1 **Physiological Responses of *Mesodinium major* to Irradiance, Prey Concentration and Prey**
2 **Starvation**

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16 **ABSTRACT**
17 Ciliates within the *Mesodinium rubrum/M. major* species complex harbour chloroplasts and other
18 cell organelles from specific cryptophyte species. *M. major* was recently described, and new studies
19 indicate that blooms of *M. major* are just as common as blooms of *M. rubrum*. Despite this, the
20 physiology of *M. major* has never been studied and compared to *M. rubrum*. In this study, growth,
21 food uptake, chlorophyll *a* and photosynthesis were measured at six different irradiances, when fed
22 the cryptophyte, *Teleaulax amphioxeia*. The results show that the light compensation point for
23 growth of *M. major* was significantly higher than for *M. rubrum*. Inorganic carbon uptake via
24 photosynthesis contributed by far most of total carbon uptake at most irradiances, similar to *M.*
25 *rubrum*. *M. major* cells contain ~four times as many chloroplast as *M. rubrum* leading to up to
26 ~four times higher rates of photosynthesis. The responses of *M. major* to prey starvation and
27 refeeding were also studied. *M. major* was well adapted to prey starvation, and 51 days without
28 prey did not lead to mortality. *M. major* quickly recovered from prey starvation when re-fed, due to
29 high ingestion rates of >150 prey predator^{-1} day^{-1}.

30 **Keywords**: Light response; growth; C-fixation; Chl. *a*; sequestered chloroplasts; sequestered
31 nucleus; *Teleaulax*

32 The ciliate genus *Mesodinium* is ubiquitous in marine and freshwaters. In marine waters, six
33 *Mesodinium* species have been formally described (Garcia-Cuetos et al. 2012; Nam et al. 2015;
34 Moestrup et al. 2012). The genus is a physiologically diverse group consisting of both heterotrophs
35 (*M. pupula and M. pulex*) and mixotrophs (*M. chamaeleon and M. coatsi*, *M. major and M. rubrum*)
36 (Smith and Hansen 2007; Garcia-Cuetos et al. 2012; Kim et al. 2019; Tarangkoon and Hansen
37 2011). Recent research using molecular techniques suggests quite a few additional species may be
38 present within this *M. rubrum*/*M. major* species complex (Kim and Park 2019; Johnson and
39 Beaudoin 2019; Johnson et al. 2016; Herfort et al. 2011).

40 The *M. rubrum/major* species complex has received the most attention, because they form non-
41 toxic “red tide” blooms worldwide (Herfort et al. 2011; Packard et al. 1978; Lindholm 1985). The
42 blooms were attributed to *M. rubrum* in older records, but recent papers have suggested that many
43 of these red *Mesodinium* blooms are due to *M. major* (Herfort et al. 2017; Johnson et al. 2016; Yang
44 et al. 2015). In fact, one paper found *M. major* to be the most widely encountered *Mesodinium*
45 species in red tides (Johnson et al. 2016). Despite this, almost all laboratory studies on the red
46 *Mesodinium* spp. have been done on *M. rubrum*, probably due to failed attempts to culture *M. major*
47 (Garcia-Cuetos et al. 2012; Rial et al. 2015). The red *Mesodinium* spp. have also received
48 considerable interest because they serve as prey for the toxic dinoflagellates, *Dinophysis* spp. (Park
49 et al. 2006). *Dinophysis* spp. produce diarrheic shellfish toxins (DST) that may damage the
50 aquaculture industry due to the accumulation DST in mussels (Reguera et al. 2012).

