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Effects of temperature on a mixotrophic dinoflagellate (*Lepidodinium* sp.) under different nutritional strategies

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ABSTRACT: Mixotrophs are widely distributed in aquatic ecosystems and play critical roles in the planktonic food web. However, how mixotrophs respond to projected ocean warming remains a debatable topic. To close the knowledge gap, we investigated the thermal responses of growth rate and functional traits of a mixotrophic dinoflagellate (*Lepidodinium* sp.) isolated from subtropical coastal waters. We found that *Lepidodinium* sp. is a facultative mixotroph with an obligate phototrophic lifestyle that adjusts its phagocytotic feeding according to inorganic nutrient concentrations. The thermal sensitivity in terms of activation energy (E_a, eV) of *Lepidodinium* sp. grown in mixotrophic mode (with sufficient prey, 0.69–0.89 eV) was significantly higher than in autotrophic mode (without prey, 0.30–0.37 eV). This finding is consistent with the results of predominantly heterotrophic mixotrophs, providing experimental evidence for the hypothesis that mixotrophs shift towards more heterotrophy with rising temperatures. Warming stimulated a higher growth rate of *Lepidodinium* sp. grown in mixotrophic conditions than in autotrophic conditions, indicating that mixotrophic dinoflagellates may benefit substantially from mixotrophy when temperature increases and prey is sufficient. Moreover, the cell size of both autotrophic and mixotrophic *Lepidodinium* sp. decreased with increasing temperature. The N:P and C:P ratios of *Lepidodinium* sp. did not vary with temperature, while the C:N ratio slightly increased. Our results suggest that mixotrophs like *Lepidodinium* sp. would become more heterotrophic with higher growth rates in warming oceans. The subsequent changes in their functional role from primary producers to consumers may affect food web dynamics and carbon and nutrient cycling.

KEY WORDS: Mixotrophs · Nutrition · Temperature · Stoichiometric ratio

1. INTRODUCTION

Marine protists are vital components in planktonic microbial food webs and are traditionally classified into 2 groups according to their nutritional modes: photoautotrophs that acquire carbon via photosynthesis, and phagotrophs that feed on bacteria and protists. However, in recent decades, many protists have been found to possess the capability of being autotrophic and heterotrophic simultaneously, and are therefore referred to as mixotrophs (Wilken et al. 2014a, Mitra et al. 2016, Selosse et al. 2017,

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Stoecker et al. 2017). Mixotrophs are widely distributed from the tropics to the polar regions and are commonly found in freshwater and marine ecosystems (Bockstahler & Coats 1993, Hartmann et al. 2012, Selosse et al. 2017). In some aquatic ecosystems, mixotrophs dominate the phytoplankton community and contribute to the majority of primary production (Stoecker et al. 2017). By contrast, some mixotrophs play an important role in bacterial loops and contribute to 40–95% of bacterial consumption, which enhances secondary production (Hartmann et al. 2012, Wilken et al. 2013). As such, acting as both primary producers and as bridges to enhance biomass transfer to larger organisms at higher trophic levels, mixotrophs are critical to marine food webs, global biogeochemical cycling, and marine ecosystem functioning (Ward & Follows 2016, Flynn et al. 2018).

In view of the ecological significance of mixotrophs, it is imperative to understand how mixotrophic protists respond to ocean warming in the context of climate change, which would have enormous implications for the effects of global warming on marine ecosystems. Yet, in contrast to numerous studies on phytoplankton and heterotrophic protists (Eppley 1972, Rose & Caron 2007, Englund et al. 2011, Chen & Laws 2017), the effects of temperature on mixotrophs have not been well documented. A recent study found that mixotrophic protists may become more heterotrophic when temperature increases (Wilken et al. 2013), because heterotrophic phagotrophic processes are more sensitive to increasing temperature than autotrophic photosynthesis processes (Brown et al. 2004, Allen et al. 2005). This shift caused by warming will change the functional role of mixotrophs from primary producers to consumers with the consequences of affecting carbon cycling (Wilken et al. 2013). However, the mixotrophic species (*Ochromonas* sp.) in that study is predominantly heterotrophic; whether other types of mixotrophs (e.g. phagotrophic 'algae' that are primarily phototrophic) would become more heterotrophic with rising temperature remains uncertain. In fact, a more recent study found that warming did not drive the freshwater mixotroph *Dinobryon* sp., which is predominantly photosynthetic, to become more heterotrophic (Princiotta et al. 2016). The effect of temperature on the balance of nutrient acquisition through photosynthesis and phagotrophy of mixotrophic protists could differ among various types and species. Therefore, studying more types of mixotrophic species and their responses to ocean warming is critical.

Temperature affects the metabolic processes and growth of mixotrophs and influences their functional traits such as cell size and intracellular nutrient stoichiometry (Atkinson et al. 2003, Yvon-Durocher et al. 2015). Temperature is a critical determinant of variations in C:N and C:P ratios of phytoplankton (Yvon-Durocher et al. 2015, Moreno & Martiny 2018). The C:N and C:P ratios of phytoplankton increase with increasing temperature because the enhanced efficiency of P-rich RNA and N-rich proteins in biochemical reactions reduces the demands of intracellular P and N contents (Woods et al. 2003, Toseland et al. 2013). The enhanced C fixation rate per unit resource at high temperature might also alter the C:N and C:P ratios of phytoplankton (Yvon-Durocher et al. 2015, Moorthi et al. 2017). Nevertheless, how the stoichiometry of mixotrophic species varies with temperature remains largely unknown, as it is more complicated than that of phytoplankton. Changes in the balance of nutrition at different temperatures may also affect the stoichiometry of mixotrophic protists because the trophic strategy (photoautotrophy or heterotrophy) determines the stoichiometry of plankton (Elser et al. 2000, Ho et al. 2020). Phytoplankton usually has higher C:N and C:P ratios than heterotrophic protists and zooplankton which can maintain the stoichiometry homeostatically (Elser et al. 2000, Sterner & Elser 2002), whereas mixotrophs have stoichiometry between phytoplankton and heterotrophic protists (Katechakis et al. 2005, Moorthi et al. 2017). If warming drives mixotrophic protists to behave more like heterotrophic protists, their intracellular nutrient stoichiometry could also vary with temperature, with lower C:N and C:P ratios at the higher temperature, which is opposite to the pattern in phytoplankton.

