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Mesodinium rubrum exhibits genus-level but not species-level cryptophyte prey selection

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ABSTRACT: The marine ciliate *Mesodinium rubrum* is known to form large non-toxic red water blooms in estuarine and coastal upwelling regions worldwide. This ciliate relies predominantly upon photosynthesis by using plastids and other organelles it acquires from cryptophyte prey. Although *M. rubrum* is capable of ingesting different species of cryptophytes, mainly *Teleaulax amphioxeia* plastids have been detected from wild *M. rubrum* populations. These observations suggest that either *M. rubrum* is a selective feeder, or *T. amphioxeia* are taken up because of higher availability. To test these hypotheses, we determined whether the ciliate showed different grazing rates, growth responses, or plastid retention dynamics when offered *Storeatula major*, *T. amphioxeia*, *T. acuta*, or a mix. When *M. rubrum* was offered the cryptophyte *S. major* as prey, no evidence was found for ingestion. In contrast, *M. rubrum* grazed both *Teleaulax* spp. equally, was able to easily switch plastid type between them, and the ratio of each in the ciliate reflected the abundance of free-living prey in the culture. *M. rubrum* grew equally well when acclimated to each plastid type or when having mixed plastids. However, when offered single prey, *T. amphioxeia* could sustain higher *M. rubrum* growth rates (μ) over longer periods. Compared to other *M. rubrum* strains, this culture had higher grazing rates, greater ingestion requirements for reaching μ_{max}, and appeared to rely more on plastid sequestration than de novo division of cryptophyte organelles. Our results suggest that while *M. rubrum* may prefer *Teleaulax*-like cryptophytes, they do not select among the species used here.

KEY WORDS: Mixotrophy · Acquired phototrophy · Kleptoplastidy · Grazing · Prey selection · Species-specific qPCR · *Mesodinium* · *Teleaulax*

INTRODUCTION

The marine ciliate *Mesodinium rubrum* Lohmann, 1908 (= *Myrionecta rubra* Jankowski, 1976) can form massive non-toxic red blooms in estuaries and coastal upwelling regions worldwide (Lindholm 1985). *M. rubrum* is an obligate mixotroph that relies primarily upon phototrophy and preys on cryptophytes in order to sequester plastids and other organelles (Yih et al. 2004, Johnson & Stoecker 2005, Johnson et al. 2006, Smith & Hansen 2007, Hansen et al. 2012). *M. rubrum* is unusual for its ability to sequester a functional cryptophyte nucleus, which it uses to control and replicate its plastids (Johnson et al. 2007, Lasek-Nesselquist et al. 2015). This elaborate prey-handling mechanism raises questions about the extent to which *M. rubrum*'s acquired photosynthetic potential depends upon the ingestion of specific prey species. In addition, *M. rubrum*-like ciliates are now known to be a species complex composed of at least 8 distinct subclades (Herfort et al. 2011, Garcia-Cuetos et al. 2012, Johnson et al. 2016). One of these clades has been officially described as a new species, *M. major* (Garcia-Cuetos et al. 2012).

To date, all stable cultures of *M. rubrum* have been established using prey of either *Teleaulax* or *Geminigera* (Gustafson et al. 2000, Hansen & Fenchel 2006, Johnson et al. 2006, Park et al. 2007), which form a

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well-supported subclade of cryptophytes along with *Plagioselmis* (Deane et al. 2002, Hoef-Emden 2008). Feeding experiments with cultured *M. rubrum* are consistent with the notion that species from this cryptophyte subclade are preferred prey for *M. rubrum*; however, it will ingest species from outside of this clade as well (Park et al. 2007, Hansen et al. 2012). Further, it is thought that the availability of suitable cryptophyte prey is important for *M. rubrum* bloom formation (Herfort et al. 2011). The preferential consumption of certain cryptophyte species by *M. rubrum* would likely shape cryptophyte community composition, especially during blooms (Park et al. 2007).

Molecular tools have enabled more precise studies on *M. rubrum* plastid origin and retention dynamics (Johnson et al. 2007, Herfort et al. 2011, Hansen et al. 2012); however, questions remain regarding whether certain plastid types are selected by the ciliate for sequestration or proliferate more efficiently once taken up. Studies of natural populations of *M. rubrum* from temperate regions, using single-cell PCR, also converge upon a perspective that although the ciliate is capable of ingesting different species of cryptophytes, mainly *T. amphioxeia* plastids are found from wild *M. rubrum* populations (Nishitani et al. 2010, Herfort et al. 2011). These observations suggest that either temperate *M. rubrum* populations selectively retain *T. amphioxeia* plastids, or they are numerically dominant because of higher encounter rates (e.g. due to abundance).

In the present study, we performed laboratory experiments with a temperate *M. rubrum* strain and 3 cryptophyte species, *Storeatula major*, *T. amphioxeia* and *T. acuta*, to test the hypothesis that *T. amphioxeia* plastids are selected over and perform better (i.e. supporting higher growth rate) than plastids from other cryptophytes. While *T. amphioxeia* is commonly encountered in coastal ecosystems, its close relative *T. acuta* is less frequently reported and its plastids are rarely found in *M. rubrum* cells (Johnson et al. 2016). The cryptophyte *S. major* was isolated from the same ecosystem as our *M. rubrum* culture, i.e. Chesapeake Bay, and has been shown to be ingested at high rates when offered to mixotrophic dinoflagellates (Li et al. 2000).

MATERIALS AND METHODS

Cultures

Two closely related cryptophyte species, *Teleaulax acuta* (Butcher) Hill, 1991 and *T. amphioxeia* (W. Conrad) Hill, 1991, as well as *Storeatula major* Butcher ex Hill, 1991 were used in this study. *T. acuta* was obtained from the Scandinavian Culture Collection of Algae and Protozoa (SCCAP; K-1486); *T. amphioxeia* (GCEP01) was isolated from Eel Pond, Falmouth, MA, USA by Dr. Mengmeng Tong; and *S. major* (strain SM or g) was isolated from the Choptank River, Cambridge, MD, a tributary of the Chesapeake Bay, USA, by Allen Lewitus. The cryptophytes were maintained in 250 ml flasks in 15 psu f/2 or f/4 medium (Guillard & Ryther 1962), under a 14 h light:10 h dark cycle at a light level of 54 µmol m^{-2} s^{-1} and at 15°C.

Mesodinium rubrum was isolated by the senior author (M.D.J.) in October 2011 from the mouth of the James River in Chesapeake Bay (strain CBJR05), and cultured in the same conditions as the cryptophytes, but in 6 well plates (12 ml $well^{-1}$) or 250 ml tissue culture flasks. The culture was originally isolated, and since maintained, upon the cryptophyte GCEP01 as prey. Strain CBJR05 is from a new clade (G) of *M. rubrum*-like ciliates (Johnson et al. 2016), based on 18S, internally transcribed spacer region (ITS), and 28S ribosomal RNA gene sequences (Herfort et al. 2011, Garcia-Cuetos et al. 2012). Depending on the purpose of the experiment, the stock cultures of *M. rubrum* were fed once a week either with *T. amphioxeia* or *T. acuta*. The acclimation period with *T. acuta* was 4 wk, and *M. rubrum* was re-isolated from that culture for Expt 3, in order to remove traces of *T. amphioxeia*. It was also verified with quantitative PCR (qPCR) that *M. rubrum* had solely *T. acuta* plastids in the beginning of that experiment (not shown).