51 Species within the *M. rubrum/M. major* complex differ from the other mixotrophic *Mesodinium*
52 spp. in their association with the ingested cryptophytes. These red *Mesodinium* spp. seem to
53 specifically utilize cryptophytes within the *Teleaulax*/*Plagioselmis*/*Geminigera* clade (Park et al.
54 2007; Peltomaa and Johnson 2017), while the other mixotrophic species, *M. coatsi* and *M.*
55 *chamaeleon*, are more flexible, and can utilize a wide range of cryptophyte species (Moeller and
56 Johnson 2018; Kim et al. 2019). *M. rubrum* and *M. major* also differ from the other mixotrophic
57 *Mesodinium* species in that they separate the prey nuclei from the rest of the ingested cell. The
58 cryptophyte chloroplasts, nucleomorph, mitochondria, ribosomes, and cytoplasm are kept together
59 as an entity (Johnson et al. 2006; Hansen et al. 2012; Kim et al. 2017; Kim et al. 2019; Nam et al.
60 2016). One of the ingested prey nuclei is made the “master”, often referred to as the “symbiont
61 nucleus” or “the centered prey nucleus”. This nucleus is transported into close proximity of the

62 ciliate nuclei (two macronuclei and a single micronucleus) and enlarged (Nam et al. 2016; Johnson
63 et al. 2007; Kim et al. 2017). Often some extra cryptophyte nuclei can be found in the periphery of
64 the *Mesodinium* cell.

65 Detailed studies of the physiology of the red *Mesodinium* species have been restricted to *M. rubrum*
66 strains from Europe (clade F), Asia (clade B) and Antarctica (clade A) (Moeller et al. 2011; Johnson
67 et al. 2016). The physiology of *M. major* and other mixotrophic members of this species complex
68 remains little explored (Moeller and Johnson 2018; Kim et al. 2019). Results from the *M. rubrum*
69 strains indicate that they acquire most of their carbon via photosynthesis, and that up to ~ 98% of
70 the carbon need in this species can be covered by photosynthesis at low prey concentrations, which
71 is enough to support high growth rates (Mitra et al. 2016; Smith and Hansen 2007). It has also been
72 shown that *M. rubrum* is able to photoacclimate, thereby allowing them to exploit low light
73 environments. Finally, it has been shown that *M. rubrum* can survive extended periods of prey
74 starvation (Smith and Hansen 2007; Johnson and Stoecker 2005)

75 Our aim was to explore the ecophysiology of *M. major*. We maintained a culture of *M. major* for >
76 two years using *Teleaulax amphioxeia* as prey and investigated: (i) The effects of different
77 irradiances on growth photosynthetic activity, cellular chl *a* and ingestion rates of *M. major*, (ii) The
78 effects of prey deprivation on the loss of the centered prey nucleus. (iii) The effects of refeeding and
79 recovery after prey starvation. These experiments allowed us to study the physiological similarities
80 and differences between *M. rubrum* and *M. major*. Our results indicate some similarities, but also
81 some differences that suggest a necessity to differentiate them with regard to physiological
82 performance.

83 **METHOD AND MATERIALS**

84 *Isolation and cultures*

85 Single cells of *Mesodinium major* (MM-DK2016) were isolated from Helsingør harbor, Denmark,
86 in 2016, using a drawn Pasteur pipette and transferred three times through 6-well multidishes
87 containing sterile filtered seawater from the location to remove all other protists. Dilute
88 concentrations of the cryptophyte, *Teleaulax amphioxeia* (SCCAP K-0434) cells were supplied as
89 prey. After some months the cultures were scaled up to blue cap glass bottles (0.5 liter, VWR,
90 Radnor, PA, USA), containing 100 ml f/4 medium based on heat-treated seawater (95 °C, 90 min)
91 with a salinity of 15. *M. major* was transferred weekly to new medium and supplied *T. amphioxeia*
92 as prey at a predator:prey ratio of ~1:5. Both species were grown at 15 °C in a temperature
93 regulated room, under a photon irradiance of 70 µmol photons m^2 s^1 (PAR, 400–700 nm), and on a
94 light:dark cycle of 16:8. Irradiance was measured at the level of incubation flasks using a light
95 meter equipped with a spherical quantum sensor (ULM and US-SQS/L, Walz GmbH, Effeltrich,
96 Germany) and pH was followed using a SenTix®41 pH electrode (WTW, Weilheim, Germany)
97 connected to a pH meter (pH 3210, WTW, Weilheim, Germany) and calibrated with pH 7 and 10
98 standard buffers. 28S and 18S gene sequences were used to confirm identification of *M. major*
99 (sequences were obtained and analyzed as described in the Supporting information).

