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Original Article

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Mixotrophs and Mixoplankton: Conceptual Integration into Aquatic Research

Effects of mixotroph evolution on trophic transfer

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ABSTRACT

Plankton form the foundation of marine food webs, playing fundamental roles in mediating trophic transfer and the movement of organic matter. Increasing ocean temperatures have been documented to drive evolution of plankton, resulting in changes to metabolic traits that can affect trophic transfer. Despite this, there are few direct tests of the effects of such evolution on predator–prey interactions. Here, we used two thermally adapted strains of the marine mixotroph (organism that combines both heterotrophy and autotrophy to obtain energy) *Ochromonas* as prey and the generalist dinoflagellate predator *Oxyrrhis marina* to quantify how evolved traits of mixotrophs to hot and cold temperatures affects trophic transfer. Evolution to hot temperatures reduced the overall ingestion rates of both mixotroph strains, consequently weakening predator–prey interactions. We found variability in prey palatability and predator performance with prey thermal adaptation and between strains. Further, we quantified how ambient temperature affects predator grazing on mixotrophs thermally adapted to the same conditions. Increasing ambient temperatures led to increased ingestion rates but declines in clearance rates. Our results for individual, pairwise trophic interactions show how climate change can alter the dynamics of planktonic food webs with implications for carbon cycling in upper ocean ecosystems.

KEYWORDS: mixoplankton; trophic transfer; thermal adaptation; grazing; prey palatability; predator–prey interactions; mixotrophy

INTRODUCTION

Plankton form the foundation of marine food webs, playing fundamental roles in mediating trophic transfer and the movement of organic matter (Fenchel, 1988; Legendre and Rassoulzadegan, 1995; Frederiksen *et al.*, 2006). Trophic transfer efficiency refers to the amount of carbon (C) in an organism that is incorporated into the biomass of higher trophic levels (Dickman *et al.*, 2008; Armengol *et al.*, 2019). High efficiencies, which may occur when prey can be captured efficiently or are of high nutritional quality and are stoichiometrically similar to their predators, may result in increased biomass transferred to higher trophic levels and greater C sequestration via the biological C pump (Dickman *et al.*, 2008; Ward and Follows, 2016; Barneche *et al.*, 2021). However, rising ocean temperatures can drive the rapid evolution of marine microbes with relatively unexplored implications for predator–prey interactions and trophic transfer (Guan *et al.*, 2017; Hutchins and Fu, 2017).

Across marine microbes, organisms experimentally evolved under increasingly higher temperatures show changes in metabolic traits that can alter trophic transfer. For example, phytoplankton experimentally evolved under increasingly higher temperatures are able to recover their growth rates and show increased C use efficiency mediated by down-regulating respiration as compared to photosynthesis (Padfield *et al.*, 2016; Schaum *et al.*, 2018; Barton *et al.*, 2020). In thermally adapted phytoplankton, evolved tolerance to high temperatures also results in changes to fatty acid profiles and cellular nitrogen (N) to phosphorus (P) ratios and C to P ratios (Schaum *et al.*, 2018; O'Donnell *et al.*, 2019; Jin *et al.*, 2020). The stoichiometry (primarily C:N:P ratios) within organisms is thought to play significant roles in controlling trophic relationships (Mitra and Flynn, 2005; Moe *et al.*, 2005; Allen and Gillooly, 2009; Sterner and Elser, 2017). Dietary value of prey varies with C:N:P ratios (John and Davidson, 2001) and fatty acid profiles (O'Donnell *et al.*, 2019; Jin *et al.*, 2020), and minor changes in prey stoichiometry can be associated with significant changes in prey quality that affect trophic interactions and subsequent ecosystem function (Mitra and Flynn, 2005, 2016). Further, rising temperatures are also widely associated with decreases in cell size in marine microbes (Garzke *et al.*, 2016; Deutsch *et al.*, 2022). Changes in cell size affect predator–prey interactions as large differences in predator–prey body sizes can result in reductions in transfer efficiency (Havens, 1998; Barnes *et al.*, 2011; Nakazawa *et al.*, 2011; García-Comas *et al.*, 2016; Mehner *et al.*, 2018; Atkinson *et al.*, 2021). Prey that are much smaller than predators may result in higher energetic costs of capture and consumption unless predators have adaptations for harvesting prey efficiently (Brose *et al.*, 2006; Branco *et al.*, 2020).

Despite evidence of changes to microbial traits driven by climate change, there are few direct tests of the effects of such evolution on trophic transfer. Here, we examine the effects of thermal adaptation on trophic transfer using microbial mixotrophs as evolved prey and heterotrophic dinoflagellates as predators. Mixotrophs are organisms that combine both heterotrophy and

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autotrophy to obtain energy and nutrients (Flynn *et al.*, 2012; Stoecker *et al.*, 2017; Leles *et al.*, 2021; Millette *et al.*, 2023). We focus on constitutive mixotrophs, which maintain their own, vertically transmitted chloroplasts while retaining the capacity for phagocytosis (Flynn *et al.*, 2012; Stoecker *et al.*, 2017; Faure *et al.*, 2019; Leles *et al.*, 2021). By using photosynthesis to compensate for respiratory losses, marine mixotrophs are speculated to increase trophic transfer efficiency which supports greater biomass among organisms on higher trophic levels (Ward and Follows, 2016). Thus, while mixotrophs play a role as consumers of bacteria, they are also instrumental as prey to heterotrophic grazers (Weithoff and Wacker, 2007; Hiltunen *et al.*, 2012; Glibert and Mitra, 2022).

One way to quantify the strength of trophic interactions in a system is through predator functional responses (Holling, 1959b, 1959a; Brose, 2010). The functional response of a predator describes the relationship between the number of prey it consumes per unit time and the abundance of prey (Holling, 1959b, 1959a; Brose, 2010). Predator functional responses can take a variety of forms, but the most common is a type II functional response (Jeschke *et al.*, 2002, 2004). Type II functional responses are determined by clearance rate (a.k.a. attack rate; effort of predator searching, detecting, encountering and successfully attacking prey) and handling time (the time a predator needs to fight, subdue, ingest and digest prey; Brose, 2010; Rall *et al.*, 2012). Similar to other biological rates, predator grazing rates of prey are affected by body size and temperature (Abrahams *et al.*, 2007; Brose, 2010; Petchey *et al.*, 2010; Rall *et al.*, 2012). Thus, as climate change results in increased temperatures and organismal trait adaptation to novel conditions, functional responses could shift via alterations in either clearance rates or handling times (Abrahams *et al.*, 2007; Petchey *et al.*, 2010; Rall *et al.*, 2012).

In this study, we quantified how mixotroph thermal adaptation affects trophic transfer by feeding thermally adapted mixotrophs to the common, generalist predator *Oxyrrhis marina*. Specifically, we quantified the functional responses of predators and measured predator performance. We used two strains of the thermally adapted mixotrophic nanoflagellate *Ochromonas* that showed evidence of increased grazing rates on bacteria, decreased photosynthesis and decreased cell size with increasing temperature (Lepori-Bui *et al.*, 2022). Our goals of this study were to understand (i) how evolved traits of mixotrophs alter their palatability to predators and (ii) how ambient temperature affects the grazing of predators on mixotrophs. We found that evolutionary adaptation to warmer temperatures generally weakened trophic interactions and, as a consequence, impacts on predator growth rates, implying cascading effects on the C cycle.