Washing prey from *Mesodinium rubrum* cells

M. rubrum cells were washed free of cryptophyte prey using 8.0 µm Transwell (Corning) inserts in 12 well plates. To wash stock cultures for setting up experiments, approximately 50–100 ml of culture was passed through the filter, and then washed with 200 ml of filtered seawater. This concentrate of *M. rubrum* cells was then enumerated and used for experiments. For qPCR sampling, 2 ml from each treatment were flushed with 100–150 ml of filtered seawater using Transwell inserts, and the removal of free cryptophyte cells was confirmed under a microscope at 100× magnification (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a078p147_supp.pdf). *M. rubrum* cells were then collected on 25 mm GF/C filters (Whatman) and stored at –20°C for not more than 24 h before DNA extraction.

Functional and numerical response of *Mesodinium rubrum* to single cryptophyte prey

Grazing experiments were conducted using *M. rubrum* acclimated to the tested prey. However, we were unable to acclimate *M. rubrum* to *S. major* and found no evidence of predation on this cryptophyte. In order to acclimate *M. rubrum* to *T. acuta,* ciliates were washed free of their original prey, *T. amphioxeia* (as described above), and grown for at least 1 mo prior to experiments with weekly additions of *T. acuta* and f/4 media. Grazing experiments were conducted in 24 well plates in triplicate, and were sampled at the same time once a day for 2 d in order to estimate grazing rates. We measured the functional grazing response of *M. rubrum* to cryptophytes by placing 500–1000 ciliates ml^{-1} with *T. amphioxeia* at concentrations of 0–30 000 cells ml^{-1}, and 1000 ciliates ml^{-1} with 0–20 000 *T. acuta* ml^{-1}. Growth (μ) and grazing rates, including the grazing constant (g), clearance rate (F), and ingestion rate (IR), were determined using equations described previously (Frost 1972, Heinbokel 1978, Jeong & Latz 1994). Grazing rates were all calculated after 24 h for assessment of the functional response to prey concentration and species, while growth rates were measured over 5–6 d. In addition, grazing rates were also determined for one experiment where plastid content was estimated and identification ensured with qPCR (see next subsection).

Mesodinium rubrum plastid dynamics when offered mixed prey

Prior to all experiments, *M. rubrum* cultures were not fed with fresh prey for 2 wk, and they were further starved for 2 additional days after washing cultures with seawater. All experiments were conducted in 6 well plates with treatments in duplicate, and in the same growth treatments detailed in 'Cultures' section with f/2 media. *M. rubrum* cells were washed (see 'Washing prey from *Mesodinium rubrum* cells' above) prior to the start of the experiments (from stock cultures) as well as at every sampling time point, in order to remove free-living prey. Only cryptophyte cultures that were in exponential growth phase were used in the experiments.

In the first plastid dynamics (PD) experiment, we examined whether *M. rubrum* sequesters plastids from a non-*Teleaulax* cryptophyte. *M. rubrum*, acclimated to *T. amphioxeia*, was fed with *T. amphioxeia* and *S. major* (predator:prey 1:7), mixed at ratios of 1:1, 1:2, 1:4, 1:8 and 1:16, respectively. This was also a short-term experiment, and the samples for qPCR were taken at time 0 (T_0), and after 6, 24 and 48 h. *M. rubrum* controls without prey as well as prey controls without *M. rubrum* were run in parallel in each experiment.

In PD Expt 2, we studied the short-term exchange of *M. rubrum* plastids and prey selection. In this experiment, *M. rubrum*, acclimated to *T. amphioxeia*, was fed with 1:0, 1:1 or 1:5 reciprocal combinations of *T. amphioxeia* and *T. acuta*, respectively (predator: prey 1:20). Samples for qPCR were taken at the following times: 0, 2, 5, 24 and 48 h.

For studying growth, grazing, and prey selection of *M. rubrum* fed 2 *Teleaulax* spp. over a longer period (2 wk; PD Expt 3), a stock culture of *M. rubrum* acclimated to *T. amphioxeia* was fed several cryptophyte prey treatments with an increasing proportion of *T. acuta*. In the experiment, *T. amphioxeia* and *T. acuta* were combined at ratios of 1:0, 0:1, 1:1, 1:2 or 1:10 to each another, respectively. The total predator:prey ratio was 1:15. Samples for cell counts were taken at T_0 and on Days 2, 4, 7, 10 and 14. Samples to evaluate plastid intake and retention by *M. rubrum* using qPCR were taken at times 0 and 4 h, and at 4, 7 and 14 d. Unlike the grazing functional response experiments (see 'Functional and numerical response of *Mesodinium rubrum* to single cryptophyte prey' above), we calculated grazing rates for this experiment over 2 d.

Finally, the effect of *M. rubrum* feeding history on their prey selection (PD Expt 4) was studied with *M. rubrum* originally acclimated to pure cultures of either only *T. amphioxeia* or *T. acuta*. The predator: prey ratio was 1:5, and the given prey ratios were 1:0, 0:1 or 1:1 combinations of *T. amphioxeia* and *T. acuta*, respectively. Samples for qPCR were taken at the following times: 0, and 3 and 7 d.

Cell enumeration and determination of rates

Samples of *M. rubrum* and cryptophyte algae were preserved with 1% acid Lugol's solution (final), and enumerated using a Sedgewick-Rafter counting chamber and a compound Zeiss AxioScope A1 at 200× magnification for cryptophytes and 100× for *M. rubrum*. In all cases, at least 100 cells were counted. Growth and grazing rates (grazing coefficient, clearance rate, and ingestion rate) were calculated using the equations of Frost (1972) and Heinbokel (1978), and refined by Jeong & Latz (1994) and Kim et al. (2008).

DNA extraction, primers and qPCR amplification

The ingestion and retention of *Teleaulax* spp. and *S. major* plastids by *M. rubrum* was detected with qPCR. All samples were washed (see 'Washing prey from *Mesodinium rubrum* cells' above) prior to extraction and qPCR analysis, and DNA was extracted from the GF/C filters using cetrimonium bromide (CTAB) (Gast et al. 2004). A qPCR assay was designed for a fragment of the plastid-encoded large subunit of RuBisCO (*rbcL*) gene for each species of cryptophyte, whereas abundance of *M. rubrum* was determined by targeting a fragment of the nuclear small subunit (SSU) rRNA (18S) gene (Table 1). All primers were synthesized by Eurofins MWG Operon (AL, USA), and were designed to target regions that had mismatches with non-target species. The specificity of the primers was crosschecked with qPCR by assessing amplification cycle threshold (C_t) and melting temperature under a range of temperature settings (not shown). Assay conditions were optimized to amplify target template sequence and to exclude non-specific amplification of other cryptophyte species. Standard curves for cryptophytes were prepared in serial dilution of duplicate DNA extractions of 50, 500, 5000, 10 000, 50 000 and 150 000 cells ml^{-1}, and for *M. rubrum* 50, 100, 500, 3000, 5000, 8000 and 13 000 cells ml^{-1}. All qPCR analyses were conducted using a Bio-Rad CFX96 Real-Time PCR detection system with SsoFast EvaGreen Supermix (Bio-Rad; reaction volume: 20 µl, primer concentration: 0.3 µM). The thermal program was as follows: 95°C for 3 min (1×); 95°C for 10 s and primer-specific temperature (Table 1) for 30 s, and a total of 40 cycles. Melting curve analysis was used to verify amplicon purity, and the copy numbers were determined from the C_t value and by using the standard curves (Bowers et al. 2000). Plastid number per cell in *M. rubrum* was then determined by taking the ratio of total cryptophyte plastids to the number of *M. rubrum* from the qPCR C_t data.