100 **Experiment 1. Effect of irradiance on cellular chlorophyll *a*, photosynthesis and growth rate**

101 *M. major* was exposed to six different irradiances (25, 38, 50, 75, 100 and 200 µmol photons m^{-2} s^{-}
102 ^{1}) at a prey concentration of 500 cells ml^{-1}, and a predator:prey ratio of ~1:15 in 0.5 L blue cap
103 flasks containing 300 ml f/4 medium. Subsamples for enumeration of *M. major* and *T. amphioxeia*
104 were withdrawn every second day for two weeks. Experiments with monocultures of *T. amphioxeia*
105 were also carried out, allowing the calculation of ingestion rates (see below). After ~three cell
106 divisions (most often after 7 days of incubation) samples for photosynthetic activity and chlorophyll

107 *a* (Chl *a*) were withdrawn. Refeeding and dilution of the cultures were carried out when
108 prey:predator ratio was around 1:1 and cultures exceeded 1,000 cells ml^{-1} of *M. major*, respectively.

109 **Experiment 2: Effect of prey depletion and starvation**
110 Experiment 2 was initiated with the cultures from experiment 1 grown at an irradiance of 100 µmol
111 photons m^2 s^1. The triplicates from experiment 1 were mixed in one bottle, diluted and transferred
112 into four new bottles, functioning as replicates. Cell numbers, photosynthetic activity, Chl *a,* and
113 number of centered prey nuclei (CPN) were monitored for 51 days. The cultures were diluted when
114 densities exceeded 1,000 cells ml^{-1} with fresh f/4 medium to avoid complications with elevated pH.

115 **Experiment 3: Effects of refeeding**
116 Experiment 3 was carried out directly following experiment 2. Subsamples from experiment 2 were
117 withdrawn after 22 days of starvation from each replicate flasks and mixed in a single bottle.
118 Subsequently, this suspension was divided into two different treatments using *Teleaulax*
119 *amphioxeia* as prey. *Treatment* 1 and 2 were initiated using a prey to predator ratio of 30:1, and
120 100:1, respectively. Each treatment was further subdivided into three new replicate bottles.
121 Measurements of photosynthetic activity, Chl *a*, number of CPNs, and cell number were made for
122 10 days. The setup was accompanied with monocultures of *T. amphioxeia*, allowing the calculation
123 of ingestion rates (see below). Dilutions of the cultures were done when densities exceeded 1,000
124 cells ml^{-1}.

125 *Cell Abundance and Growth Rate*
126 Aliquots (2 ml) withdrawn from each flask were fixed with acid Lugol's solution (final
127 concentration 1%). Abundances of *M. major* and *T. amphioxeia* were enumerated using a
128 Sedgewick-Rafter chamber under an inverted microscope (Olympus CK40, Olympus, Center
129 Valley, PA, USA) at 100X and 200X. At least 200 cells were enumerated each time. Growth rates
130 were calculated during the exponential portion of the growth phase using the following exponential
131 growth equation: $\mu = \ln(N_2/N_1)/(t_2 - t_1)$, where N_1 and N_2 are cell concentrations at time 1 and time
132 2, respectively.

133 *Ingestion rate*
134 The ingestion rate of *M. major* was calculated from the reduction in prey concentrations over 48-72
135 h periods as compared with prey control cultures according to (Jakobsen and Hansen 1997). This
136 method assumes that the prey grows exponentially at the same rate as in predator-free prey controls.
137 Ingestion rate (U) was calculated for each sample point using following equation: If $\mu_x \neq \mu_y$, then
138 $U = (\mu_y - \mu_x)(X_0 - X_T e^{\mu_x T})/Y_0(e^{\mu_x T} - e^{\mu_y T})$, where prey ($X$) are ingested by grazer (Y), μ_y are
139 exponential growth of *M. major*, μ_x are exponential growth of *T. amphioxeia* without predator, X_0
140 and X_T are cell concentrations at time 0 and time T, respectively.

