157	2.991	0.029

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Figures

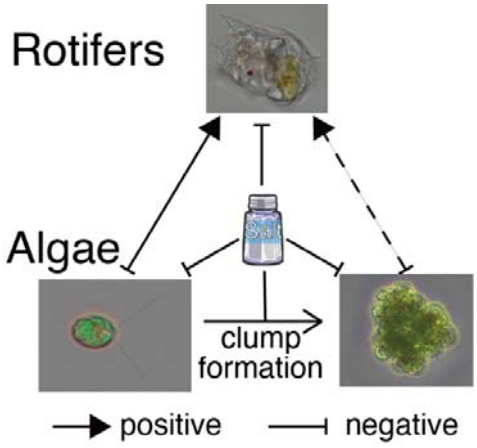
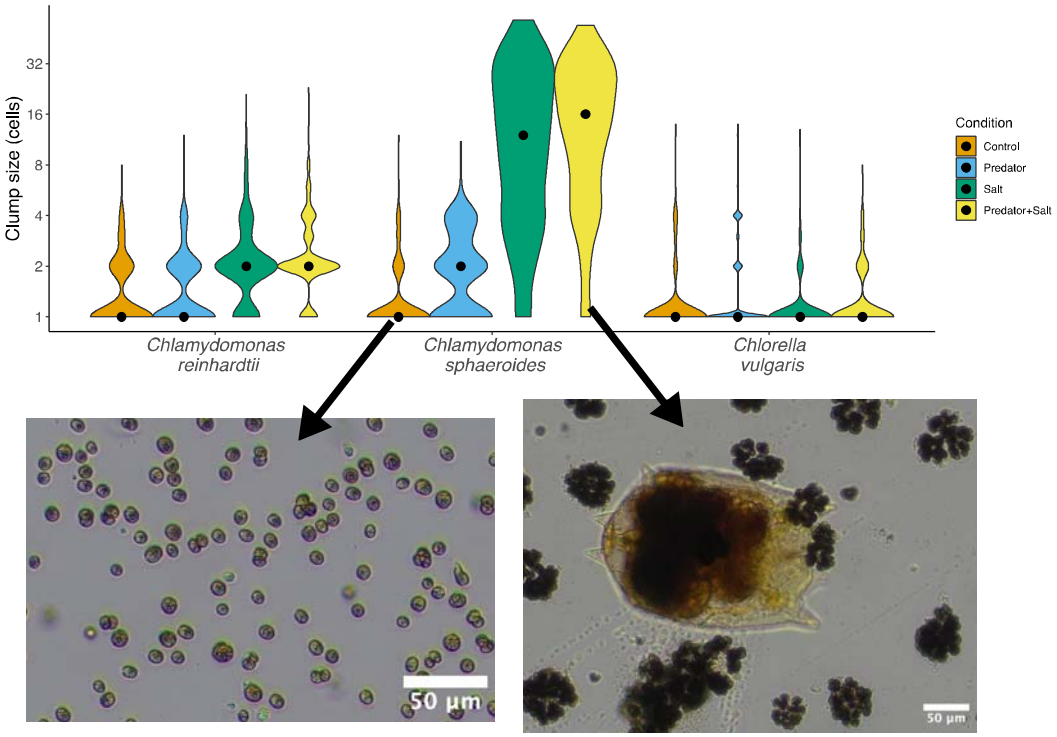


Figure 1. Schematic representation of the system.
The green algae *Chlamydomonas sphaeroides* is consumed by rotifers *Brachionus calyciflorus* (the solid and dashed oblique arrows between them). While the salinity directly decreases the growth of both species (the three arrows from the salt), it also induces the clump formation of the green algae (the horizontal arrow). The clumps weaken the predator-prey interaction because rotifers are gape-limited predators (i.e., the trait-mediated indirect effect of salinity stress: the dashed oblique arrows). The salt icon by Servier https://smart.servier.com/ is licensed under CC-BY 3.0 Unported https://creativecommons.org/licenses/by/3.0/. Photos were taken by S. Shibasaki.