Statistical analysis

The effect of the 2 prey species and their different proportions on *M. rubrum* clearance and ingestion rates, and grazing coefficients were analysed with ANOVA, whereas *M. rubrum* growth rates were examined with the nonparametric Kolmogorov-Smirnov test. The possible prey selection due to previous feeding history was analysed with ANOVA, and the effect of different proportions of *T. acuta* and *T. amphioxeia* was tested with the nonparametric Jonckheere-Terpstra test, in which the abundance of *T. acuta* was considered as an ordering alternative. All statistical tests were conducted with IBM SPSS Statistics 22.

RESULTS

Functional and numerical response of *Mesodinium rubrum* to single cryptophyte prey

We were unable to find evidence that *Mesodinium rubrum* grazed on *Storeatula major*, and it failed to support growth of the ciliate, so no results are shown for this prey species. *M. rubrum* grazing coefficients (g) declined exponentially with increasing prey concentrations with *Teleaulax amphioxeia*, while the decline was linear for *T. acuta* (Table 2). Maximum g for *T. amphioxeia* and *T. acuta* prey were both high, at 3.8 ± 0.06 and 3.2 ± 0.52 d^{-1}, respectively (Table 2), but overall population-level grazing on *T. acuta* was significantly higher (ANOVA, $F_{1,8}$ = 5.97, p = 0.0239). However, the cell-specific grazing rates for clearance (F) (ANOVA, $F_{1,8}$ = 10.93, p = 0.0035) and ingestion (IR) (ANOVA, $F_{1,8}$ = 11.51, p = 0.0029) were greater for *T. amphioxeia* prey. Maximum F and IR for *M. rubrum* were 6.8 ± 1.9 and 3.0 ± 0.4 µl cell^{-1} d^{-1} (Fig. 1a), and 13.0 ± 0.7 and 8.2 ± 0.7 prey cell^{-1} d^{-1} (Fig. 1b) for *T. amphioxeia* and *T. acuta*, respectively. Growth rates of *M. rubrum* were nearly identical

Table 1. Primers used for quantitative PCR assays in this study to enumerate cryptophyte plastids

Primer	Sequence	Temperature (°C)
Teleaulax acuta 683F	5′-TGC TGA GTT CGG TAA AGA GCT-3′	64.2
Teleaulax acuta 830R	5′-ACG AGC GTA TGT AGA GTT ACC T-3′	
Teleaulax amphioxeia F1	5′-CTT CCT TAA AGA TGA CGA GAA CAT T-3′	62.8
Teleaulax amphioxeia R1	5′-TGA CCT TTA ATT TCA CCT GTA GCT-3′	
Storeatula major 600F	5′-AGA GCT GCT GCT GGT ACT G-3′	64.6
Storeatula major 780R	5′-CTG TTC TTA CGA GCC CAG ATA C-3′	
Mesodinium rubrum 405F	5′-TAC CCA ATG CAG ACA CTG TGA G-3′	64.5
Mesodinium rubrum 524R	5′-CCA GAC TTT CCC ATC AGT TGC TA-3′	

Table 2. Functional grazing response of *Mesodinium rubrum* (MR) to *Teleaulax amphioxeia* (Tam) and *T. acuta* (Tac). Prey and predator concentrations at the beginning of the experiment (T_0), growth rate (μ) of predator and prey, and grazing constant (g) after 24 h. Rates are mean ± SD (n = 3)

Prey	Carbon in prey at T_0 (ng C ml^{-1})	Prey at T_0 (cells ml^{-1})	MR at T_0 (cells ml^{-1})	Prey:MR ratio at T_0	MR μ (d^{-1})	Prey μ (d^{-1})	g (d^{-1})
Tam	0	0	610	0	0.03 ± 0.05	–	–
	55	600	530	1	0.21 ± 0.09	0.44 ± 0.13	3.81 ± 0.06
	124	1500	710	2	0.12 ± 0.03	0.52 ± 0.07	2.37 ± 0.23
	285	3200	620	5	0.27 ± 0.09	0.60 ± 0.05	1.27 ± 0.21
	634	7000	630	11	0.31 ± 0.04	0.60 ± 0.07	1.08 ± 0.21
	1120	12500	670	19	0.33 ± 0.05	0.65 ± 0.06	0.69 ± 0.10
	2540	28000	630	44	0.37 ± 0.12	0.72 ± 0.11	0.28 ± 0.03
Tac	0	0	930	0	0.14 ± 0.01	–	–
	73	730	990	1	0.16 ± 0.05	0.02 ± 0.13	3.19 ± 0.52
	150	1500	810	2	0.23 ± 0.07	0.20 ± 0.03	2.72 ± 0.11
	330	3300	840	4	0.34 ± 0.06	0.26 ± 0.05	2.87 ± 0.29
	680	6800	890	8	0.32 ± 0.02	0.24 ± 0.05	2.01 ± 0.27
	1200	12000	940	13	0.40 ± 0.02	0.23 ± 0.13	1.10 ± 0.29
	2150	21500	880	24	0.36 ± 0.04	0.25 ± 0.04	0.42 ± 0.04

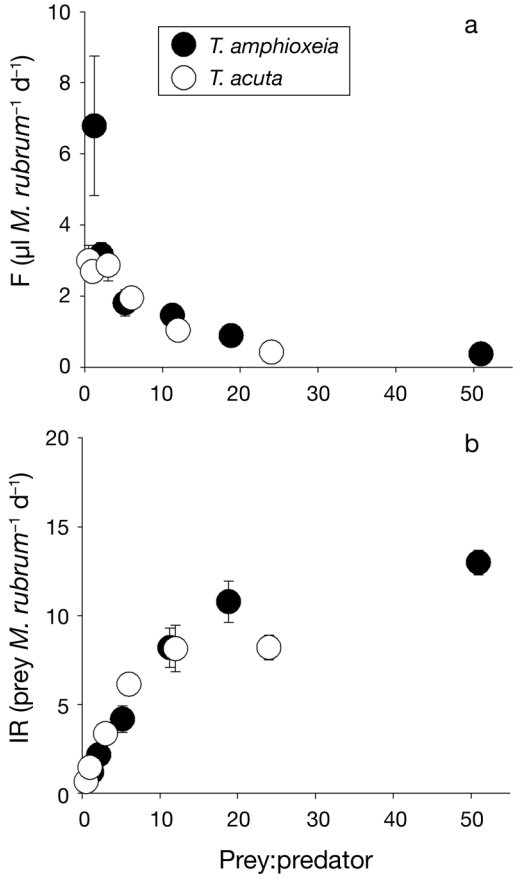

Fig. 1. *Mesodinium rubrum* grazing response (n = 3) as a function of *Teleaulax amphioxeia* and *T. acuta* prey:predator ratio (at T_0). (a) Clearance (F) and (b) ingestion (IR) rates of *M. rubrum* on prey. Error bars are SD