141 *Photosynthetic Activity (^{14}C)*
142 Two 2-ml aliquots were withdrawn from each flask, transferred to each of two 23-ml glass
143 scintillation vials. Twenty microliter of $NaH^{14}CO_3^-$ stock solution (specific activity 100 µCi ml^{-1})
144 was added to each vial. One vial of each pair was incubated for 3 h in the same place as the
145 experimental flask, and the other vial was kept in complete darkness by wrapping in aluminum foil.
146 After incubation, a 100 ml sub-sample was withdrawn from each vial and added to a new vial
147 containing 200 ml phenylethylamine for measurements of specific activity (Skovgaard et al. 2000).
148 The remaining 1.9 ml was acidified with 2 ml 10% glacial acetic acid in methanol, and evaporated
149 overnight at 60 °C to remove all inorganic carbon. The residue in the vial was re-dissolved in 2 ml
150 Milli-Q water before adding 10 ml of scintillation cocktail (Insta-Gel Plus, PerkinElmer Waltham,
151 Massachusetts, USA). All vials were analyzed using a liquid scintillation counter (Tri-Carb 2910

152 TR, PerkinElmer Waltham, MA, USA). Rates of photosynthetic activity PA (µgC × ml^{-1} × h^{-1}) were
153 calculated from the equation: PA $= (DPM \times IC)/(^{14}C_a \times h \times N_t)$, where *DMP* is disintegrations
154 min^{-1} ml^{-1}, *IC* is the concentration of inorganic carbon (µgC × ml^{-1}), $^{14}C_a$ is the specific activity in
155 disintegrations min^{-1} ml^{-1}, h is the incubation time in hours and N_t is the total number of cells in the
156 vail. *IC* was measured using a Shimadzu Total Organic Carbon (TOC) analyzer (Shimadzu, Kyoto,
157 Japan).

158 *Chlorophyll a measurements*
159 2 ml subsample was filtered onto a 24 mm GF/F (Whatman Sigma-Aldrich, Maidstone, GB) glass
160 fiber filter, which was subsequently transferred to 5 ml ethanol (96%) in 23-ml glass scintillation
161 vials. The vials were wrapped with tinfoil and left overnight in the refrigerator at 4 °C. Aliquots
162 were transferred to 2 ml glass vials and measured on a Turner ® Trilogy Fluorometer (Turner
163 designs, San Jose, CA, USA) using non-acidification method. Throughout all handling of these
164 samples extraction and measurements, light was eliminated.

165 *Enumeration of centered prey nuclei and imaging of Mesodinium major*
166 Prey nuclei and chloroplasts were stained using the fluorescent nuclear stain Hoechst 33258
167 (Invitrogen, Carlsbad, CA, USA) and plasma membrane stain using CellMask Green (Life
168 technologies, Carlsbad, CA, USA). Two ml of culture were fixed in 4% glutaraldehyde in filtered
169 seawater and stored cold (4 °C). The samples were stained with a mix of 25 mg ml^{-1} Hoechst and
170 0.25 mg ml^{-1} Cell mask, and left for 15 min, before collected on a 0.2 µm black polycarbonate
171 membrane filter (Frisenette, Knebel, Denmark) using filtration. A single drop of immersion oil was
172 added to a microscope slide and the filter was placed on top of this. Another drop of immersion oil
173 was added to the top of the filter, before a cover slip was added. The slides were kept at 4 °C in the
174 dark, before analysis. Epifluorescence micrographs of stained *M. major* cells were taken at 1,000X
175 magnification using a digital camera coupled to an Olympus BX51 microscope equipped with
176 differential interference contrast. 3D images were generated using IMARIS software program
177 (Bitplane, Zürich, Switzerland) to assess the number of chloroplasts of *M. major*.