To close the knowledge gap regarding the effect of temperature on mixotrophic protists, we conducted experiments using *Lepidodinium* sp., a mixotrophic dinoflagellate recently isolated from subtropical coastal waters that has rarely been studied (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m678p037_supp.pdf). We investigated how increasing temperature affects the balance of nutrition (more phototrophic or heterotrophic) of this mixotrophic dinoflagellate and its physiological and stoichiometric traits. Based on the metabolic theory of ecology (Brown et al. 2004), we hypothesized that this mixotrophic dinoflagellate should become more heterotrophic at higher temperatures with higher temperature sensitivity when grown in mixotrophic mode than in autotrophic mode. In addition, the C:N and C:P ratios of the dinoflagellate should decrease with increasing temperature when this mixotroph becomes more heterotrophic.

2. MATERIALS AND METHODS

2.1. Culture conditions

The mixotrophic dinoflagellate (*Lepidodinium* sp.) used in this study was isolated from Port Shelter in the eastern waters of Hong Kong in the spring (April) of 2014. The water temperature when the single-cell isolation was conducted was about 21°C. Species identification was conducted by microscopic observation and 18s rDNA sequencing. Based on a BLAST search of GenBank, our species showed 99% similarity to *Lepidodinium* sp. (Strain MH360, accession number: AB686255) (Ng et al. 2017). Mixotrophic *Lepidodinium* sp. was grown in 0.2 µm filtered seawater and provided with the cryptomonad *Rhodomonas salina* as prey. *R. salina* was grown in filtered seawater with F/2 nutrient medium (Guillard & Ryther 1962). The cultures were maintained at 22°C with a light intensity of 100 µmol photons m^{-2} s^{-1} and a light:dark cycle of 12:12 h. Before each experiment, the *Lepidodinium* sp. cultures were acclimated in the designated conditions for at least 2 wk.

2.2. Ingestion rate

To explore the effect of nutrient availability on ingestion in mixotrophic *Lepidodinium* sp. and identify its functional type, we conducted grazing experiments under 3 different nutrient conditions using *Lepidodinium* sp. as the predator and *R. salina* as the prey. The 3 nutrient conditions were 80% (700 µmol l^{-1} NO_3^-, 29 µmol l^{-1} PO_4^{3-}), 50% (438 µmol l^{-1} NO_3^-, 18 µmol l^{-1} PO_4^{3-}), and 20% (175 µmol l^{-1} NO^{3-}, 7.2 µmol l^{-1} PO_4^{3-}) F/2 medium, respectively, which were sufficient for photosynthesis of *Lepidodinium* sp.

Prior to the experiments for measuring ingestion rates, *Lepidodinium* sp. was cultivated in the 3 designated nutrient conditions with sufficient prey to acclimate for at least 2 wk. We used semi-continuous cultures (i.e. transfer every 4 d) during the acclimation to ensure that nutrients would not be depleted and to keep *Lepidodinium* sp. growing in the exponential growth phase. Culture conditions were monitored every day by microscopic observation to confirm that *Lepidodinium* sp. grew well, and prey was added when needed during the acclimation.

To set up the grazing experiments under 3 designated nutrient conditions, we removed the nutrients and prey from the previous *Lepidodinium* sp. cultures. *Lepidodinium* sp. was filtered by 10 µm membranes and resuspended in sterile artificial seawater without nutrients. To remove the nutrients in the prey cultures, *R. salina* was centrifuged at 800 rpm for 5 min at 22°C, then washed and resuspended in sterile artificial seawater. Both *Lepidodinium* sp. and *R. salina* were observed under the microscope to guarantee that they were in good condition after the treatments. *Lepidodinium* sp. at a final concentration of 1000 cells ml^{-1} was transferred to 10 ml of medium (with designated nutrient concentrations) with 20 000 cell ml^{-1} of *R. salina*. The prey concentration was previously determined to be saturating for *Lepidodinium* sp. (Fig. S2). Cultures of *R. salina* at the same density as above were incubated under the same condition as controls for calculating the net growth rate of *R. salina* during grazing experiments. All treatments and controls were run in triplicates. The grazing experiments lasted for 6 h with a light intensity of 100 µmol photons m^{-2} s^{-1}. Samples (triplicates) for determining the cell abundance of *Lepidodinium* sp. and *R. salina* were collected and fixed in Lugol's solution (final concentration 2%) at 0 and 6 h of the experiment. The samples were observed using an inverted microscope (Olympus CK30, 100×) within 1 wk.

We conducted the above experiments at 25, 28, and 31°C to evaluate the effect of temperature on the ingestion activities of *Lepidodinium* sp. and how the interaction of nutrients and temperature affects the ingestion rate of *Lepidodinium* sp. The temperature range was set according to the temperature variations of the isolation site (www.seatemperature.org).

The ingestion rates of *Lepidodinium* sp. (I, cells Lep^{-1} h^{-1}) in grazing experiments were calculated as follows (Harris et al. 2000):

$$I = \frac{C_0 - C_t + [C] \times (e^{kt} - 1)}{n \times t} \quad (1)$$

where C_0 and C_t are the food concentrations at the beginning and end of the experiment; $[C]$ is the mean food concentration, calculated by averaging the initial and final concentration of *R. salina*; t is incubation time; k is the instantaneous growth coefficient of the food organisms; and n is the cell concentration of *Lepidodinium* sp. at the beginning of experiments, as the increase of *Lepidodinium* sp. was marginal during the short experimental time.

2.3. Growth rate and thermal trait

The growth rates of *Lepidodinium* sp. in 2 nutrition modes (autotrophic and mixotrophic) were measured

at 4 temperatures (19, 25, 28, and 31°C) to estimate the thermal traits of *Lepidodinium* sp. We distinguished the mixotrophic mode from autotrophic mode by adding sufficient prey to the cultures. For autotrophic growth, *Lepidodinium* sp. was cultivated under 3 nutrient conditions mentioned above (80, 50, and 20 % F/2 medium), while for mixotrophic growth, sufficient prey (20 000–30 000 cells ml^{-1}) was added to all treatments with 3 nutrient concentrations. Before the experiments, cultures of *Lepidodinium* sp. grown in the 2 nutrition modes and 3 nutrient concentrations were acclimated under the designated temperatures for 2 wk using semi-continuous cultures (i.e. transfer every 4 d). The cell densities of *Lepidodinium* sp. and *R. salina* in the cultures were monitored every other day under the microscope to ensure sufficient prey supply for *Lepidodinium* sp. After acclimation, the *Lepidodinium* sp. cultures with an initial concentration of 500 cells ml^{-1} were incubated at the designated conditions with a light intensity of 100 µmol photons m^{-2} s^{-1} and a light:dark cycle of 12:12 h. A subsample (1 ml) was collected from each bottle every 24 h, fixed with Lugol's solution (final concentration 2%), and observed under the microscope to determine cell abundance. The concentration of *R. salina* was also measured after 48 h. Additional prey was added into culture bottles (mixotrophic treatments) to maintain the prey concentration around 20 000–30 000 cells ml^{-1} and ensure the prey was sufficient for the growth of *Lepidodinium* sp. The experiments lasted for 4 d, and *Lepidodinium* sp. grew in the exponential growth phase during the experiments (Fig. S3).