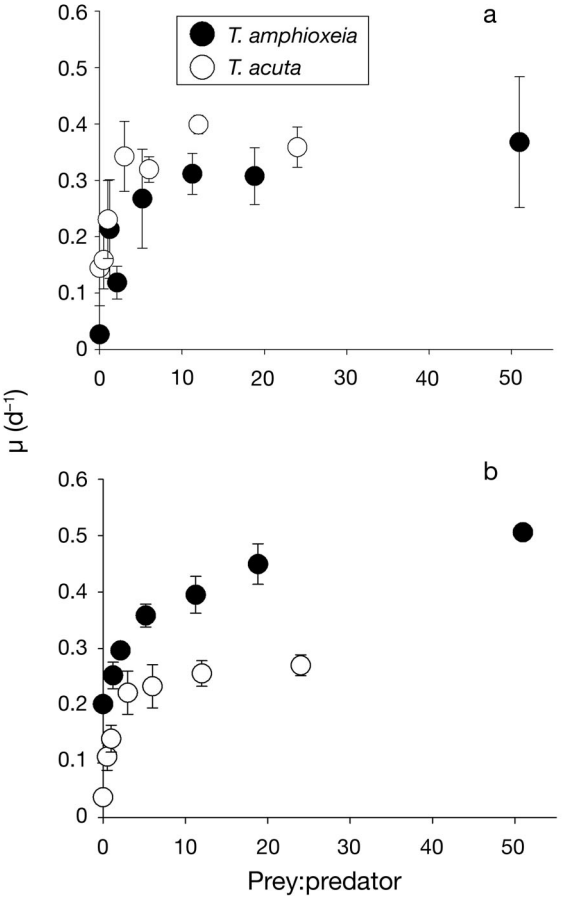

Fig. 2. Growth rate (μ) response (n = 3) of *Mesodinium rubrum* as a function of *Teleaulax amphioxeia* and *T. acuta* prey:predator ratio (at T_0) after (a) 2 d and (b) 5–6 d. Error bars are SD

over the first 2 d (Fig. 2a), with maximum growth rates reaching 0.62 ± 0.07 and 0.56 ± 0.14 d^{-1} during that time (from Day 1 to Day 2; not shown) when grown with *T. amphioxeia* and *T. acuta*, respectively. Over longer periods (5–6 d), however, μ of *M. rubrum* was significantly higher in *T. amphioxeia* (Fig. 2b).

Mesodinium rubrum growth, grazing, and PD with mixed cryptophyte prey

While we found no evidence of ingestion of *S. major* prey or retention of its plastids during the functional response experiment (see previous subsection), we decided to use it in a mixed-prey experiment with *T. amphioxeia* in order to see if we could detect ingestion of *S. major* using qPCR (PD Expt 1). All treatments that included *S. major* resulted in negative growth rates of *M. rubrum*, even if *T. amphioxeia* was also added (Table 3), and no evidence of *S. major* was detected in *M. rubrum* using qPCR in any of our treatments. Thereafter, all mixed-prey experiments utilized *T. amphioxeia* and *T. acuta*.

In order to determine if mixed *Teleaulax* prey has an effect upon *M. rubrum* grazing or growth rates, we measured prey selection and PD of *M. rubrum* grazing on *T. amphioxeia*, *T. acuta*, or mixtures of the 2 at different ratios, but identical prey concentrations. PD Expt 2 had the highest predator:prey ratio (1:20), and after 48 h, this resulted in the greatest average number of plastids per cell (16–17) that we observed in this *M. rubrum* strain (Fig. 3). *M. rubrum* plastid intake began soon after they were introduced to new prey, as *T. acuta* plastids were detected in *M. rubrum* after 2 h (earliest time point) and total plastid numbers more than doubled following their introduction. This change in the number of plastids from a different prey source was rapid and *M. rubrum* replaced half of its plastids with *T. acuta* after 24 h when fed with a 5:1 ratio (Fig. 3). *M. rubrum* did not strongly discriminate between *Teleaulax* plastid sources, and the proportion of *T. amphioxeia* and

Table 3. Plastid dynamics (PD) experiments to assess if *Mesodinium rubrum* (MR) selects prey between *Teleaulax amphioxeia* (Tam), *T. acuta* (Tac), and *Storeatula major* (SM), and if prey organelle acclimation state affects prey selection. Average growth rates (μ_{avg}, ±SD) were estimated over the entire time of the experiment, while maximum growth rates (μ_{max}, ±SD) were between 2 time points. TPG: *Teleaulax/Plagioselmis/Geminigera*. *Significant difference ($p < 0.05$)

MR (cells ml^{-1})	Prey (acclimation)	Prey (cells ml^{-1})	Treatment	MR:prey ratio	Tam:Tac/SM ratio	MR μ_{avg} (d^{-1})	MR μ_{max} (d^{-1})
PD Expt 1: plastid sequestration from a non-TPG clade cryptophyte (Tam vs. SM; 2 d)							
400	Tam	3000	Single prey	1:7	1:0	0.06 ± 0.02	0.06 ± 0.02
400	Tam	3000	Single prey	1:7	0:1	−0.15 ± 0.13	−0.07 ± 0.09
400	Tam	3000	Mixed prey	1:7	1:1	−0.21 ± 0.06	−0.21 ± 0.06
400	Tam	3000	Mixed prey	1:7	1:2	−0.16 ± 0.20	−0.16 ± 0.20
400	Tam	3000	Mixed prey	1:7	1:4	−0.26 ± 0.12	−0.08 ± 0.27
400	Tam	3000	Mixed prey	1:7	1:8	−0.41 ± 0.15	−0.39 ± 0.59
400	Tam	3000	Mixed prey	1:7	1:16	−0.28 ± 0.18	−0.28 ± 0.18
PD Expt 2: short-term (2 d) exchange of MR plastids and prey (Tam vs. Tac) selection							
300	Tam	6000	Single prey	1:20	1:0	0.22 ± 0.04	0.22 ± 0.04
300	Tam	6000	Single prey	1:20	0:1	0.02 ± 0.08	0.02 ± 0.08
300	Tam	6000	Mixed prey	1:20	1:1	0.04 ± 0.07	0.05 ± 0.19
300	Tam	6000	Mixed prey	1:20	1:5	0.02 ± 0.03	0.02 ± 0.03
300	Tam	6000	Mixed prey	1:20	5:1	0.14 ± 0.08	0.14 ± 0.08
PD Expt 3: long-term (14 d) exchange of MR plastids and prey (Tam vs. Tac) selection							
300	Tam	4500	Single prey	1:15	1:0	0.07 ± 0.01	0.25 ± 0.06
300	Tam	4500	Single prey	1:15	0:1	0.12 ± 0.03	0.29 ± 0.06
300	Tam	4500	Mixed prey	1:15	1:1	0.07 ± 0.02	0.32 ± 0.02
300	Tam	4500	Mixed prey	1:15	1:2	0.09 ± 0.02	0.19 ± 0.07
300	Tam	4500	Mixed prey	1:15	1:10	0.11 ± 0.02	0.26 ± 0.02
PD Expt 4: effect of MR feeding history on their prey (Tam vs. Tac) selection (7 d)							
300	Tam	1500	Single prey	1:5	1:0	0.08 ± 0.02	0.14 ± 0.06
300	Tam	1500	Single prey	1:5	0:1	0.12 ± 0.06	0.12 ± 0.06
300	Tam	1500	Mixed prey	1:5	1:1	0.18 ± 0.02	0.25 ± 0.03*
300	Tac	1500	Mixed prey	1:5	1:0	0.10 ± 0.02	0.10 ± 0.02
300	Tac	1500	Mixed prey	1:5	0:1	0.15 ± 0.06	0.15 ± 0.06
300	Tac	1500	Mixed prey	1:5	1:1	0.07 ± 0.02	0.07 ± 0.02*