178 **Statistical analyses**
179 Rates of photosynthetic activity (P) and growth rate (µ) of *M. major* as functions of irradiance and
180 the effect of irradiance (T) were fitted to the Michelis-Menten kinetics using the software GraphPad
181 Prism 9.0.1: $P(d^{-1}) = P_{max}(x)/(K_m + (x))$, where P_{max} ís the maximal cellular rate of
182 photosynthesis in *M. major* (pg C $cell^{-1}$ h^{-1}), x is the irradiance (µmol photons m^{-2} s^{-1}) and K_m is
183 irradiance sustaining ½ P_{max}, and: $\mu(d^{-1}) = \mu_{max}(x - x_0)/(K_m + (x - x_0))$, where μ_{max} is the
184 maximal growth rate of *M. major*, x is the irradiance (µmol photons m^{-2} s^{-1}), x_0 is the threshold
185 irradiance for growth (where $\mu_y = 0$) and K_m is irradiance sustaining ½ μ_{max}, and: $\mu(d^{-1}) =$
186 $\mu_{max}(p - p_0)/(K_m + (p - p_0))$, where μ_{max} is the maximal growth rate of *M. major*, p is the
187 photosynthetic activity (pg C $cell^{-1}$ h^{-1}), p_0 is the threshold photosynthetic activity for growth (where
188 $\mu_y = 0$) and K_m is irradiance sustaining ½ μ_{max}.

189 Rates of declining photosynthetic activity and numbers of centered prey nucleus of *M. major* as a
190 function of prey deprivation were fitted to One phase decay kinetics using the software GraphPad
191 Prism 9.0.1: $Y = (Y_0 - Plateau) \times (\exp(-K \times x) + Plateau)$, where Y_0 is the maximal
192 photosynthetic activity or numbers of centered prey nucleus, x is the time (d^{-1}) and K is the time
193 sustaining ½ Y_0.

194 **RESULTS**

195 **Microscopic observations**

196 Large variations in size and number of chloroplasts were observed in *Mesodinium major* (Fig. 1A-C
197 and F) under the epifluorescence microscope. Cells ranged from 30-80 µm in length, and the
198 chloroplasts were mainly located in the periphery of the cell (Fig. 1B and C). Attempts were made
199 to enumerate the chloroplasts using confocal microscopy together with the Software "Imaris".
200 However, the large size of cells, and the large number of chloroplasts retained, made it difficult to
201 observe the chloroplasts because the chloroplasts on the top of the preparation shadowed the
202 chloroplasts located on the other side of the cell (Fig. 1A). Nevertheless, assuming that the
203 chloroplasts are equally distributed in the cell, estimated number of chloroplasts ranged from 40-80
204 chloroplasts. Well-fed cells of the ciliate had two macronuclei, one micronucleus and a centered
205 prey nucleus (CPN) (Fig. 1D and E). Extra prey nuclei were rarely observed.

206 **Experiment 1. Effect of irradiance on the physiology of *Mesodinium major***

207 The growth rates of *Mesodinium major* in well-fed cultures were highly affected by irradiance and
208 the growth rates as a function of irradiance fitted Michaelis-Menten kinetics very well ($R^2 = 0.84$;
209 Fig. 2A). Maximum growth rates of 0.39 d^{-1} were achieved > 75 µmol photons m^{-2} s^{-1}. At lower
210 irradiances the growth was reduced, and a growth rate of only 0.07 d^{-1} was achieved at an irradiance
211 of 25 µmol photons m^{-2} s^{-1}. Photosynthetic activity also increased as a function of irradiance from
212 79.2 pg C $cell^{-1}$ h^{-1} at 25 µmol photons m^{-2} s^{-1} to 323 pg C $cell^{-1}$ h^{-1} at an irradiance of 200 µmol
213 photons m^{-2} s^{-1}. The data also fitted Michaelis-Menten kinetics well ($R^2 = 0.76$, Fig. 2B). The
214 photosynthetic activity of the prey, *Teleaulax amphioxeia* could not be fitted to Michaelis-Menten
215 kinetics, because it kept increasing with irradiance in the investigated range (Fig. S1).