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672

673 **Figure 2**. Large clump formation was induced by salinity stress in *Chlamydomonas*
674 *sphaeroides*.
675 Top: The distributions of clump sizes of *Chlamydomonas reinhardtii* (left),
676 *Chlamydomonas sphaeroides* (middle), and *Chlorella vulgaris* (right) under four
677 conditions (orange: control, blue: with rotifers, green: with 0.06M NaCl, and yellow:
678 with rotifers and 0.06M NaCl) after seven days. The black dots represent the median
679 clump sizes of 900 clumps. Bottom: examples of clump sizes of *Chlamydomonas*
680 *sphaeroides* in the control condition (left) and in the presence of salinity stress and
681 rotifers (right). The white bars represent the scale (50 µm). Photos were taken by S.
682 Shibasaki.

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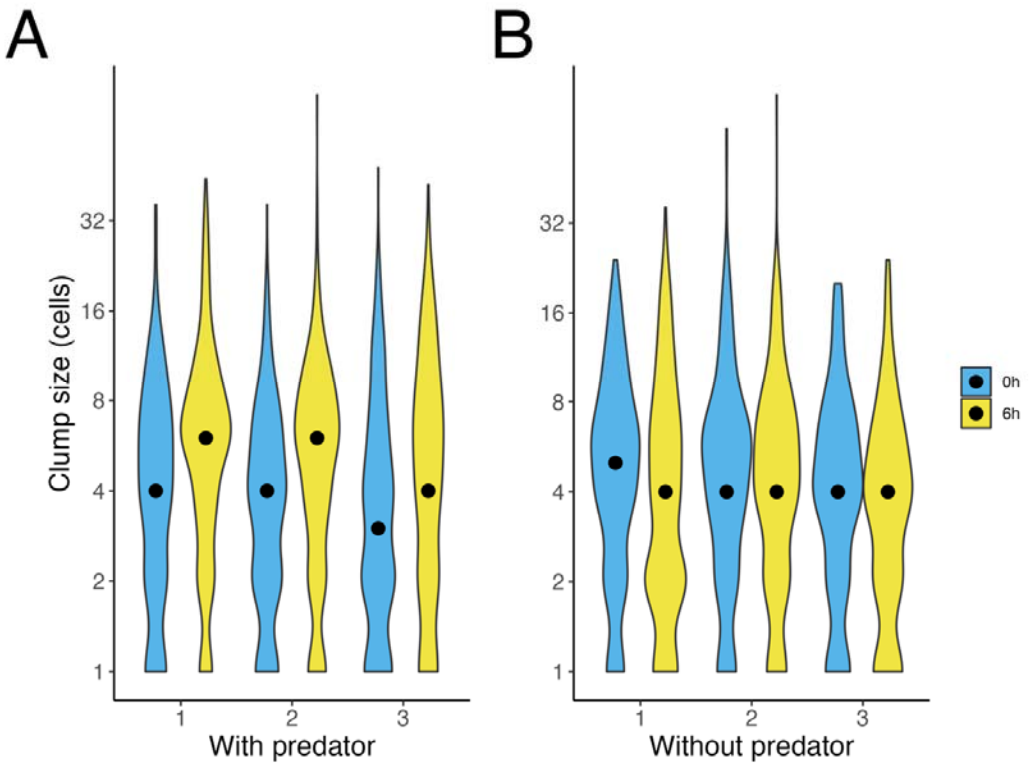


Figure 3. Large clumps were not consumed by rotifers.

The distributions of clump sizes of *Chlamydomonas sphaeroides* in the presence (A) or absence (B) of rotifers in 0.06M NaCl C medium with three replicates (flasks). By comparing the size distributions before (blue) and after (yellow) the exposure to rotifers for six hours, it is clear that rotifers consumed smaller clumps (A) and salinity stress cannot explain the difference (B). The black dots represent the median clump size of 300 clumps.