METHOD

Experimental cultures and growth conditions

To test the effects of thermal adaptation on grazing, we used experimentally evolved lineages of the marine mixotrophic nanoflagellate *Ochromonas* (Lepori-Bui *et al.*, 2022) as prey. We used thermally adapted lineages from two *Ochromonas* strains representing different degrees of mixotrophy: Strain CCMP 1391 is obligately phototrophic and requires sunlight to grow, although growth rates increase with phagotrophy on bacterial prey (Moeller *et al.*, 2019; Barbaglia *et al.*, 2024). Strain CCMP 2951 is facultatively phototrophic, requiring bacteria but not light for growth (Wilken *et al.*, 2020; Barbaglia *et al.*, 2024).

The *Ochromonas* cultures used in this study have experienced long-term selection for adaptation to temperature (cold = 18°C, ancestral = 24°C and hot = 30°C) and light (low = 50 μmol quanta m^{-2} s^{-1}; high = 100 μmol quanta m^{-2} s^{-1}). Full information about the creation of these evolutionary lineages can be found in Lepori-Bui *et al.* (2022). All six replicate lineages from each evolutionary condition and strain (= 2 *Ochromonas* strains × 6 replicate lineages × 3 temperatures × 2 light levels, for a total of 72 evolved lineages) were used in this experiment. Cultures were maintained in K medium (Keller *et al.*, 1987) made by adding pre-mixed nutrients (National Center for Marine Algae and Microbiota, NCMA, Bigelow Laboratory, Easy Boothbay, ME) to 0.2 micron filtered, autoclaved coastal seawater from the Santa Barbara channel. These cultures are xenic, so co-occurring bacteria provide *Ochromonas* with a food supply and no additional food supplementation was given.

This study was conducted two years after the data reported in Lepori-Bui *et al.* (2022), as maintenance of the *Ochromonas* cultures in exponential growth at the evolutionary temperatures and light levels has continued during this time. The evolution of these cultures appeared to have plateaued after approximately 300 generations (Lepori-Bui *et al.*, 2022), so we suspect that traits of *Ochromonas* have not significantly changed. Analyses of growth rates (reported in this study) and chlorophyll content (H. Moeller, Santa Barbara, personal communication) suggest that phenotypes continue to be consistent. We also report data on cellular C, N and C to N ratios collected from these experimental cultures at the time of Lepori-Bui *et al.*, (2022; Fig. 5).

For the predator in this experiment, we used the cosmopolitan heterotrophic dinoflagellate *O. marina* (NCMA CCMP 3375). *O. marina* is a widely distributed heterotrophic dinoflagellate known to graze on a variety of prey, including strains of *Ochromonas* (Roberts *et al.*, 2011). While *O. marina* can vary in size, CCMP 3375 grown in similar conditions to our cultures have been measured to be from 18 to 21 μm in equivalent spherical diameter (Franzè and Menden-Deuer, 2020). *O. marina* cultures were maintained at a light level of 20 μmol quanta m^{-2} s^{-1} at a temperature of 18°C in 0.2 micron filtered, autoclaved coastal seawater from the Santa Barbara Channel. *O. marina* were routinely fed the phytoplankton *Isochrysis galbana* strain CCMP 1323 and transferred each week to 50 mL culture flasks with fresh filtered seawater and *I. galbana*. *I. galbana* cultures were maintained at 18°C at 20 μmol quanta m^{-2} s^{-1} in f/2-Si medium (Guillard, 1975) made by adding pre-mixed nutrients (NCMA) to 0.2 micron filtered, autoclaved coastal sweater from the Santa Barbara Channel. For grazing experiments assayed at the higher 24°C temperature (see "Grazing assays" below), the *O. marina* cultures were acclimated at a light level of 50 μmol quanta m^{-2} s^{-1} and a temperature of 24°C for at least 3 weeks prior to beginning experiments. For these grazing assays, we also acclimated the *I. galbana* culture used to feed the *O. marina* to 50 μmol quanta m^{-2} s^{-1} and 24°C. *Prior* to initialization of grazing experiments, we confirmed elimination of extra *I. galbana* prey using light microscopy.

Grazing assays

To assess how predators responded to increasing prey concentrations, we fed evolved *Ochromonas* to *O. marina* in target concentrations of 0, 25 000, 50 000, 75 000, 100 000 and 125 000 *Ochromonas* cells per mL. We used 1000 *O. marina* cells per mL as a predator target population. We conducted all grazing assays in 12-well tissue culture plates (VWR International, Radnor, PA, USA; Part No. 10062-89). The target concentrations of *Ochromonas* in K media (to a total volume of 3.5 mL) were added to each well and incubated for 24 hours to allow *Ochromonas* cultures to overcome the initial mortality of being transferred to fresh media (Moeller *et al.*, 2019; Lepori-Bui *et al.*, 2022) prior to the addition of the predators. To accurately assess how evolved traits of *Ochromonas* altered predation, we avoided acclimating the *Ochromonas* cultures to the assay temperature to preserve their evolved phenotypes. 24 hours later, 1.5 mL of *O. marina*-containing filtered seawater (or sterile filtered seawater, in control wells) was added to bring the total volume to 5 mL. *Ochromonas* and *O. marina* populations were then counted daily for 4 days. *Ochromonas* populations were counted using a Guava easyCyte flow cytometer (Luminex Corporation). *O. marina* populations were fixed using Lugol's solution (final concentration 1%) and at least 100 cells per sample were counted on a compound light microscope at 100× magnification using a Sedgwick Rafter chamber.

We conducted grazing assays for all evolutionary lineages (= 72 grazing assays) at an assay light level of 50 μmol quanta m^{-2} s^{-1} and an assay temperature of 18°C. All grazing assays were maintained under a 12:12 h light:dark cycle with illumination from above. We used 18°C as a common assay temperature and 50 μmol quanta m^{-2} s^{-1} as a common assay light level to control for assay conditions in order to distinguish changes in grazing based on evolutionary traits and because our stock population of *O. marina* is maintained at this temperature and close to this light level. To test the effects of temperature on grazing, we also conducted a secondary set of grazing assays at an assay light level of 50 μmol quanta m^{-2} s^{-1} and an assay temperature of 24°C. Here, we only conducted experiments with *Ochromonas* strains evolved at the control evolutionary temperature (24°C; 2 strains × 2 light levels × 6 replicates = 24 grazing assays) in order to control for evolutionary effects and isolate the effects of the grazing temperature assay. For these experiments, we temporarily acclimated *O. marina* to the assay temperature (see "Experimental cultures and growth conditions" above).