216 The cellular Chl *a* concentration of ~75 pg $cell^{-1}$ was found to be the same within irradiances of
217 35-200 µmol photons m^{-2} s^{-1}. However, at an irradiance of 25 µmol photons m^{-2} s^{-1} the cellular Chl
218 *a* was significantly higher, 135 pg $cell^{-1}$ ($p < 0.005$) (Fig. 2C). *M. major* ingested *T. amphioxeia* at a
219 rate of 9-15 prey $predator^{-1}$ d^{-1}, and ingestion rates were not affected by irradiance in the range of
220 25-200 µmol photons m^{-2} s^{-1} (Fig. 2D). Growth rates as a function of photosynthetic activity could
221 be fitted to Michaelis-Menten kinetics ($R^2 = 0.90$, Fig. 3A). The relationship indicated that
222 maintenance requirements were ~70 pg C $cell^{-1}$ h^{-1}, which is equivalent 25-33 % of photosynthesis
223 at its maximum growth rate (Fig. 3A). Comparison of the carbon uptake via ingestion and
224 photosynthesis of *M. major* indicates that the primary carbon source in *M. major* exclusively comes
225 from photosynthesis at irradiances ≥ 50 µmol photons m^{-2} s^{-1} (Fig. 3B). At lower light levels (< 50
226 or 25, 38 µmol photons m^{-2} s^{-1}) the contribution of carbon from ingestion was half of the amount of
227 photosynthetic carbon fixation.

228 The Chl *a*-specific photosynthetic capacity (pg C pg Chl a^{-1} h^{-1}) of *M. major* was similar to that of
229 *Teleaulax amphioxeia* (Fig. 4A) at irradiances of ≤100 µmol photons m^{-2} s^{-1}, but higher at
230 irradiances above 100 µmol photons m^{-2} s^{-1}. If the number of chloroplasts inside *M. major* are
231 estimated using measured photosynthetic activity and Chl *a* for *M. major*, and scaled to values for a
232 *T. amphioxeia* cell (Fig. 4B), we would then estimate that they harbour between 200-350
233 chloroplasts per cell. This was, however, not supported from the direct observations, where
234 numbers were in the range of 40-80 chloroplasts.

235 **Experiment 2: Effect of prey depletion/starvation on *Mesodinium major***

236 *Mesodinium major* divided 5 times during 51 days of prey deprivation (Fig. 5A). Cell divisions
237 occurred mostly in the beginning of starvation period (16 days) and only one division was recorded
238 during the remaining period (last 40 days of the experiment). During the first 10 days, cells with a
239 central prey nuclei (CPNs) fell from 50 % to 15% and this number remained stable at 10% after day

240 17 (Fig. 5B). Similarly, the photosynthetic activity fell from 119 pg C cell^{-1} h^{-1}, during the first 10
241 days and leveled out at ~70 pg C cell^{-1} h^{-1} during the remaining experimental period (Fig. 5C).
242 Levels of cellular Chl *a* were in the range of 60-90 pg Chl *a* cell^{-1}, and no significant relationship
243 with time was observed (Fig. 5D). This resulted in an increase Chl *a*-specific photosynthetic
244 capacity for the first two measurements (Day 0 and 1), and hereafter an unchanged capacity during
245 the rest of the experimental period (Fig. 6A). An exponential relationship between photosynthetic
246 activity and numbers of cells with CPNs (Fig. 6B) was found and the data could be fitted to
247 exponential function (R^2 = 0,784).