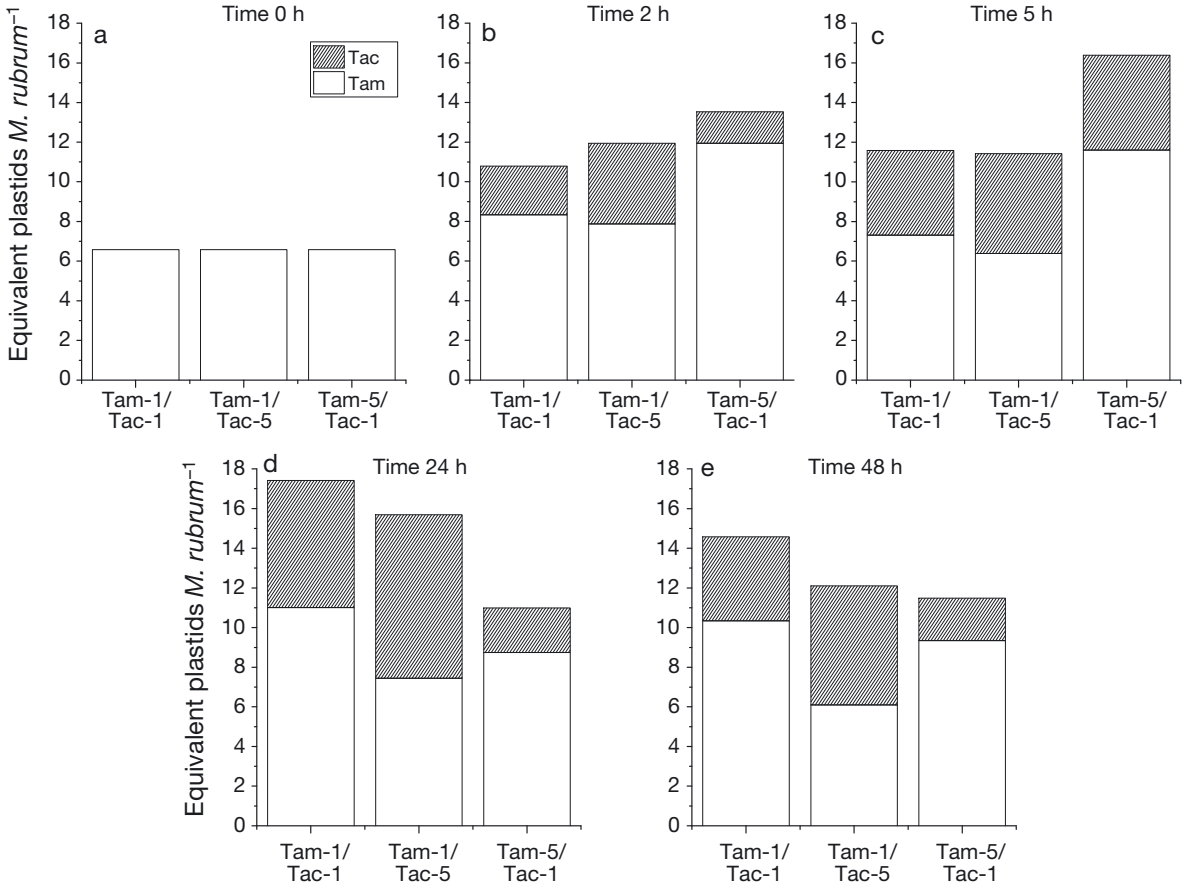

Fig. 3. Short-term (48 h) plastid dynamics (PD) in *Mesodinium rubrum* as determined by qPCR (PD Expt 2) at times (a) 0, (b) 2 h, (c) 5 h, (d) 24 h, and (e) 48 h. *M. rubrum* was acclimated to *Teleaulax amphioxeia* (Tam) prey and fed Tam and *T. acuta* (Tac) at a prey:predator ratio of 20:1. Three treatments varied the ratio of Tam:Tac prey at 1:1 (Tam-1/Tac-1), 1:5 (Tam-1/Tac-5), and 5:1 (Tam-5/Tac-1). Tac plastids could be detected in *M. rubrum* 2 h after their introduction (ANOVA, $p < 0.05$). Stacked bar graphs are means (n = 2)

T. acuta plastids in *M. rubrum* seemed largely dependent on the given proportions of these 2 cryptophytes when *M. rubrum* was fed with mixed prey (Jonckheere-Terpstra, $p < 0.01$; Expt 2; Fig. 3). However, the 1:5 *T. amphioxeia*:*T. acuta* treatment only resulted in about 30–40% more *T. acuta* plastids in *M. rubrum* compared to the 1:1 prey mix after 48 h, indicating that either IR was low (not measured here), the ciliate became satiated in this experiment, or that they selected against *T. acuta* prey. Regardless, *Teleaulax* prey species did not affect *M. rubrum* growth rate (μ) (Kolmogorov-Smirnov, $p > 0.05$), which after 2 d ranged between 0.19 and 0.27 d^{-1} (Table 4).

Table 4. Growth (μ) and grazing parameters (g: grazing constant, F: clearance rate, IR: ingestion rate) for *Mesodinium rubrum* (MR) fed various *Teleaulax* (Tam: *T. amphioxeia*, Tac: *T. acuta*) prey combinations during plastid dynamics Expt 3 after 48 h. Values are mean ± SD (n = 2). Predator:prey ratio at T_0 was 1:15

Treatment (Tam:Tac ratio)	Prey μ (d^{-1})	MR μ (d^{-1})	g (d^{-1})	F (μl MR^{-1} d^{-1})	IR (prey MR^{-1} d^{-1})
Tam only (1:0)	0.44 ± 0.08	0.25 ± 0.06	1.01 ± 0.12	2.05 ± 0.07	7.64 ± 0.90
Tac only (0:1)	0.53 ± 0.04	0.24 ± 0.04	0.71 ± 0.04	1.64 ± 0.44	5.85 ± 1.51
Tam-1/Tac-1 (1:1)	0.57 ± 0.07	0.27 ± 0.04	0.49 ± 0.10	1.16 ± 0.10	4.11 ± 1.99
Tam-1/Tac-2 (1:2)	0.46 ± 0.02	0.19 ± 0.07	0.65 ± 0.08	1.56 ± 0.04	6.25 ± 0.34
Tam-1/Tac-10 (1:10)	0.45 ± 0.07	0.26 ± 0.02	0.43 ± 0.33	0.96 ± 0.77	6.23 ± 5.55

In PD Expt 3, we simultaneously measured the functional grazing and growth response as well as PD of *M. rubrum* in response to our mixed-prey treatments. No grazing parameters differed between the various prey ratio treatments (ANOVA, $p > 0.05$; Table 4) and, averaged across all treatments and combinations of *Teleaulax* prey, IRs in PD Expt 3 were 6.1 ± 1.9 prey predator^{-1} d^{-1}. Also, *M. rubrum* growth kinetics were similar across treatments (Table 3; Fig. S2 in the Supplement at www.int-res.com/articles/suppl/a078p147_supp.pdf). Calculations based on IR and cell abundance revealed that in this experiment ingestion, rather than plastid division, was sufficient to explain plastid content per cell during the first 4 d. The prey:predator ratio in this experiment was 15:1, and plastids per cell in *M. rubrum* increased from 3 at T_0 to between 4–10 after 4 h, and reached a maximum of 10–14 after 4 d (Fig. 4). Total *M. rubrum* plastid replacement from one species (*T. amphioxeia*) to the other (*T. acuta*) took ~2 wk and occurred when *M. rubrum* was fed with only the other *Teleaulax* species (Figs. 3–5).