Cellular C and N content

Cellular C and N content (and resulting C:N ratios) for all *Ochromonas* lineages were collected by Lepori-Bui *et al.* (2022). Briefly, known numbers of *Ochromonas* and bacterial prey cells were collected on pre-combusted GF/F filters, acidified to remove inorganic carbonates and analyzed on an elemental analyzer (Model CEC 440HA; Exeter Analytical). Lepori-Bui *et al.* (2022) accounted for contributions of bacteria to *Ochromonas* biomass by separately measuring bacterial C and N content, and subtracting these contributions from total C and N.

Statistical analyses

We quantified grazing and ingestion rates following the methods of Jeong and Latz (1994). Briefly, the method contrasts prey growth rates in the presence and absence of predators to estimate the prey consumed by predators. To make this (and other) calculations, we used the software package R (version 4.3.0). We estimated prey and predator growth rates by fitting a linear model to the log of population size (R function *lm*). For prey, we calculated growth rates over the first 48 hours of data to minimize plate effects, while predator growth rates were calculated over the entire duration of the experiment (72 hours) to compensate for noisier predator population data and better capture long-term effects on predator performance. We calculated grazing rates and ingestion rates using methods of Jeong and Latz (1994) for each *Ochromonas* target concentration. We fit ingestion rate data with both Holling Type I (linear):

$$\text{Ingestion} = a * [\text{Prey Concentration}]$$

and Holling Type II (saturating):

$$\text{Ingestion} = \frac{a * [\text{Prey Concentration}]}{1 + a * h * [\text{Prey Concentration}]}$$

functional response curves, where a is the clearance rate (a.k.a. attack rate, in units of microliters pre predator per day) and h is the handling time (in units of predator hours per prey; handling times for linear Type I functional responses are per definition 0). We used Akaike information criterion values to determine which functional response curves fit best and used the associated estimates of clearance rates and handling times in downstream analyses. To assess predator performance on prey at each assay temperature, we calculated normalized predator growth rates and predator growth sensitivity. We normalized predator growth rates to those of the unfed (starved control) predators in each grazing experiment. This normalization allowed us to control for small differences in feeding history or temperature that would result in different baseline predator growth rates and extract the relative impact of the presence of (increasing amounts of) *Ochromonas* prey. We calculated predator growth sensitivity by subtracting the control predator growth rate for each evolutionary lineage of prey from the average predator growth rates to indicate whether predators were performing better or worse in the presence of prey.

We tested for differences between clearance rates, handling times, predator growth sensitivity and cellular C, N and C:N ratios among *Ochromonas* lineages experimentally evolved at different temperatures (analysis of variance (ANOVA), corrected with Tukey's honestly significant difference (Tukey HSD) tests; R functions *aov* and *TukeyHSD*).

For ingestion rates (Figs 1 and 4; Figs S1 and S3) and predator normalized growth rates (Fig. 2; Fig. S2), we used linear models (R function *lm*) to fit the displayed data. We fit linear models to individual biological replicates ($n = 6$ per evolutionary lineage) as well as across all unpooled replicates for each evolutionary temperature at each light level ($n = 1$ per evolutionary lineage). To assess the strength of predator–prey interactions, we visualized ingestion rates with predator growth rates (Fig. 3). For this data, we fit linear models to only individual biological replicates. Additionally, we calculated ingestion rates, handling times, predator normalized growth rate and predator sensitivity in units of prey C (Fig. S1–S3).

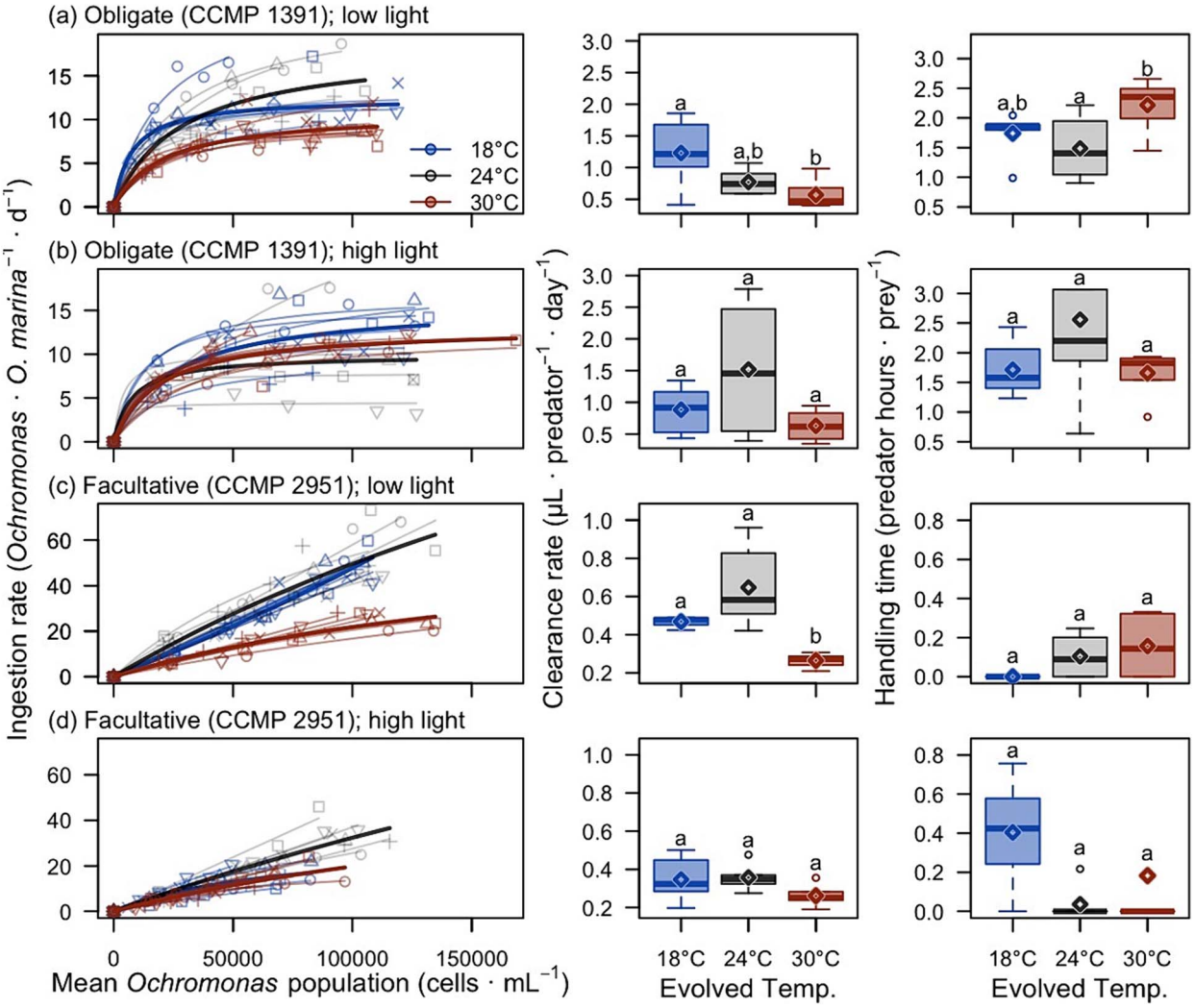

Fig. 1. Ingestion rates (left column), clearance rates (middle column) and handling times (right column) of *O. marina* when fed cold-evolved (18°C), hot-evolved (30°C) and control (24°C) strains of *Ochromonas* to simulate climate change. Ingestion rates (left column) are plotted across mean *Ochromonas* populations. Symbols and thin lines indicate biological replicates ($n = 6$) fit with Holling Type I or Type II functional response curves (thin lines). The thick lines (one per evolutionary temperature) indicate the functional response curve fit across unpooled replicates. On the box plots, the horizontal line indicates the median, and the symbol indicates the mean. The different letters indicate statistically significant differences between clearance rates or handling times from Tukey HSD tests between evolved temperatures of *Ochromonas* within one strain and light level. (**a**) Results for the obligate phototroph (CCMP 1391) evolved at low light. (**b**) Results for the obligate phototroph evolved at high light. (**c**) Results for the facultative phototroph (strain CCMP 2951) evolved at low light. (**d**) Results for the facultative phototroph evolved at high light.