Specific growth rate (µ) during exponential growth was calculated as:

$$\mu = \frac{\ln(N_1) - \ln(N_2)}{t_1 - t_2} \quad (2)$$

where N_1 and N_2 denote the abundances at time t_1 and t_2, respectively.

The thermal sensitivity of *Lepidodinium* sp. growth was then estimated according to the metabolic theory of ecology (Brown et al. 2004):

$$\mu = \mu_0 e^{-E_a/kT} \quad (3)$$

where µ is the specific growth rate of *Lepidodinium* sp. at either autotrophic or mixotrophic modes at temperature T, μ_0 is a pre-exponential constant independent of temperature, k is Boltzmann's constant (8.62×10^{-5} eV K^{-1}), and E_a is the activation energy of growth rate, which describes how fast the growth rate increases with increasing temperature. When calculating, the Boltzmann temperature $1/kT$ was standardized with a reference temperature (T_c = 15 °C): $1/kT_c - 1/kT$.

To investigate the optimal growth temperature as another thermal trait of *Lepidodinium* sp. in different nutritional modes (autotrophic vs. mixotrophic), we applied a unimodal extension of the Boltzmann-Arrhenius model to determine the optimal growth temperature of *Lepidodinium* sp. (Johnson & Lewin 1946, Chen & Laws 2017, Liu et al. 2019). As we did not observe a peak in the data of growth rate vs. temperature, we added an experiment to measure the growth rate of autotrophic and mixotrophic *Lepidodinium* sp. at 33 and 35°C. In this experiment, given that we confirmed that *Lepidodinium* sp. was mixotrophic under the 3 nutrient conditions mentioned above with sufficient prey, we only cultivated the *Lepidodinium* sp. using 50 % F/2 medium (with and without prey). These experiments also lasted 4 d, and a subsample (1 ml) was collected daily. The subsamples were fixed with Lugol's solution for microscopic observations. The data on the growth rates at different temperatures were then used to fit the model below:

$$\mu = \mu_0 \frac{e^{\frac{E_a}{k_b}\left(\frac{1}{T_0} - \frac{1}{T}\right)}}{1 + \frac{E_a}{E_h - E_a} e^{\frac{E_a}{k_b}\left(\frac{1}{T_{opt}} - \frac{1}{T}\right)}} \quad (4)$$

where T_{opt} is the optimal temperature at which the rate reaches the maximum value, and E_h is added to describe the 'steepness' of the decrease of the rate when the temperature exceeds T_{opt}. Other terms are the same as in Eq. (3).

2.4. Chlorophyll *a* content, cell size, and elemental composition

To examine the variation of cellular elemental contents and stoichiometry ratio, chlorophyll *a* (chl *a*) content, and cell size of *Lepidodinium* sp. in 2 nutrition modes at different temperatures, we cultivated *Lepidodinium* sp. in 50 % F/2 medium with and without prey at different temperatures. Before collecting the samples, the cultures were maintained semi-continuously in the 50 % F/2 medium with and without prey at the designed temperatures with a light intensity of 100 µmol photons m^{-2} s^{-1} under a light:dark cycle of 12:12 h for at least 2 wk (i.e. transfer every 4 d for both autotrophic and mixotrophic cultures, and prey was added to the mixotrophic cultures when needed). For chl *a* content and cell size analysis, the cultures were incu-

bated at 19, 25, and 31°C, while for C and N content analysis, 4 temperatures (i.e. 22, 25, 28, and 31°C) were chosen.

To collect the chl *a* samples, the cultures (40 ml) were filtered through 10 µm polycarbonate membrane filters to remove prey, because *R. salina* is about 6~10 µm in length and could theoretically pass through the membrane. It is worth noting that this method cannot remove prey already inside the *Lepidodinium* sp. The filters were then soaked in 90% acetone at 4°C for 24 h in darkness for pigment extraction. Chl *a* concentration was measured using a Turner Designs fluorometer with a non-acidification module (Model No. Trilogy 040) (Ducklow & Dickson 1994, Welschmeyer 1994). Meanwhile, a 1 ml sample was collected and fixed with Lugol's solution for cell counting, and an additional 1 ml sample was collected for cell size measurement.

The cell size of *Lepidodinium* sp. was estimated by the area-based diameter (ABD) which is one output of the Flow Cytometer And Microscope (FlowCAM®) (Álvarez et al. 2014). The living cells pass through a prismatic glass chamber at a flow rate of 0.15 ml min^{-1}, and 22 frames are recorded per second. All pictures are processed by the image-processing software of FlowCAM (Visual Spreadsheet). Based on the ABD algorithm of the FlowCAM, the diameter of each cell is measured by the number of greyscale pixels of the binary image of the target cell and automatically converted to a circle with the same number of pixels. The total biovolume is generated from the pixel volumes of the images, and the diameter is then calculated. It has been reported that the cell size estimated based on the ABD algorithm of FlowCAM is more accurate than that estimated from traditional microscopy (Karnan et al. 2017).

For C and N content analysis, *Lepidodinium* sp. cultures (120 ml) were filtered through 10 µm polycarbonate membrane filters to remove prey and bacteria and then resuspended in 65 ml of sterile artificial seawater. Subsamples (30 ml) of the resuspended cultures were filtered onto pre-combusted GF/F filters (Whatman, 550°C, 5 h). After filtration, samples were dried in a 60°C oven for 24 h and analyzed using a CHNS elemental analyzer (Thermo Fisher Scientific). Another 30 ml of resuspended culture were filtered on pre-combusted GF/F filters (Whatman, 550°C, 5 h) for P content. Cellular P content of *Lepidodinium* sp. was extracted and analyzed using the wet oxidation method (Pujo-Pay & Raimbault 1994). Triplicate samples were collected for each treatment. The same cultures used here were also used in the ingestion experiment described in Section 2.2.