In PD Expt 4, we tested whether prey acclimation state would affect uptake or selection of prey when offered separately or mixed. We found no differences in growth rate or total plastids per cell with prey acclimation state or treatment (Fig. 5). However, *M. rubrum* was more efficient at replacing *T. amphioxeia*

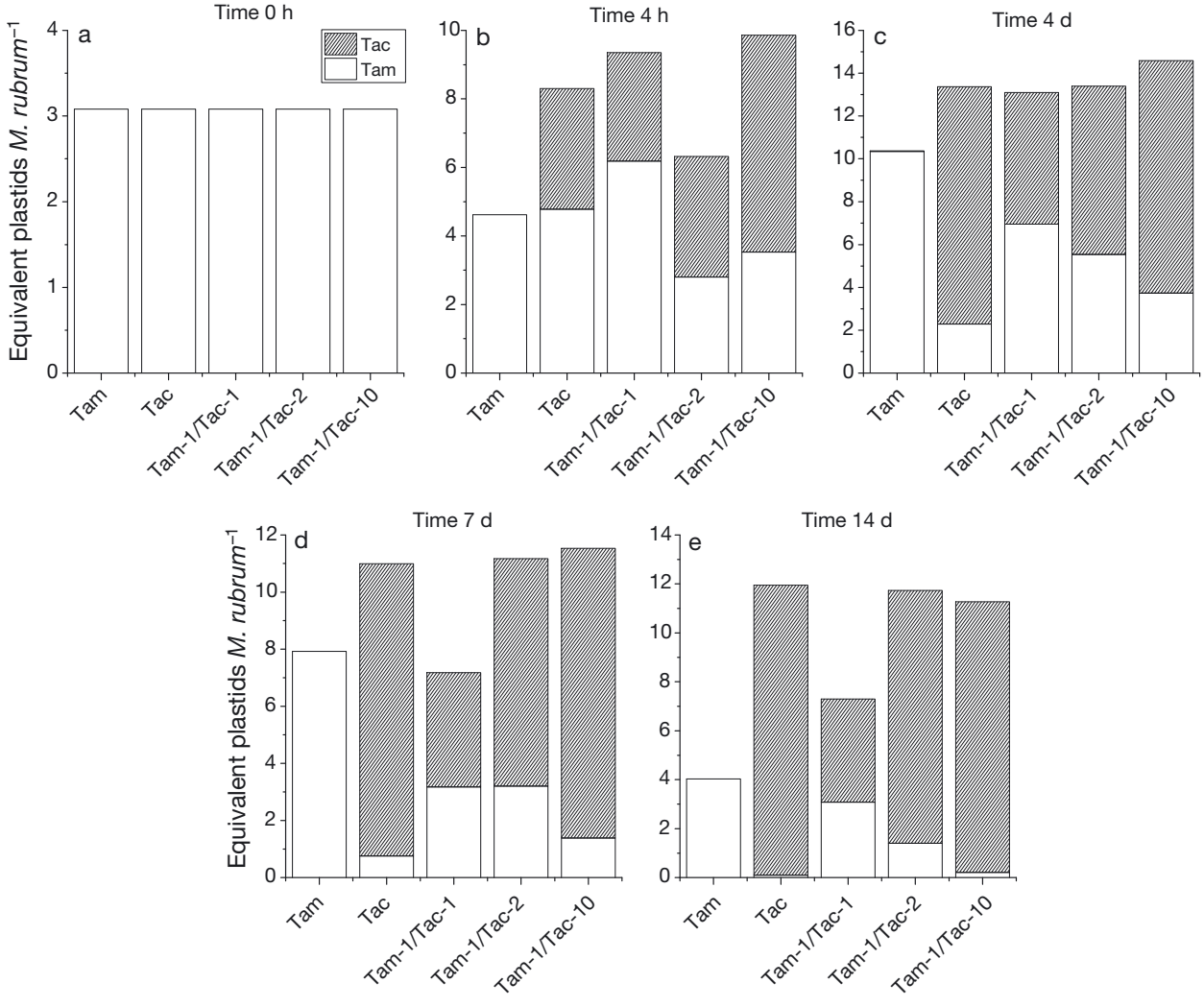

Fig. 4. Plastid dynamics (PD) in *Mesodinium rubrum* acclimated to *Teleaulax amphioxeia* (Tam) and fed Tam or *T. acuta* (Tac), or a 1:1 (Tam-1/Tac-1), 1:2 (Tam-1/Tac-2), or 1:10 (Tam-1/Tac-10) mix of the two (PD Expt 3), at time points (a) 0, (b) 4 h, (c) 4 d, (d) 7 d, and (e) 14 d. Total prey:predator ratio in all treatments was 15:1. Plastid number and type determined using qPCR. The number of Tam and Tac plastids in *M. rubrum* was dependent on the given proportions of these 2 cryptophytes (Jonckheere-Terpstra test, $p < 0.01$). Stacked bar graphs are means (n = 2)