RESULTS

Variation in prey palatability with evolutionary temperature

Ochromonas palatability was affected by evolutionary temperature (Fig. 1). Between strains, ingestion rates were higher on average for predators feeding on the facultative phototroph (*Ochromonas* strain CCMP 2951) than feeding on the obligate phototroph (*Ochromonas* strain CCMP; Fig. 1, left column). In general, ingestion rates were higher for cold-evolved than hot-evolved *Ochromonas* lineages, with the exception of the facultative phototroph at high light where ingestion rates were similar. Further, contrasts between thermally evolved *Ochromonas* and control evolution lines (evolved at the ancestral temperature of 24°C) were also variable: in most cases, control and cold-evolved lineages were eaten at similar rates, suggesting that evolution to hot temperatures had the greatest impact on *Ochromonas* palatability.

In most cases, Holling Type II (saturating) functional responses were the best fit to the experimental data. Clearance rates tended to decrease with increasing evolutionary temperature (though control lineages were more variable), and this trend was significant at the low evolutionary light level (Fig. 1, middle column). Handling times tended to increase with evolutionary temperature, although these findings were generally not statistically significant (i.e. no significant differences between evolutionary temperatures within a strain; Fig. 1, right column).

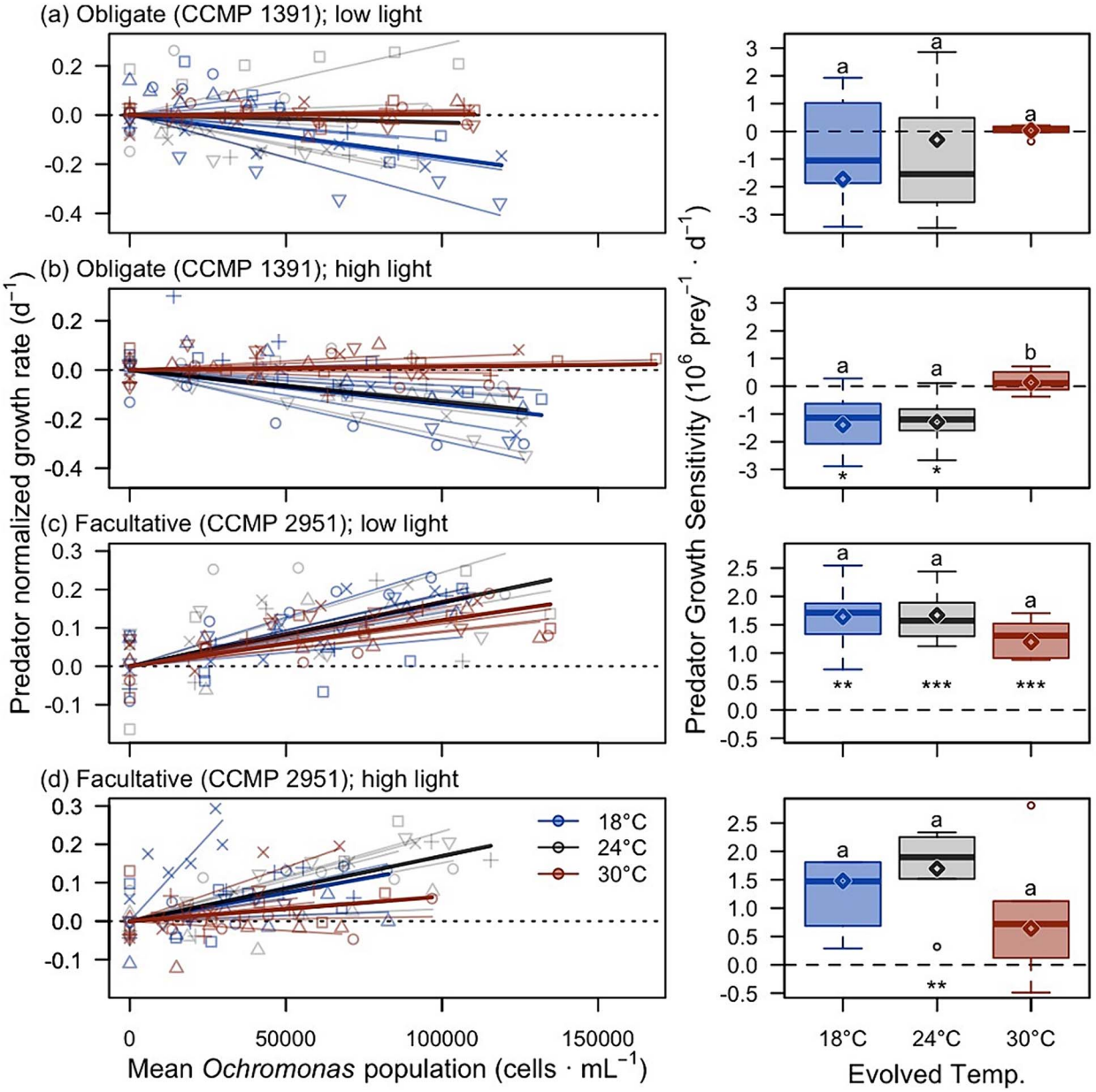

Fig. 2. Predator (*O. marina*) normalized growth rate (as compared to the starved predators; left column) and predator growth sensitivity (right column) when fed thermally adapted *Ochromonas* evolved at cold (18°C), ancestral (24°C) and hot (30°C) temperatures. Predator normalized growth rate is plotted across mean prey populations. Symbols and thin lines indicate biological replicates ($n = 6$) fit with a linear model by evolutionary temperatures. The thick lines (one per evolutionary temperature) indicate the linear model fit across unpooled replicates. Negative predator growth sensitivities indicate that predators are performing worse in the presence of prey. On the box plots, the line indicates the median and the symbol indicates the mean. The different letters indicate statistically significant differences between predator sensitivities from Tukey HSD tests between evolved temperatures of Ochromonas within one strain and light level. (**a**) Results for the obligate phototroph (CCMP 1391) evolved at low light. (**b**) Results for the obligate phototroph evolved at high light. (**c**) Results for the facultative phototroph (CCMP 2951) evolved at low light. (**d**) Results for the facultative phototroph evolved at high light. Asterisks indicate significant differences from zero (*t*-test; $* = P$-value < 0.05; $** = P$-value < 0.01; $*** = P$-value < 0.001).