2.5. Statistical analyses

All data are expressed as mean ± SD unless otherwise indicated. Differences in the slopes of linear regressions on growth rate vs. temperature were tested using ANCOVAs. Differences among treatments were tested using 1-way ANOVAs, 2-way ANOVAs, and Tukey's (HSD) post hoc comparisons. All analyses were considered significant at $p < 0.05$ and were conducted using GraphPad Prism (Version 8.3.0.).

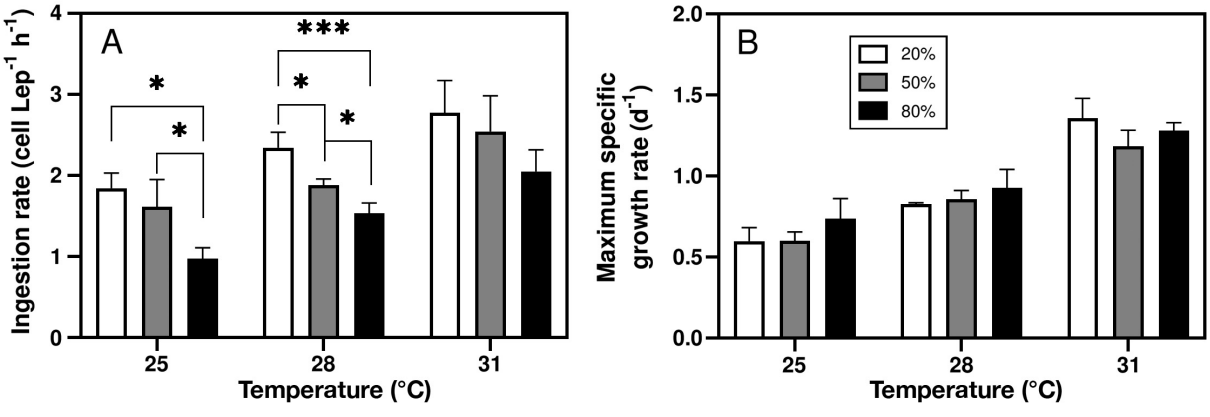

Fig. 1. (A) Ingestion rate and (B) specific growth rate of *Lepidodinium* sp. at 3 temperatures (25, 28, 31°C) and 3 nutrient concentrations (80, 50, and 20% F/2 medium). Asterisks indicate the significance of the correlations (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Error bars are SD

3. RESULTS

3.1. Effects of nutrient and temperature on *Lepidodinium* sp. ingestion rates

Lepidodinium sp. fed on *Rhodomonas salina* when both prey and inorganic nutrients were provided (Fig. 1A). At 25 and 28°C, the ingestion rate of *Lepidodinium* sp. significantly increased with decreasing inorganic nutrient concentration in the presence of the same prey levels (1-way ANOVA, $p < 0.05$ for 25°C and $p < 0.01$ for 28°C, Fig. 1A), while the ingestion rate did not vary with nutrient concentration at 31°C (1-way ANOVA, $p > 0.05$, Fig. 1A). In contrast, at the 3 temperatures (25, 28, and 31°C), the maximum specific growth rate of mixotrophic *Lepidodinium* sp. remained unchanged under different inorganic nutrient concentrations (Fig. 1B, 1-way ANOVA, $p > 0.05$). Temperature influenced both the ingestion rate and the maximum specific growth rate of mixotrophic *Lepidodinium* sp. Ingestion rate increased with increasing temperature under 3 nutrient conditions (Fig. 1A). The thermal sensitivities of ingestion rate in 20, 50, and 80% F/2 medium treatments were 0.97 ± 0.12, 0.60 ± 0.17, and 0.53 ± 0.11 eV (mean ± SE), respectively.

3.2. Thermal traits of *Lepidodinium* sp. in autotrophic and mixotrophic modes

In both autotrophic and mixotrophic modes, the growth of *Lepidodinium* sp. increased with increasing temperature, albeit at different rates (Fig. 2A). The activation energies of *Lepidodinium* sp. grown in mixotrophic mode under 3 nutrient conditions were significantly higher (0.70 ± 0.07, 0.74 ± 0.06, and 0.89 ± 0.07 eV for 80, 50, and 20% F/2 medium, respectively) than those grown at autotrophic mode (0.3 ± 0.05, 0.37 ± 0.04, and 0.3 ± 0.04 eV, respectively) (Fig. 2, ANCOVA, $p < 0.001$). Specifically, the growth rate of *Lepidodinium* sp. in the 2 nutritional modes showed no significant difference at low temperature (19°C), whereas the growth rate of mixotrophic *Lepidodinium* sp. exceeded that of autotrophic *Lepidodinium* sp. when temperature increased to 28°C. In addition, the activation energy of mixotrophic *Lepidodinium* sp. showed an increasing trend when nutrient concentration decreased from 80 to 20% F/2 medium. In contrast, the activation energy of autotrophic *Lepidodinium* sp. did not vary with nutrient concentrations (Fig. 2A).

To examine whether the *Lepidodinium* sp. grown in different nutritional modes had different optimal growth temperatures, we also kept the cultures in 50% F/2 medium (with and without prey) at 33 and 35°C and measured their growth rate. We observed that *Lepidodinium* sp. did not grow at 35°C, and the growth rate at 33°C was significantly lower than at 31°C (Fig. 3). We found that the optimal growth temperature for mixotrophic growth was 32.04°C, which was not significantly different from that of autotrophic growth (31.95°C, Fig. 3). Nevertheless, the maximum growth at optimal temperature was significantly higher for mixotrophic *Lepidodinium* sp. than for autotrophic *Lepidodinium* sp. (Fig. 3).