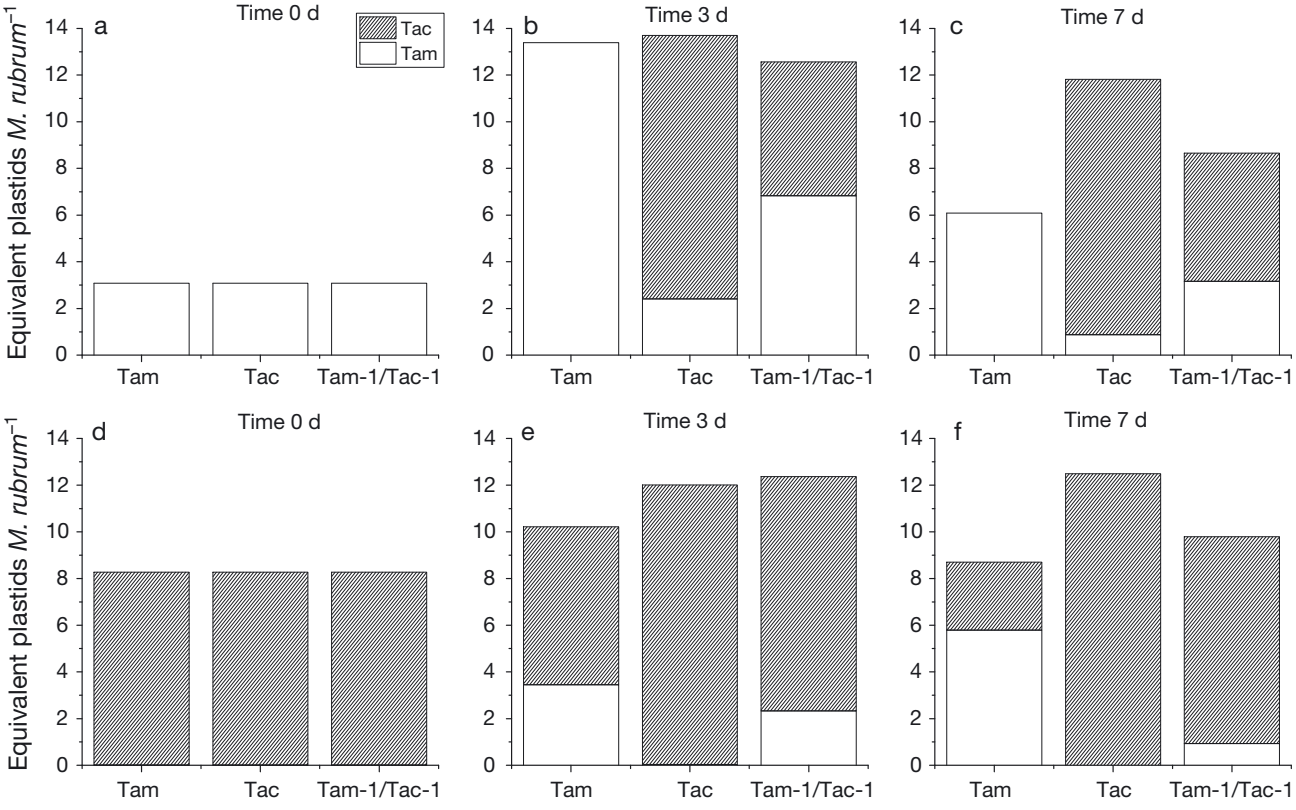

Fig. 5. Plastid dynamics (PD) in *Mesodinium rubrum* acclimated to either (a–c) *Teleaulax amphioxeia* (Tam) or (d–f) *T. acuta* (Tac) and fed those or a 1:1 mix (Tam-1/Tac-1) of them (PD Expt 4), at time points (a) 0, (b) 3 d, and (c) 7 d. Total prey:predator ratio in all treatments was 5:1. Plastid number and type were determined by qPCR. *M. rubrum* did not discriminate between the 2 *Teleaulax* plastid sources, and the feeding history of *M. rubrum* did not cause prey selection (ANOVA, $p > 0.05$). Stacked bar graphs are means (n = 2)

plastids with those from *T. acuta* than vice versa (ANOVA, $p < 0.05$). When fed only the other prey source, ~70% of plastids were replaced with *T. amphioxeia* when acclimated to *T. acuta*, while >90% were replaced by *T. acuta* when acclimated to *T. amphioxeia*. When fed a mix (1:1), the difference in replacement efficiency was similar, with *T. acuta*-acclimated cells replacing only ~15% of their plastids, while *T. amphioxeia*-acclimated cells replaced ~35%. In the mixed-prey treatment, these differences could be explained by competitive interactions between the prey, which appear to favor *T. acuta* (see PD Expt 3).

Prey growth responses and evidence for competitive interactions from *Mesodinium rubrum*-free controls

During the functional response experiments, *T. amphioxeia* (μ_{max} = 0.72 ± 0.11 d^{-1}) had significantly higher growth rates (ANOVA, $p < 0.01$) than *T. acuta* (μ_{max} = 0.26 ± 0.05 d^{-1}) within the predator-free controls (Table 2). However, we found no difference in the growth rates of the 2 *Teleaulax* species (*T. amphioxeia*: μ = 0.73 d^{-1} and *T. acuta*: μ = 0.77 d^{-1}; ANOVA, $p > 0.05$) within our no-predator controls during the PD experiments. The effect of grazing on *Teleaulax* abundance was clear: the prey populations grew fast without *M. rubrum*, but remained at a constant level with *M. rubrum* (ANOVA, $p < 0.001$; Fig. S3 in the Supplement). Interestingly, the 2 *Teleaulax* species revealed evidence for competitive interactions during co-culture in PD Expt 3. The growth rates of *T. amphioxeia* and *T. acuta* were similar when grown separately, as were the combined prey growth rates in all mixed-prey treatments when they were grown without *M. rubrum* (Table 4). However, qPCR analysis of no-grazer controls revealed that the relative proportions of these prey species changed during co-culture, with *T. acuta* taking over in all mixed treatments within 4 d.

DISCUSSION

A preference for *Teleaulax*

Both field and laboratory studies on *Mesodinium rubrum* suggest that cryptophytes from the *Teleaulax/Plagioselmis/Geminigera* (TPG) clade support higher growth and are compatible for plastid sequestration (Yih et al. 2004, Johnson et al. 2006, Park et al. 2007, Nishitani et al. 2010, Herfort et al. 2011, Myung et al. 2011, Hansen et al. 2012, Rial et al. 2015). In temperate regions, both natural populations and cultured isolates of *M. rubrum* are almost always associated with *T. amphioxeia*-like plastids/prey (Nishitani et al. 2010, Herfort et al. 2011, Garcia-Cuetos et al. 2012, Riobó et al. 2013). In this study, we assessed the grazing functional response, growth, and plastid sequestration dynamics of *M. rubrum* when fed single or mixed populations of cryptophytes. Using species-specific qPCR assays for a plastid-encoded gene, we determined that the temperate *M. rubrum* strain CBJR05 did not ingest *Storeatula major*, but consumed both *Teleaulax* species and retained their plastids with essentially equal efficiency, with no short-term (1–2 d) difference in growth rates. The growth rates of our *M. rubrum* were similar to the growth rates of the other temperate *M. rubrum* strains (Park et al. 2007, Hansen et al. 2012). However, over longer periods (5–6 d), growth of *M. rubrum* on *T. amphioxeia* was higher than on *T. acuta* in one of our experiments.

It is reported that *M. rubrum* cells may simultaneously contain chloroplasts from different cryptophyte species (Nishitani et al. 2010, Myung et al. 2011, Hansen et al. 2012). With our quantitative analysis, we confirmed that *M. rubrum* can indeed have mixed plastids, and, if prey species are suitable, the retained plastid ratio reflects the proportions of the given prey. This suggests that the field observations of *M. rubrum* containing mainly *T. amphioxeia* plastids (Nishitani et al. 2010, Herfort et al. 2011) is perhaps due to this prey species being more abundant than other compatible TPG cryptophytes. Our results are also consistent with *Teleaulax* spp. having unique phenotypic traits compared to other cryptophyte taxa, e.g. *S. major*, that make them more attractive to *M. rubrum* as prey. Even if *M. rubrum* were ingesting but not retaining *S. major* plastids, we would expect to see some signal from qPCR of washed ciliates after a brief exposure period. The absence of both a qPCR signal as well as any detectable depression in *S. major* growth rate strongly suggests that *M. rubrum* did not ingest them. In addition to chloroplasts, *M. rubrum* also retains prey nuclei (kleptokaryon), which are transcriptionally active and apparently control photosynthesis in the ciliate (Johnson et al. 2007, Lasek-Nesselquist et al. 2015). While we did not study the role of the kleptokaryon here, our results imply that in certain circumstances, *M. rubrum* may simultaneously possess nuclei of multiple *Teleaulax* species as well as mismatched nuclei-plastid combinations. Since this likely occurred in our study with no apparent effect on growth rate of the ciliate, it raises intriguing questions regarding the molecular control, coordination and interaction of mixed foreign organelles in *M. rubrum*.

A role for *Mesodinium rubrum* in shaping cryptophyte populations

Like other *M. rubrum* strains (Gustafson et al. 2000), our culture began to feed immediately after their introduction to optimal cryptophyte prey; however, ingestion rates were higher than those reported previously (Yih et al. 2004, Hansen & Fenchel 2006, Smith & Hansen 2007). Factors explaining differences in reported *M. rubrum* ingestion rates are unclear; however, strain/variant type of both the ciliate and prey as well as feeding history of the ciliates likely play a role. Our results, together with results from these previous studies, suggest that *M. rubrum* may exert substantial grazing pressure (5–12 cryptophytes *M. rubrum*$^{-1}$ d^{-1}) on natural populations of *Teleaulax* cryptophytes, and suggest that when they encounter high concentrations are able to quickly ingest and assimilate prey organelles, fueling their population growth. In natural populations of *M. rubrum* within the Columbia River Estuary, North Pacific coast, USA, a novel mechanism of apparent rapid prey uptake has been observed using FlowCAM analysis of natural samples (Peterson et al. 2013). While this result was not likely a factor, these observations suggest that certain variants of the *M. major/rubrum* species complex may achieve even higher ingestion rates when cryptophyte prey are abundant. Our results also imply that grazing pressure exerted by *M. rubrum* may play a role in shaping cryptophyte diversity. The apparent prevalence of *T. amphioxeia* both in *M. rubrum* cells (Nishitani et al. 2010, Herfort et al. 2011) and in natural communities (Johnson et al. 2016) suggests that *T. acuta* may be a poor competitor in nature. However, our qPCR results of predator-free mixed *Teleaulax* prey controls suggests the opposite, with *T. acuta* becoming dominant within 4 d. Reconciliation of these observa-