For the facultative phototroph at the high light level, lower experimental *Ochromonas* densities inhibited our ability to quantify the saturation of ingestion rates (Fig. 1d, left column).

Overall, the facultative phototroph was more palatable than the obligate phototroph, with higher maximum ingestion rates and lower handling times for the facultative phototroph. However, clearance rates were lower, and thus the acceleration of predation with increased *Ochromonas* availability was less pronounced in the facultative phototroph.

Predator performance on thermally adapted prey

The effects of thermal evolution on predator growth varied by *Ochromonas* strain (Fig. 2). Overall, predators only exhibited positive growth when grazing on the facultative phototroph

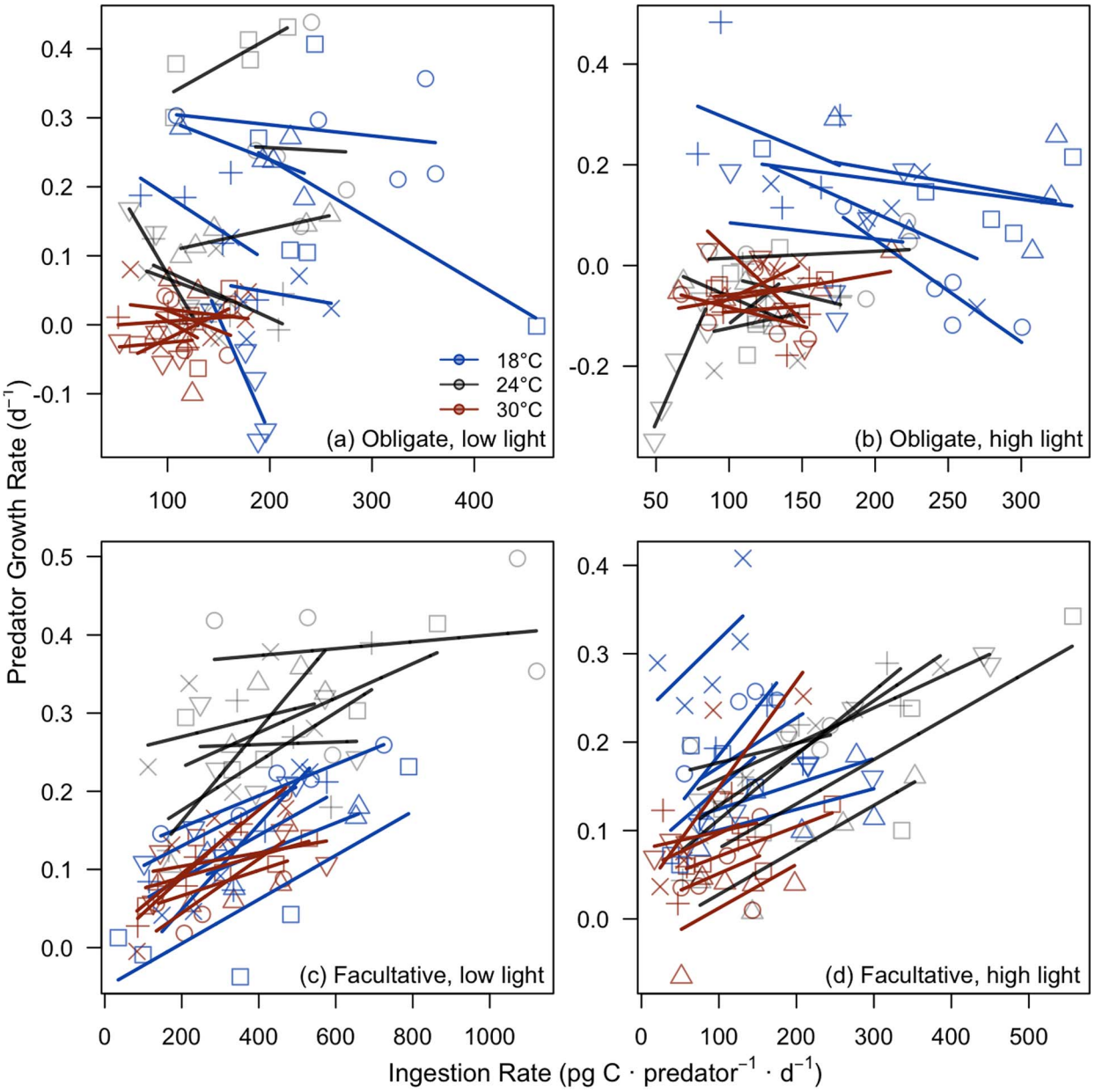

Fig. 3. Predator (*O. marina*) ingestion rates and growth rates when fed thermally adapted strains of *Ochromonas* evolved at cold (18°C), control (24°C), or hot (30°C) temperatures to show the strength of predator–prey interactions. Lines and symbols indicate biological replicates for each evolutionary temperature ($n = 6$) fit with a linear model by evolutionary temperature, indicated by lines. (**a**) Results for the obligate phototroph (CCMP 1391) evolved at low light. (**b**) Results for the obligate phototroph evolved at high light. (**c**) Results for the facultative phototroph (CCMP 2951) evolved at low light. (**d**) Results for the facultative phototroph evolved at high light.

(Fig. 2c and d), but evolution at hot temperatures reduced these growth benefits at the high light level (Fig. 2d). In contrast, predators fed the obligate phototroph exhibited negative growth rates, but these mortality effects were reduced when the *Ochromonas* strains were evolved at hot temperatures (Fig. 2a and b).

Effects of prey ingestion amount on predator growth

We observed that, in some cases (e.g. facultative phototroph at high light), predator growth benefits were linked to prey ingestion (Fig. 3). Indeed, when we compared predator growth rate to prey ingestion rate (converted to units of C using previous measurements of *Ochromonas* C content, Lepori-Bui *et al.*, 2022), we found that for the facultative phototroph, increases in the number of ingested prey were generally linked to increases in *O. marina* growth rate (Fig. 3c and d). However, these relationships were more muddled for the obligate phototroph (Fig. 3a and b). For the cold-evolved obligate phototroph, ingestion rates (in terms of C per day) extended to higher values and were linked with decreasing growth rates, suggesting that increased ingestion of this *Ochromonas* strain led to mortality in *O. marina* (Fig. 3a and b). For lineages evolved at

hot temperatures, ingestion rates were lower and varied over a smaller range, so there was no clear sign (increase or decrease) of impact on *O. marina* growth rates.

Functional response curves across assay temperatures

Control lineages of *Ochromonas* were fed to predators at the cold (18°C) and ancestral (24°C) temperatures to see how grazing rates varied with the temperature at which they occur (Fig. 4). Across both strains, grazing rates were sensitive to temperature and generally higher with the higher temperature (Fig. 4, left column), with the exception of the facultative phototroph at low light. Results for clearance rate (Fig. 4, middle column) were generally lower when assayed at the ancestral temperature (significantly lower for the facultative phototroph at low light), with the exception of the facultative phototroph at high light. Handling time was also generally lower at the ancestral temperature (Fig. 4, right column) and this pattern was significant for the obligate phototroph at high and low light.