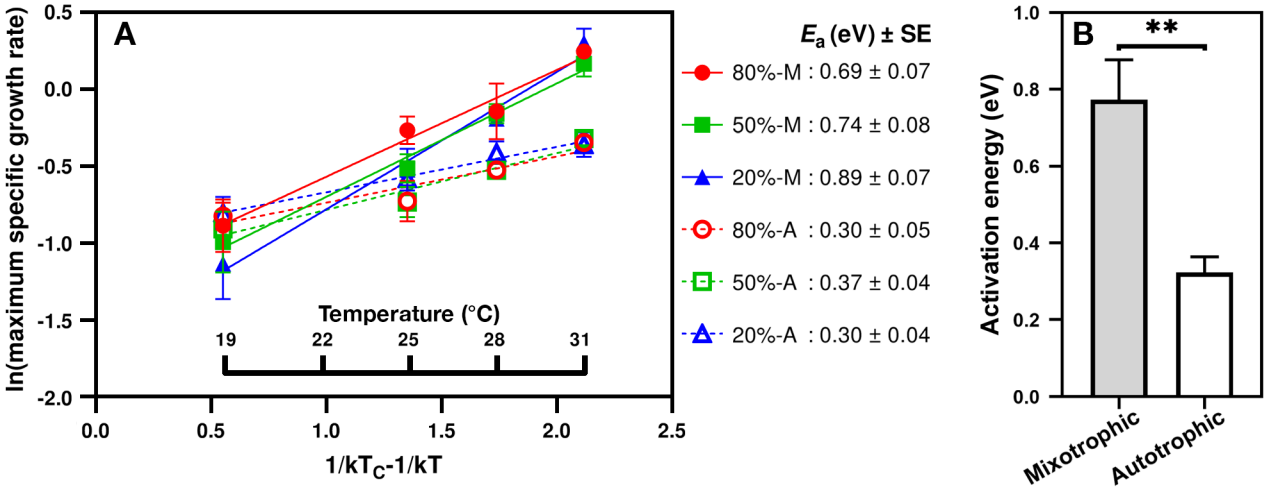

Fig. 2. (A) Relationship between the growth rate of *Lepidodinium* sp. and temperature (standardized Boltzmann temperature with a reference temperature T_c = 15°C: $1/kT_c - 1/kT$) under different nutrient treatments (80, 50 and 20% F/2 medium cultures) yielding different activation energy (E_a) values. M: mixotrophic, A: autotrophic. (B) Average activation energy of *Lepidodinium* sp. under mixotrophic and autotrophic treatments. Asterisks indicate the significance of the difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Error bars are SD

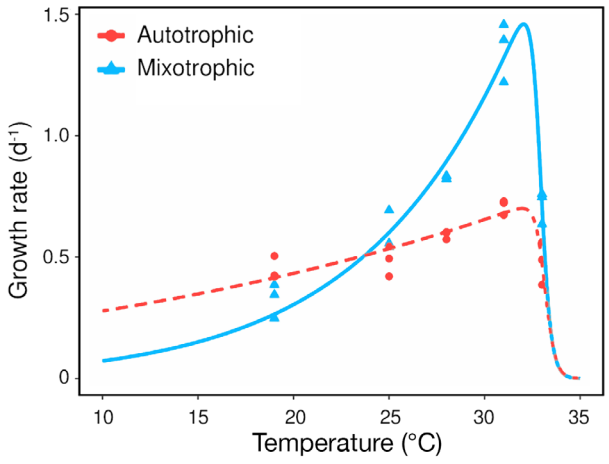

Fig. 3. Thermal performance curves for the growth rates of *Lepidodinium* sp. in autotrophic and mixotrophic modes. The cultures were maintained in 50 % F/2 medium

3.3. Functional traits and stoichiometric composition of *Lepidodinium* sp.

The effects of temperature and nutritional modes on the mean cell size of *Lepidodinium* sp. were significant (2-way ANOVA, $p < 0.001$). The cell size of both mixotrophic and autotrophic *Lepidodinium* sp. showed a decreasing trend as temperature increased (2-way ANOVA, $p < 0.001$, Fig. 4A). The mean cell size of autotrophic *Lepidodinium* sp. decreased by 11 %, from 16.09 ± 1.84 µm (95 % CI: 16.02–16.17 µm) to 14.75 ± 1.95 µm (95 % CI: 14.61–14.88 µm), and the mean cell size of mixotrophic *Lepidodinium* sp. reduced by 14 %, from 16.09 ± 1.99 µm (95 % CI: 16.93–17.05 µm) to 14.55 ± 1.79 µm (95 % CI: 14.49–14.60 µm) when temperature increased from 19 to 31°C. The mixotrophic *Lepidodinium* sp. was larger than autotrophic *Lepidodinium* sp. at 31 and 19°C (Tukey's [HSD] post hoc, $p < 0.01$).

The cellular chl *a* content of *Lepidodinium* sp. grown in autotrophic mode was not significantly different from that grown in mixotrophic mode at 19, 25, and 31°C (2-way ANOVA, $p > 0.05$, Fig. 4B), although we observed that the mixotrophic *Lepidodinium* sp. had a slightly lower chl *a* content. In both autotrophic and heterotrophic modes, the cellular chl *a* content of *Lepidodinium* sp. slightly declined when temperature increased (2-way ANOVA, $p < 0.05$, Fig. 5B). The mean cellular chl *a* content of autotrophic *Lepidodinium* sp. decreased from 22 ± 0.2 ng (19°C) to 15 ± 0.3 ng (31°C), and it decreased from 18 ± 0.3 to 12 ± 0.2 ng when grown in mixotrophic mode.

For the stoichiometric composition of *Lepidodinium* sp., the C:N ratio was slightly higher when grown in mixotrophic mode than autotrophic mode (2-way ANOVA, $p < 0.001$, Fig. 5A). When temperature increased, the C:N ratio of both mixotrophic and autotrophic *Lepidodinium* sp. slightly increased (2-way ANOVA, $p < 0.05$, Fig. 5A). The C:N ratio of autotrophic *Lepidodinium* sp. increased by 6 %, from 6.22 ± 0.095 to 6.65 ± 0.314, and in mixotrophic *Lepidodinium* sp. it increased by 12 %, from 6.45 ± 0.275 to 7.30 ± 0.275, when temperature increased from 22 to 31°C. In both mixotrophic and autotrophic *Lepidodinium* sp., C:N increased with growth rate (Fig. 6A). In particular, the C:N ratio of mixotrophic *Lepidodinium* sp. was significantly positively correlated with ingestion rates (Fig. 6C, $p < 0.05$). In comparison, the C:P and N:P ratios showed no significant difference between these 2 nutritional modes at differ-