tions may be found in the growth rates of *T. amphioxeia* and *T. acuta*, which, averaged across all experiments, were 0.61 and 0.38 d^{-1}, respectively. The reason for lower growth rates in *T. acuta* during functional response experiments is unclear, and the higher growth rates measured during the PD experiment have not been repeated. Taken together, differences in *Teleaulax* growth rates, combined with extremely high grazing rates on both *Teleaulax* spp. by *M. rubrum*, may help to explain the apparent dominance of *T. amphioxeia* in nature (Johnson et al. 2016). This difference in population survival was manifested during our grazing functional response experiments to prey concentrations, where the grazing constant (g) averaged across all prey:predator ratios was greater on *T. acuta* than on *T. amphioxeia*. However, numerous other factors could explain patterns of cryptophyte genetic diversity in natural ecosystems, and additional experiments on multiple strains of each *Teleaulax* sp. would be needed in order to test this conclusion.

Comparisons of CBJR05 with other strains

Our *M. rubrum* strain had on average 8–14 plastids $cell^{-1}$ when recently fed, which is consistent with other studies (Hansen & Fenchel 2006, Johnson et al. 2006, 2007, Garcia-Cuetos et al. 2012), and it took 2 wk to replace all sequestered plastids from one *Teleaulax* species to another. This time frame contrasts with the observations of Hansen et al. (2012), whose Danish *M. rubrum* strain needed a much longer time (i.e. >35 d) for full plastid turnover when switching from *T. amphioxeia* to *T. acuta*. The slower plastid turnover time for the Danish *M. rubrum* strain was most likely due to the much lower concentrations of prey used in that study (prey:predator ratio of 1), which likely resulted in a greater role of plastid division during the transition of *T. amphioxeia* to *T. acuta* plastids (Hansen et al. 2012). Our results demonstrate for the first time that at least one *M. rubrum* variant is capable of quickly turning over its plastids with those from another *Teleaulax* spp. by ingesting large amounts of prey and apparently sequestering their plastids, rather than de novo organelle division. These results suggest that the *M. rubrum* clade G (Johnson et al. 2016) strain used here may have less control over sequestered organelles compared to other strains. Further research is needed to directly compare plastid uptake, replacement, and de novo division of sequestered organelles among different *M. rubrum* clades in order to conclude how these processes differ within the group. One potential issue could be how well *M. rubrum* exploits organelles from different strains within *T. amphioxeia* as well as other species. Previous studies have shown that *M. rubrum* has variable growth rates when fed different *T. amphioxeia* strains (Park et al. 2007) and *Teleaulax* spp. (Rial et al. 2015).

Another important difference between the present study and Hansen et al. (2012) is the methodological approach for quantifying plastids. While we used qPCR to enumerate plastid types, Hansen et al. (2012) used PCR and cloning. When dealing with mixed templates, the use of qPCR with species-specific primers is less prone to biases that may affect traditional PCR, such as variations in template concentration, primer annealing efficiency due to base pair composition, and amplification efficiency due to amplicon size. The choice of gene to target for PCR and cloning is also important. While divergent gene sequences may provide a strong phylogenetic signal, they are also likely to possess sequence variations that mediate PCR bias. These concerns are particularly valid here, since Hansen et al. (2012) targeted the plastid nucleomorph SSU rRNA genes in cryptophytes, which are highly divergent and often possess unalignable regions (Hoef-Emden et al. 2002). While this result was unlikely a factor when discerning between the closely related *Teleaulax* sequences, it may have played a role with some of their negative results with non-TPG cryptophytes. However, their conclusions are also supported by transmission electron micrographs of *M. rubrum* fed non-TPG cryptophytes, which suggest that the ciliate digests these prey within food vacuoles rather than sequestering their organelles (Hansen et al. 2012). In contrast, a Korean *M. rubrum* strain (MR-MAL01) has been reported to ingest non-TPG clade cryptophytes and sequesters their plastids (Park et al. 2007, Myung et al. 2011). While we found no evidence of our *M. rubrum* strain ingesting *S. major*, this was the only non-*Teleaulax* cryptophyte that we tested in this study. While certain TPG cryptophytes clearly support optimal growth in *M. rubrum* and appear to be selected over other groups, more research is needed to determine if they can sequester and physiologically exploit organelles from other cryptophyte groups.

A potential role for grazing in bloom formation

It is suggested that *M. rubrum* bloom formation depends on the availability of suitable cryptophyte

prey (Herfort et al. 2011). High concentrations of cryptophyte algae have been shown to co-occur with growing *M. rubrum* populations in the Columbia River Estuary, and decline as the bloom reaches its peak levels (Herfort et al. 2012). In Chesapeake Bay, high concentrations of cryptophyte algae were observed to occur within a tributary preceding localized blooms of *M. rubrum*, and decline when concentrations of the ciliate increase (Johnson et al. 2013). In coastal Korean waters, peaks in *M. rubrum* abundance appear to be positively correlated with that of cryptophytes (Kim et al. 2007, Yih et al. 2013). Our experiments demonstrate that *M. rubrum* is extremely effective in controlling *Teleaulax* prey populations, with grazing coefficients exceeding 1 d^{-1} at prey:predator ratios as high as 10:1. However, previous studies have also noted that certain *M. rubrum* strains are extremely efficient at exploiting foreign organelles by regulating and dividing them (Johnson & Stoecker 2005, Johnson et al. 2007, Moeller et al. 2011), and that ingestion rates as low as 1 cryptophyte *M. rubrum*$^{-1}$ d^{-1} are sufficient for supporting maximum growth (Yih et al. 2004, Smith & Hansen 2007). The latter 2 research groups have also reported the percent carbon contributed from ingested prey (CCP) in *M. rubrum* cultures. A culture from Korean waters (clade B) was found to have a CCP of 0.06–5.54 (Yih et al. 2004), while a strain from coastal Denmark (clade F) was found to have values between 0.5–22 (Smith & Hansen 2007). Our calculations using the same caveats discussed in Smith & Hansen (2007) were 6–32% for both *Teleaulax* species. While these results imply different physiological dependencies upon carbon through phagotrophic mixotrophy, we feel that such estimations in *M. rubrum* are inappropriate, given that much of the ingested carbon is retained as intact organelles. Rather, we interpret these differences as various clades of *M. rubrum* perhaps having different capacities for dividing cryptophyte organelles and therefore possessing different requirements for acquiring them (i.e. feeding rates). Further work is needed with different strains of *M. rubrum* and *Teleaulax* prey in order to better understand these dynamics.

Taken together, these results provide an emerging view that *M. rubrum* blooms may be fueled by 'luxury' uptake of cryptophyte organelles when optimal prey (e.g. *Teleaulax* spp.) are abundant, coupled with their highly adapted and extremely efficient mode of acquired phototrophy. Further studies that directly compare the functional response and physiological performance of *M. rubrum* variants to different cryptophyte prey species and concentrations are needed in order to better understand and contextualize these results. Clearly, the nature of this relationship is also sensitive to both prey species and ingestion rate, and suggests that when optimal prey are available, high ingestion rates in *M. rubrum* are a key factor in their ecological success.

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