DISCUSSION

Climate change can drive evolution of marine organisms as they adapt in response to rapidly changing abiotic conditions (Thomas *et al.*, 2016; Raven and Beardall, 2021). Such evolutionary responses can alter organismal traits in ways that affect predator–prey interactions and subsequent trophic transfer (DeWitt and Langerhans, 2003; Johnson and Agrawal, 2003; Friman *et al.*, 2014). In this study, we used two thermally adapted strains of the constitutive marine mixotroph *Ochromonas* and the common, generalist predator *O. marina* to quantify how evolved traits of mixotrophs to hot and cold temperatures affects trophic transfer. Evolution to hot temperatures tended to reduce the overall ingestion rates of both *Ochromonas* strains (Fig. 2), resulting in weakening of predator–prey interactions (Fig. 3). We also found variability in prey palatability and predator performance with prey thermal adaptation and between *Ochromonas* strains (Figs 1 and 2). Further, we quantified how ambient temperature affects the grazing of predators on mixotrophs thermally adapted to the same conditions as shifts in ambient temperature can affect predator–prey interactions (Kathol *et al.*, 2009; Vázquez-Domínguez *et al.*, 2012). We found that increasing ambient temperatures led to increased ingestion rates but declines in clearance rates (Fig. 4).

One mechanism potentially underlying lower ingestion rates of mixotrophs evolved at hot temperatures is changes in mixotroph cell size. In the thermally adapted mixotroph prey, cellular C content (a metric for cell size) decreased by 19% in hot evolved lineages (Fig. 5, top row; Lepori-Bui *et al.*, 2022), with the exception of the facultative phototroph at low light. Consistent with a decline in cell size, cellular N content also decreased with increasing evolutionary temperature (Fig. 5, middle row; Lepori-Bui *et al.*, 2022). Although other studies have found that changes in prey stoichiometry can influence predator behavior and ingestion rates (John and Davidson, 2001; Mitra and Flynn, 2005, 2016), our thermally adapted *Ochromonas* lineages had similar C:N ratios across evolutionary temperatures (Fig. 5, bottom row; Lepori-Bui *et al.*, 2022), so this likely does not explain the differences we saw in predator responses. However, changes to prey body size, which impacts the total amount of C or N ingested per prey, may have affected ingestion. It is more likely that alterations to cell size explain our results as the predator *O. marina* exhibits selective feeding, typically preferring larger prey (Roberts *et al.*, 2011). Further, predator–prey theory also suggests that when prey are not optimally sized, ingestion rate of the predator slows (Wirtz, 2013). The lower ingestion rates coupled with higher handling times for hot adapted lineages (Fig. 1 left column, right column) could have resulted from their smaller cell size as predators have not evolved behavioral or physiological adaptations to maximize search efficiency or handling time for smaller prey.

Overall, our results indicated variability in palatability and predator growth across *Ochromonas* strains. Because the *Ochromonas* genus is polyphyletic and highly diverse (Lie *et al.*, 2018; Wilken *et al.*, 2020; Barbaglia *et al.*, 2024), it is unsurprising that predator responses would also vary. Between our two focal strains, the facultative phototroph appeared to be more palatable to and supported positive growth of the predator *O. marina*. In contrast, *O. marina* fed the obligate phototroph ingested less (in both mixotroph cells and C per day) and exhibited negative growth rates. Indeed, the more of the obligate phototroph that *O. marina* cells consumed, the more negative their growth rates. Our findings are indicative that the obligate phototroph may be toxic to *O. marina* (Weithoff and Wacker, 2007; Hiltunen *et al.*, 2012; Chapman *et al.*, 2019). Strains of *Ochromonas* and other species of mixotrophs have been documented to be similarly toxic to consumers (Hambright *et al.*, 2014; Flynn *et al.*, 2018) with severity depending on metabolic strategy and predator species (Fu *et al.*, 2012; Hiltunen *et al.*, 2012). Interestingly, the obligate phototroph evolved at hot temperatures was consumed less, preserving *O. marina* biomass. This suggests that a weakening of ecological interactions may, in some cases, preserve predator performance by reducing toxic effects.

Aside from differences across *Ochromonas* strains, predator responses also varied with thermal adaptation within respective *Ochromonas* strains. Within the obligate phototroph, predator growth increased with hot-evolved lineages as compared to cold-evolved and control lineages while the opposite was true for the facultative phototroph. This variability in grazer responses is perhaps expected given that mixotrophs become more heterotrophic with warmer temperatures (Wilken *et al.*, 2013; Lepori-Bui *et al.*, 2022) and the mode of nutrition of mixotrophs determines food quality for their consumers through compositions of consumer-relevant compounds such as fatty acids (Weithoff and Wacker, 2007; Hiltunen *et al.*, 2012). For example, when cultured in autotrophic, heterotrophic (via the consumption of dissolved organic matter) and mixotrophic conditions, the chlorophyte *Chlamydomonas* exhibited decreasing polyunsaturated fatty acid (PUFA) concentrations from autotrophs, mixotrophs, to heterotrophs (Heifetz *et al.*, 2000; Poerschmann *et al.*, 2004) and higher protein contents under a mixotrophic metabolic strategy (Lalibertè and de la Noüie, 1993). Within one strain of the genus *Ochromonas* specifically, autotrophs had the highest PUFA concentrations while mixotrophs and heterotrophs had higher concentrations of saturated fatty acids (SFAs; Boëchat *et al.*, 2007). PUFA content is widely linked to nutritional value and limitation of PUFAs can decrease