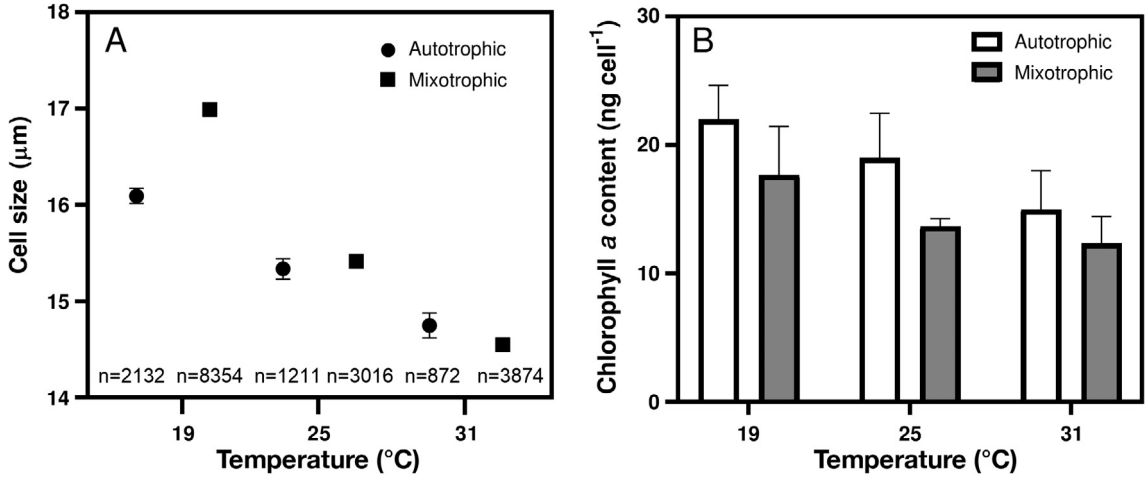

Fig. 4. (A) Average cell diameter of *Lepidodinium* sp. in 2 nutrition modes at 3 temperatures (19, 25, 31°C). (B) Cellular chl *a* concentration of autotrophic and mixotrophic *Lepidodinium* sp. at 3 temperatures (19, 25, and 31°C). Error bars are SD

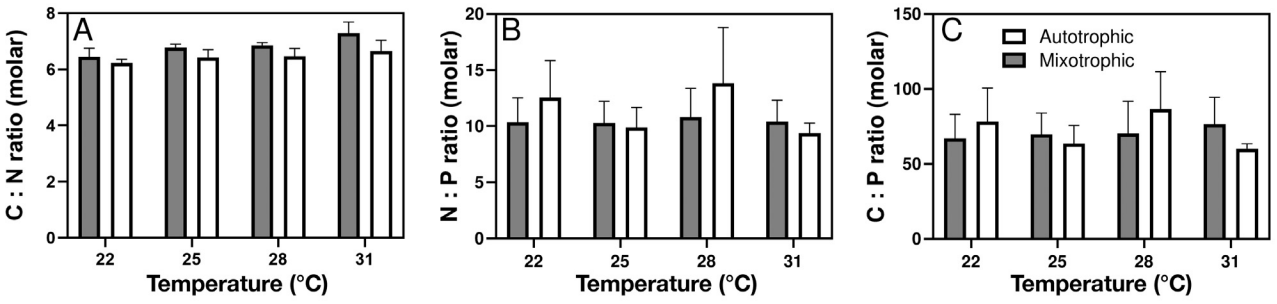

Fig. 5. Mean C:N, N:P, and C:P ratios of the mixotrophic and autotrophic *Lepidodinium* sp. at 4 temperatures (22, 25, 28, and 31°C). Error bars are SD

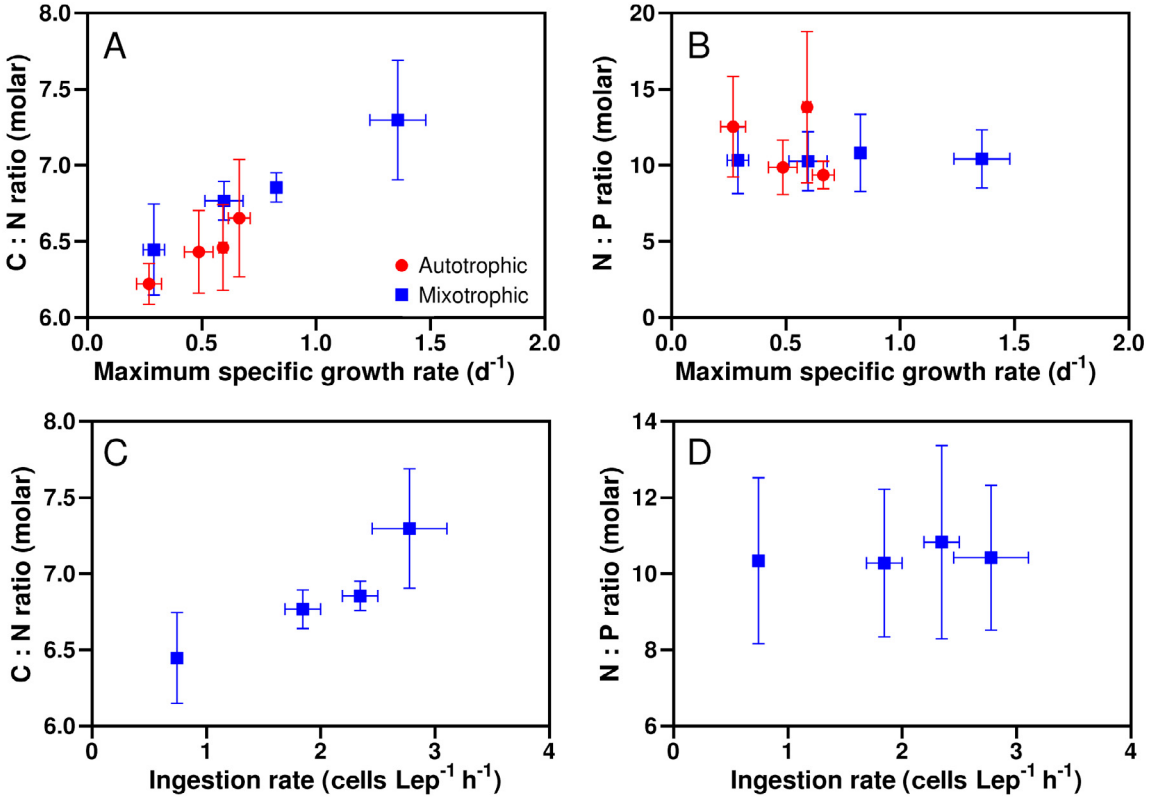

Fig. 6. Relationship between maximum specific growth rate and (A) C:N and (B) N:P ratios, and between ingestion rate and (C) C:N and (D) N:P ratios of *Lepidodinium* sp. Error bars are SD

ent temperatures (2-way ANOVA, $p > 0.05$, Fig. 5B,C). The N:P and C:P ratios of both mixotrophic and autotrophic *Lepidodinium* sp. did not vary with temperature and ingestion rate (2-way ANOVA, $p > 0.05$, Fig. 6).