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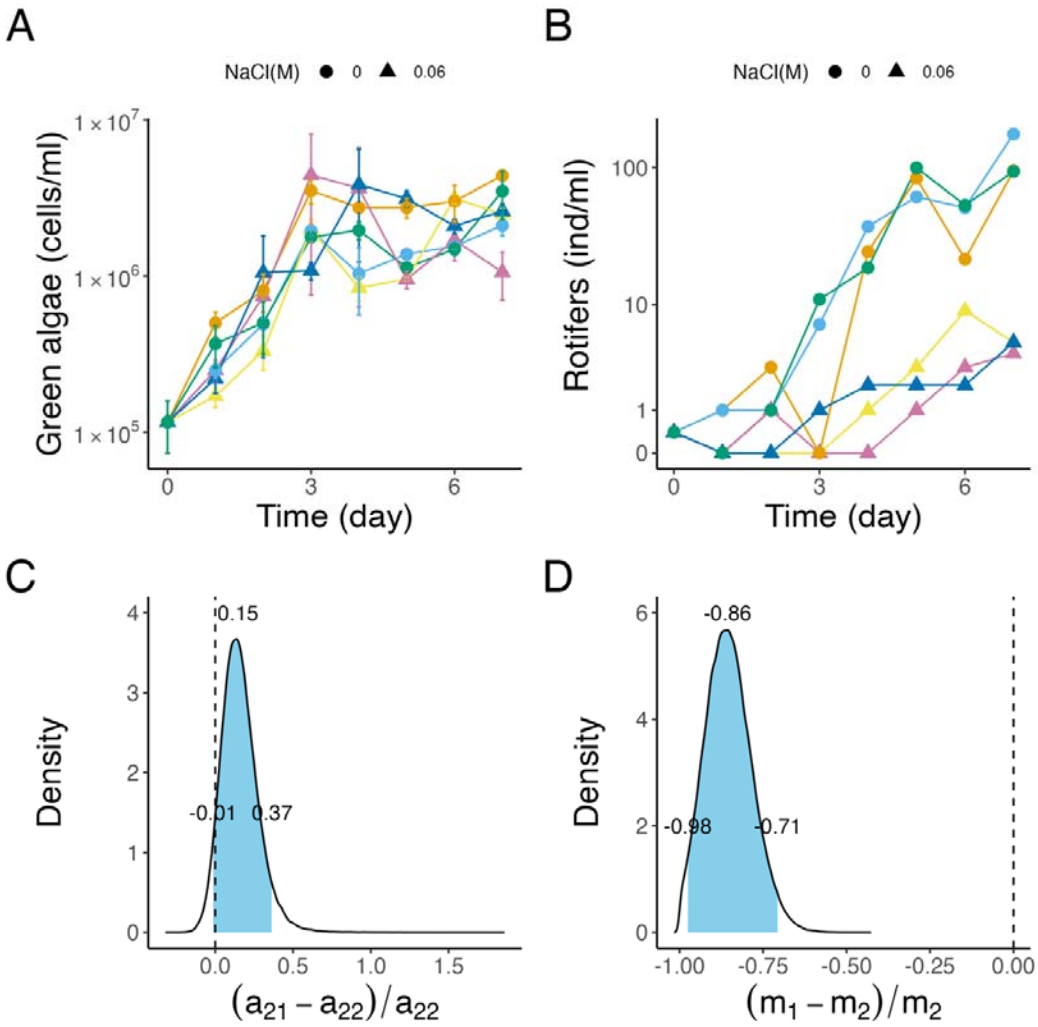


Figure 4. Predator-prey dynamics with or without salinity stress.
A-B: Population dynamics of *Chlamydomonas sphaeroides* (A) and rotifers (B) in the presence (triangle: 0.06M NaCl) or absence (circle: 0M NaCl) salinity stress with three replicates (shown by different colors). The error bars in A represent the standard errors while the markers show the mean values. C-D: Posterior distributions of the relative differences in the attack rate on *Chlamydomonas sphaeroides* (a_{2j}: C) and the mortality rate (m_2: D) of rotifers in the presence (j = 2) and absence (j = 1) of salinity stress. The shaded areas represent 95% credible intervals (CIs). The three digits indicate the boundaries of the 95% CIs and the median values.