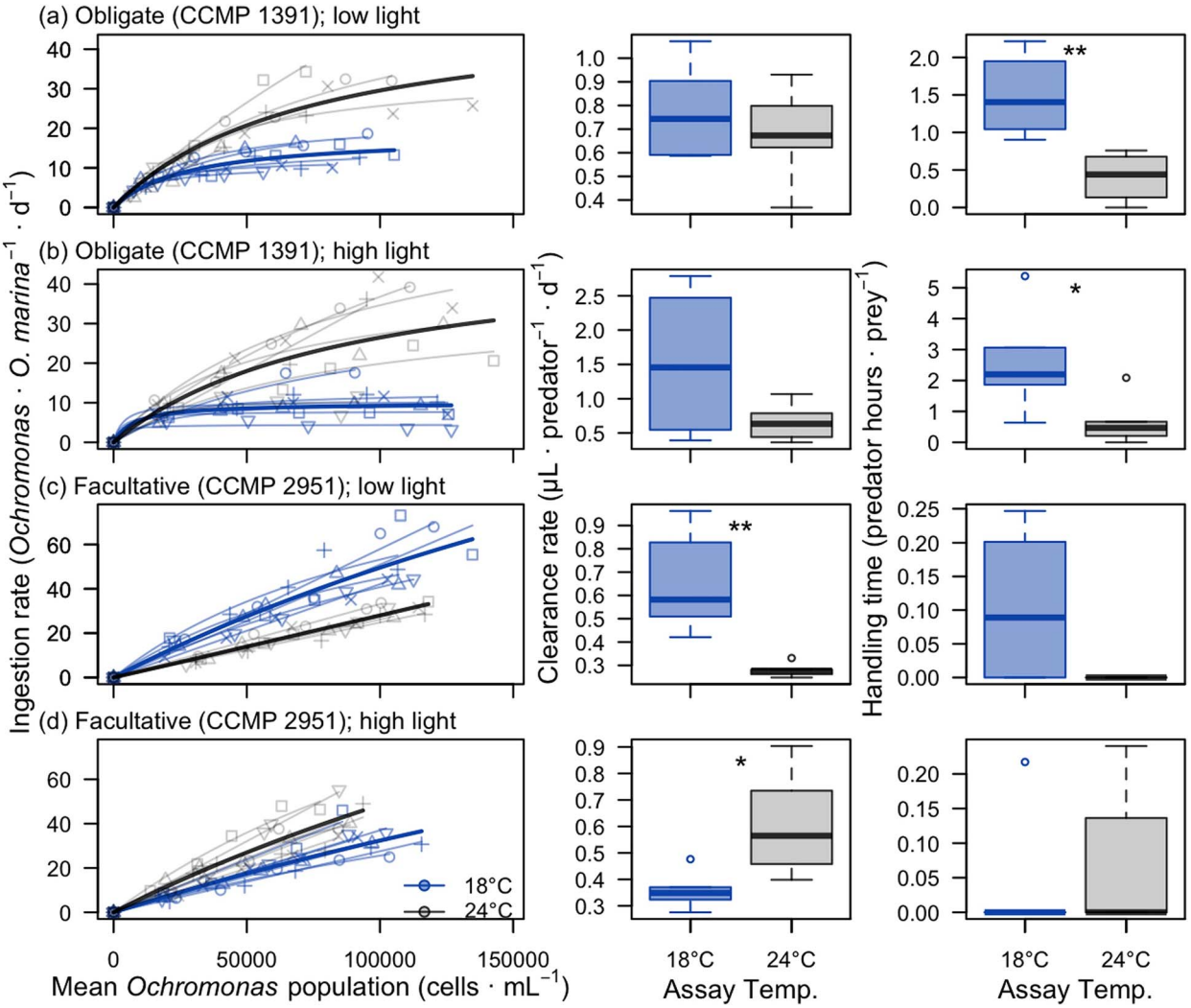

Fig. 4. Ingestion rates (left column), clearance rates (middle column) and handling times (right column) of *O. marina* fed control lineages of prey when assayed at 18°C and 24°C to evaluate how grazing is altered by temperature. Ingestion rates (left column) are plotted across mean *Ochromonas* populations. Symbols and thin lines indicate biological replicates ($n = 6$) fit with Holling Type I or Type II functional response curves (thin lines). The thick lines (one per assay temperature) indicate the functional response curve fit across unpooled replicates. On the box plots, the horizontal line indicates the median and the symbol indicates the mean. (**a**) Results for the control obligate phototroph (CCMP 1391) evolved at low light. (**b**) Results for the control obligate phototroph evolved at high light. (**c**) Results for the control facultative phototroph (CCMP 2951) evolved at low light. (**d**) Results for the control facultative phototroph evolved at high light. Asterisks indicate significant differences in clearance rates or handling times between assay temperatures (*t*-test; $* = P$-value < 0.05; $** = P$-value < 0.01).

consumer growth (Sikora *et al.*, 2014; Twining *et al.*, 2016, 2021). Warming temperatures have been shown to affect fatty acid composition in microbes. In phytoplankton, acclimation and evolution to higher temperatures led to increased SFAs at the expense of PUFAs to maintain cell rigidity (Sinensky, 1974; Maazouzi *et al.*, 2008; O'Donnell *et al.*, 2019; Jin *et al.*, 2020; Lau *et al.*, 2021). This change in fatty acid profiles can result in inefficient energy transfer and limits secondary production in taxa unable to adjust their physiology and nutrient storage capacities (Twining *et al.*, 2016). While we did not quantify biochemical compounds of our evolved *Ochromonas* lineages, it is likely that profiles of consumer relevant compounds like fatty acids and thus prey quality varied between the strains as well as between the thermally adapted lineages within strains, potentially explaining the differences in predator responses we found between thermally adapted lineages.

We also observed differences in *O. marina* functional responses across the two evolutionary light levels considered in this study. Specifically, trends in evolutionary effects were more consistent and pronounced for *Ochromonas* lineages evolved at lower light than higher light. Light availability affects rates of both photosynthesis and phagotrophy (Caron *et al.*, 1993; Fischer *et al.*, 2022; Schenone *et al.*, 2022), including in the *Ochromonas* strains used in our study (Barbaglia *et al.*, 2024). Generally, when resources are limiting, *Ochromonas* invest more in resource acquisition (Barbaglia *et al.*, 2024), consistent with, for example, higher chlorophyll content in low-light evolved *Ochromonas* (Lepori-Bui *et al.*, 2022). Although not measured in our study,