4. DISCUSSION

Mixotrophs are being increasingly recognized as important components of the plankton community and planktonic ecosystems (Stoecker et al. 2017). Equipped with alterable trophic strategies, mixotrophs are deemed to have great potential to be favored in a changing ocean. However, more knowledge of how they respond to the changing conditions of oceans, especially to projected ocean warming, is critical. In this study, we explored the effects of temperature on a newly isolated mixotrophic dinoflagellate regarding the balance between autotrophic and heterotrophic nutrition as well as their functional traits, which have added to our knowledge on how mixotrophs will respond to future climate changes.

4.1. Inorganic nutrients affect the ingestion rate of *Lepidodinium* sp.

Lepidodinium spp. are constitutive mixotrophs which are also called 'phytoplankton that eat', as they constitutively synthesize and maintain their own chloroplasts (Mitra et al. 2016). They have the innate ability to photosynthesize and engage in phagotrophy; as such, their phototrophy is obligate, and mixotrophy is facultative. These constitutive mixotrophs are suggested to be abundant in aquatic systems where nutrients are low or unbalanced and where light is limited (Ghyoot et al. 2017). The phagocytotic feeding of such constitutive mixotrophs may be initiated when nutrients or light are limited. For instance, ingestion activities of *Prorocentrum minimum* are induced by low inorganic nutrient concentrations (Stoecker et al. 1997), and they do not feed much under nutrient-replete conditions. Similarly, many bloom-forming mixotrophic dinoflagellates start to feed on prey when the inorganic nutrients become limiting in the late bloom period, which is instrumental in maintaining their dominance (Mitra et al. 2016, Stoecker et al. 2017). However, it is intriguing to find that *Lepidodinium* sp. was able to feed substantially when inorganic nutrients were in excess (Fig. 1A). Unlike many mixotrophic dinoflagellates, *Lepidodinium* sp. used its facultative mixotrophic abilities to supplement its main nutrition even when nutrients were not limited. In this case, it engaged in phagocytotic feeding when prey was available. Through feeding, *Lepidodinium* sp. will uptake and use the ammonium from the degradation of prey, which facilitates growth.

More interestingly, *Lepidodinium* sp. was able to regulate its ingestion activities according to the inorganic nutrient concentrations even when nutrients were available in excess (Fig. 1A). The ability to adjust phagotrophy according to resource availability such as light and nutrients is one characteristic of most constitutive mixotrophs (Mitra et al. 2016, Stoecker et al. 2017). For instance, the ingestion rates of *Fragilidium subglobosum* and *Karlodinium veneficum* vary with inorganic nutrient concentrations (Skovgaard et al. 2000, Calbet et al. 2011). Nevertheless, the ingestion rate of *Lepidodinium* sp. was much higher when grown in 20% F/2 medium than in 80% F/2 medium at 25 and 28°C, although enrichment of nutrients from 20 to 80% F/2 medium did not further promote the growth of *Lepidodinium* sp. (Fig. 1A,B), which indicates that *Lepidodinium* sp. may increase ingestion rates to maintain growth when inorganic nutrient supply decreases. However, the mechanisms underlying such regulations of *Lepidodinium* sp. remain substantially understudied.

4.2. Warming shifts *Lepidodinium* sp. towards more heterotrophy

Our results revealed that the activation energy (E_a) of *Lepidodinium* sp. grown in autotrophic mode (0.30~0.37 eV) was significantly lower than in mixotrophic mode (0.69~0.89 eV), indicating that mixotrophic *Lepidodinium* sp. are more responsive to increasing temperature (Fig. 2). When growing in autotrophic mode, the growth of *Lepidodinium* sp. is determined solely by photosynthesis, which has lower temperature sensitivity than heterotrophic metabolic processes (Allen et al. 2005, Chen et al. 2012). The lower temperature sensitivity of photosynthesis is likely because the high temperature constrains photosynthesis rates by enhancing photorespiration and weakening carboxylation reactions (Allen et al. 2005). Similarly, the autotrophic growth of *Lepidodinium* sp. seems to be constrained at high temperatures, as we found that the autotrophic growth rate was significantly lower than the mixotrophic growth rate at high temperatures (i.e. 28 and 31°C, Fig. 2A).

At low temperatures (19 and 25°C), the growth rates of *Lepidodinium* sp. in autotrophic and mixotrophic modes were not significantly different (Fig. 2), which indicates that the carbon gained through photosynthesis could fulfil its metabolic and growth requirements. At the same time, its ingestion activities may act as a supplement for maintaining stoichiometric balance and can be adjusted according to the inorganic nutrient concentrations (Fig. 2). By contrast, the growth rate of mixotrophic *Lepidodinium* sp. was significantly higher than that autotrophic *Lepidodinium* sp. at 31°C, which suggests that the role of photosynthesis and phagocytotic feedings might have been shifted when the temperature increased. As autotrophic growth may be constrained by high temperature, ingestion activities may take the leading role in carbon and nutrient uptake, which drives the much higher growth in the mixotrophs than in the autotrophs. In such a state, the ingestion rate of *Lepidodinium* sp. no longer varies with inorganic nutrient concentration (Fig. 1B). Therefore, when temperature increases, the balance of autotrophic and heterotrophic nutrition of *Lepidodinium* sp. shifts towards more heterotrophy, leading to a higher temperature sensitivity that equals heterotrophic processes (Brown et al. 2004, López-Urrutia et al. 2006). Nevertheless, when the temperature further increased, the growth

rate of both the autotrophic and mixotrophic *Lepidodinium* sp. decreased, as their optimal growth temperatures were similar (about 32°C, Fig. 3).

Our results are largely consistent with a previous study on chrysophytes of the genus *Ochromonas* that are primarily phagotrophic (Wilken et al. 2013), illustrating that not only the primarily phagotrophic mixotrophs but also primarily phototrophic mixotrophs might become more heterotrophic at high temperature. In addition, we also found that, unlike *Ochromonas*, the mixotrophic *Lepidodinium* sp. attained much higher growth rates than autotrophic ones at high temperatures, which might make the former more competitive in the warming ocean.

A shift towards more heterotrophy with increasing temperature alters the functional role of mixotrophic protists from primary producers to consumers with a potentially profound influence on aquatic ecosystems. It could be expected that overall primary production would decline, since becoming more heterotrophic may reduce the contribution of mixotrophs to autotrophic biomass and primary production. As our results show, the chl *a* concentration of mixotrophic *Lepidodinium* sp. was lower than that of the autotrophs (Fig. 4B). Meanwhile, warming spurs constitutive mixotrophs such as *Lepidodinium* sp. to enhance their grazing activities on phytoplankton when the prey is sufficient, which would also lead to the decline of primary production (Chen et al. 2012, Liu et al. 2019). Nevertheless, mixotrophs are intraguild predators, which prey upon autotrophic and heterotrophic protists while also competing with them for resources (Polis & Holt 1992, Thingstad et al. 1996). Shifting the trophic positions of mixotrophs towards becoming predators will simultaneously weaken their role as competitors, which may favor phytoplankton by relieving them from fierce resource competition; at the same time, however, the phytoplankton may face more predation pressure from the intraguild mixotrophs, which will also affect the community dynamics (Wilken et al. 2014b, Moeller et al. 2019). Therefore, warming affects the interactions and dynamics of the plankton food web involving mixotrophs, whereas the ultimate effects on primary production resulting from the shift towards more heterotrophy of mixotrophs requires further investigation.

4.3. Effects of temperature on physiological traits and stoichiometric ratios of *Lepidodinium* sp.

In addition to the balance of autotrophic and heterotrophic nutrition, temperature also influences the physiological traits of mixotrophic protists. We found that the cell size of both autotrophic and mixotrophic *Lepidodinium* sp. decreased slightly as temperature increased (Fig. 4A). This finding is consistent with the temperature–size rule for protists, which posits a decline in plankton cell size with increasing temperature (Atkinson et al. 2003). Especially for phytoplankton, the smaller cells show superior competitiveness to larger ones by higher nutrient uptake rates due to the large surface area to volume ratio (Rasconi et al. 2015). While comparing the cell size of the 2 nutrition modes of *Lepidodinium* sp., we found that the mean cell size of mixotrophic *Lepidodinium* sp. was slightly larger than that of autotrophic ones, which is likely due to the endocytosis of *Rhodomonas salina* with a similar size (6–10 µm in length).

The stoichiometric traits of mixotrophs may also be influenced by temperature. The effects of temperature on the elemental stoichiometry of phytoplankton have been extensively studied with several hypotheses (Yvon-Durocher et al. 2015, Moreno & Martiny 2018). The 'growth-rate hypothesis' predicts that the C:P and N:P ratios should decline with increasing temperature because the high growth rate requires more P-rich ribosomes (Sterner & Elser 2002, Gillooly et al. 2005). Conversely, another hypothesis suggests that the demand for ribosomal cellular P should decrease with enhanced ribosome efficiency, which results in higher C:P and N:P ratios at warmer temperatures (Toseland et al. 2013). However, our results do not align with either hypothesis, as C:P and N:P ratios of both autotrophic and mixotrophic *Lepidodinium* sp. did not vary with temperature (Fig. 5). The slight increase in C:N ratio with rising temperature may be attributed to the increasing growth rate but is not directly linked to temperature (Fig. 6). As such, the effect of temperature on the stoichiometry of the mixotrophic dinoflagellate *Lepidodinium* sp. is very limited. However, further investigation on other mixotrophic protists is still in critical need. The slight variation in C:N:P ratios of *Lepidodinium* sp. may be due to the ample nutrient and food supply in our study. It has been reported that the effects of temperature on stoichiometry are much weaker under high rather than low nutrient conditions (De Senerpont Domis et al. 2014, Verbeek et al. 2018). Under replete nutrient conditions, the elevated metabolic rates by warming are invested in growth and biomass accumulation. Nevertheless, when nutrients are limited, although warming enhances the metabolic rates, as growth is constrained, the unlimited elements would be accumulated in the cell, leading to a shift in stoichiometry (Schulhof et al. 2019). Thus, we did not

observe a clear pattern between the C:N:P ratios and temperature under nutrient-replete conditions in our study.

Aside from the direct effect of temperature on the stoichiometric traits, the shift towards more heterotrophy along with warming could also affect the C:N:P ratios of mixotrophs. Nevertheless, whether mixotrophs regulate their cellular elemental stoichiometry when they behave more like heterotrophic protists has never been explored before. Since the trophic strategy determines the stoichiometry of plankton (Elser et al. 2000, Ho et al. 2020), the C:N and C:P ratios of mixotrophic *Lepidodinium* sp. are expected to be lower than autotrophic ones. Contrary to our expectations, their C:N ratio is a bit higher when grown in mixotrophic mode at all experimental temperatures (Fig. 5). The higher C:N ratio of mixotrophic *Lepidodinium* sp. could arise from the ingestion of *R. salina*, which has a relatively high C:N ratio (8.42 ± 0.43 during stationary phase, Johannesen 2018), because we cannot remove the prey inside the *Lepidodinium* sp. in practice. However, we found that the C:N ratio of mixotrophic *Lepidodinium* sp. significantly increases with increasing ingestion rate (Fig. 6C), which implies that the stoichiometric ratios may be affected by stoichiometric ratios of the ingested prey, as in some heterotrophic protists (e.g. *Oxyrrhis marina*) (Hantzsche & Boersma 2010, Malzahn et al. 2010). Thus, ingesting more high-C:N ratio prey may elevate the C:N ratio of mixotrophs, indicating that the stoichiometric homeostasis regulation capacity of mixotrophs is as weak as that of phytoplankton and some heterotrophic dinoflagellates (Golz et al. 2015). Due to the weak homeostasis, the cellular C:N:P ratio of mixotrophs should be easily altered by environmental inorganic nutrient conditions as well as by the nutrient ratios of ingested food. It could be speculated that the higher C:N ratio of mixotrophs may put them under less predation pressure from higher trophic levels, as they would be less favorable for zooplankton that prefer prey with a low C:N ratio (John & Davidson 2001, Wickham & Wimmer 2019).

5. CONCLUSIONS

The recently isolated dinoflagellate *Lepidodinium* sp. is a facultative mixotroph with an obligate phototrophic lifestyle. It feeds on prey substantially even under replete nutrient conditions and adjusts its feeding according to inorganic nutrient concentrations. For such constitutive mixotrophs, warming shifts their nutrition balance of autotrophy and heterotrophy towards more heterotrophy and spurs mixotrophic growth much more than autotrophic growth. Thus, mixotrophic dinoflagellates may benefit substantially from mixotrophy and become more competitive under sufficient prey conditions than their autotrophic counterparts in the warming ocean. Moreover, our results revealed that the cell size of *Lepidodinium* sp. decreased with rising temperature, and the stoichiometric ratio (C:N) varied between mixotrophic and autotrophic modes. As mixotrophs are ubiquitous in aquatic ecosystems and play a critical role in the plankton food web, becoming more heterotrophic and shifting their functional roles from primary producers to consumers in warming oceans will profoundly influence species interactions, food web dynamics, and carbon and nutrient cycling of marine ecosystems.

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