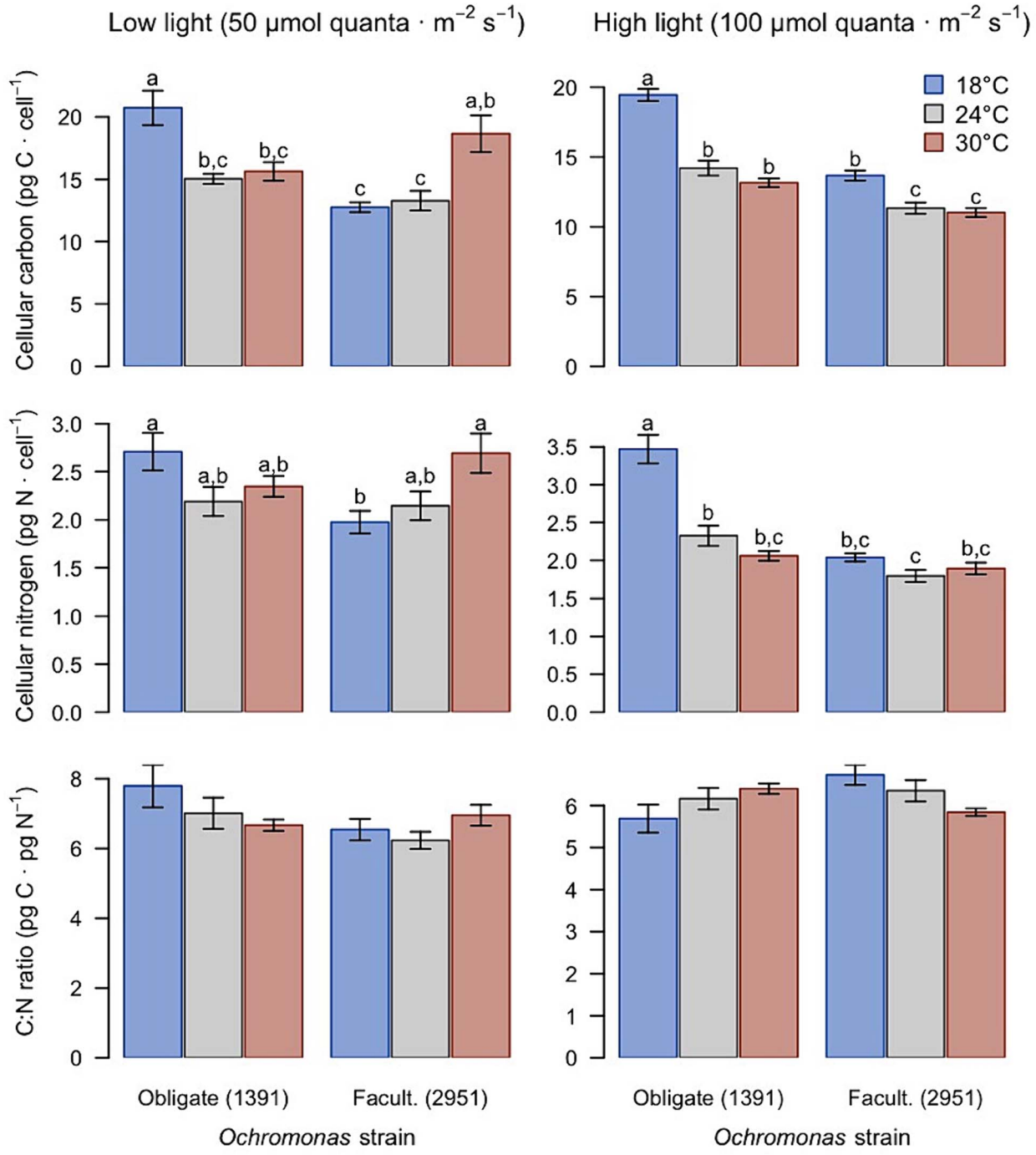

Fig. 5. Cellular C (top row), N (middle row) and C:N ratios (bottom row) of evolved lineages of *Ochromonas* (CCMP 1391, 2951) used as prey. Lineages were evolved in low light conditions (left column) and high light conditions (right column). *Ochromonas* were evolved at cold temperatures (18°C), hot temperatures (30°C), or ancestral temperatures (24°C). Error bars indicate ±1 standard error. Letters indicate statistically significant differences between C content or N content at the $P < 0.05$ level from Tukey HSD-corrected ANOVA between evolved temperatures of *Ochromonas* within one strain and light level. We found no significant differences in C:N ratios. Data were collected by Lepori-Bui *et al.* (2022).

changes in these rates may also produce differences in cell stoichiometry, size and metabolite profiles which have cascading effects on grazer functional responses. However, we urge caution in interpreting these results, because our experiments were all conducted at a common light level and temperature. While this allows us to control for *in situ* conditions to isolate the impacts of evolutionary history, this also means that some of our *Ochromonas* lineages may have been growing in suboptimal conditions (e.g. at lower light levels or colder temperatures than those at which they had been evolved). For example, at the cold assay temperature used in this experiment, hot-evolved *Ochromonas* exhibited lower growth rates (Lepori-Bui *et al.*,

2022), and this reduced metabolic activity may account for some of the weakening in predator–prey interactions when *O. marina* was fed hot-evolved prey. Future work on this topic could include grazing assays at the evolutionary temperature and light level of the prey for complete comparison.

O. marina clearance rates and handling times varied between *Ochromonas* strains and with thermal adaptation within each *Ochromonas* strain. Between the two strains, the obligate phototroph had relatively higher clearance rates and handling times than the facultative phototroph (Fig. 1, middle and columns). While this could be due to fundamental morphological, biochemical, or behavioral differences between these two prey types (Wilken *et al.*, 2020), this could also be explained by the fact that, in some cases for the facultative phototroph (Fig. 1c and d; Fig. 4c and d), *O. marina* grazing did not reach saturation because some *Ochromonas* lineages did not grow to sufficiently high densities for more dense experimental inoculation. In these instances, a Type I functional response was the best fit, and handling time is defined as zero and thus lower than for the obligate phototroph where grazing saturated (Fig. 1a and b; Fig. 4a and b). Although *O. marina* ingestion would likely saturate at sufficiently high densities beyond those used in this study, variation in grazer response is nonetheless evident within each *Ochromonas* strain through generally lower clearance rates and higher handling times with hot-evolved lineages as compared to cold-evolved or control lineages.

We additionally quantified how ambient temperature affects the grazing of predators on mixotrophs within one evolutionary temperature. When *O. marina* grazed on control lineages of *Ochromonas* at the cold and ancestral temperatures, we generally found that ingestion rates increased with temperature. Our result coincides broadly with similar theoretical and empirical predictions of thermal scaling (Montagnes *et al.*, 2001; Wilken *et al.*, 2013; Xiang *et al.*, 2017; Pomati *et al.*, 2020). Together, this evidence suggests that under warmer temperatures, the increased ingestion and respiration of predators should result in stronger predator–prey interactions that could potentially destabilize food webs (Gilbert *et al.*, 2014; West and Post, 2016; Schaum *et al.*, 2018; Robertson and Hammill, 2021). Thus, with increased temperatures, clearance rates are expected to increase and handling times are expected to decrease (Rall *et al.*, 2012). Our results for handling time concur with this pattern, but we found decreasing clearance rates with warmer temperatures. These unexpected results may be explained by *O. marina* being at the limit of their metabolic equilibrium and experiencing lower growth rates at higher temperatures (Calbet *et al.*, 2022; Calbet and Saiz, 2022), despite acclimation to warmer temperatures (Calbet and Saiz, 2022).

CONCLUSION

In conclusion, our results highlight how thermal adaptation of mixotrophs can affect traits that alter their palatability and trophic transfer to dinoflagellate predators. Evolution to hotter temperatures generally reduced ingestion rates, resulting in the subsequent weakening of predator–prey interactions. Predator performance varied between mixotroph strains and with prey thermal adaptation. Explanations for these results include possible toxicity of one mixotroph strain or changes in cell size and biochemical composition driven by thermal adaptation. Irrespective of thermal adaptation, increasing ambient temperature led to increased ingestion rates but decreased clearance rates of mixotroph prey. Our results coincide with evidence from natural and experimental microbial systems that show weakening or total loss of trophic interactions under warming conditions (Winder and Schindler, 2004; O'Gorman and Emmerson, 2009; Ullah *et al.*, 2018). Such decoupling of trophic interactions has led to shifts in planktonic communities (Francis *et al.*, 2012) as well as expansion of primary producers and loss of biomass to higher trophic levels (Ullah *et al.*, 2018). Understanding how mixotroph adaptation to climate change can alter trophic transfer is essential to understand the role that mixotrophs and microbes as a whole will play in the changing dynamics in the microbial loops, the classical food chain and C cycling in marine ecosystems.

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SUPPLEMENTARY DATA

Supplementary data can be found at *Journal of Plankton Research* online.

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DATA AVAILABILITY

Data and analysis code are available at https://zenodo.org/doi/10.5281/zenodo.13831287.

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