248 **Experiment 3: Refeeding of *Mesodinium major***
249 After 22 days of prey deprivation *M. major* was fed two different predator:prey ratios, 1:30 and
250 1:100, respectively, and cell abundances of both *M. major* and *Teleaulax amphioxeia* were then
251 monitored over 10 days (Fig. 7A). *T. amphioxeia* was completely depleted in both treatments at day
252 8, and *M. major* divided 5 times in both treatments during the 10 days of observation. Ingestion
253 rates for treatment 1:30 varied ingestion rates increased from initially ~20-25 preys predator^{-1} d^{-1}
254 during the first 3 days of incubation, but as the prey got depleted, these rates decreased, and at day 8
255 no prey cells were left (Fig. 7B). Initial ingestion rates were low (not measurable) in the 1:100
256 treatment. However, the following days, ingestion rates increased to reach 85 and 167 preys *M.*
257 *major*$^{-1}$ d^{-1}. No differences in photosynthetic activity, Chl *a*, or percentage of cells with CPNs were
258 found between the two treatments (Fig. 8A, B and C). The photosynthetic activity and cellular Chl *a*
259 increased from 50 to 200 pg C cell^{-1} h^{-1} and 60 to 120 pg Chl *a* cell^{-1}, respectively, from day 0 to
260 day 8, in both treatments. The percentage of cells with CPNs increased over the first four days, as
261 long as prey was available; thereafter the number of CPNs decreased (Fig. 8C).

262 **DISCUSSION**
263 **The ecophysiology of *Mesodinium major***
264 *Dependence on light for growth*
265 The sustained growth of *Mesodinium major* depended on irradiance. At 25 µmol photons m^{-2} s^{-1} *M.*
266 *major* was barely able to sustain itself with a growth rate of 0.07 d^{-1}, even when offered prey in
267 excess. Maximum growth rate of ~ 0.35 d^{-1} was achieved at irradiances > 75 µmol photons m^{-2} s^{-1} at
268 15°C. The data fitted very well to Michaelis-Menten kinetics (r^2 = 0.86; Fig. 2A). This strain of *M.*
269 *major* was not able to grow in complete darkness. Only few data are available on the light responses
270 of other planktonic *Mesodinium* spp., which utilize cryptophytes within the *Teleaulax*/*Geminigera*
271 clade as prey and donor of cell organelles. A temperate strain of *M. rubrum* (clade F) had a
272 maximum growth rate of 0.23 and 0.49 d^{-1} at irradiances of 20 and 100 µmol photons m^{-2} s^{-1},
273 respectively (see Table 1 for a comparison of *M. major* and *M. rubrum*) (Smith and Hansen 2007).
274 An Antarctic strain of *M. rubrum* (clade A) was able to grow at irradiances as low as ~2.5 µmol
275 photons m^{-2} s^{-1} with a growth rate of ~ 0.11 d^{-1} at a temperature of 4°C (Johnson and Stoecker
276 2005). None of the investigated *M. rubrum* strains could sustain growth in complete darkness. Thus,
277 even though both the investigated *M. major* and *M. rubrum* strains required light for growth, the
278 requirements for light was higher for this isolate of *M. major* compared to the closely related *M.*
279 *rubrum* strains studied. The light compensation point (interception with x-axis) for growth was
280 found to be 21.5 µmol photons m^{-2} s^{-1} for *M. major* (Fig. 2A). For the Arctic strain of *M. rubrum*
281 (clade A) the light compensation point was found to be as low as 0.5 µmol photons m^{-2} s^{-1} (Table 1;
282 (Moeller et al. 2011).

283 Data on light responses are also available for other *Mesodinium* spp., like the benthic heterotrophic
284 *M. pulex* and the mixotrophic *M. coatsi* and *M. chamaeleon*. The heterotrophic *M. pulex* has been
285 shown to grow in both light (Irradiance of 100 µmol photons m^{-2} s^{-1}) and in complete darkness at

286 growth rates of 1.41 d^{-1} and 1.19 d^{-1}, respectively, when supplied food in excess at 15 °C
287 (Tarangkoon and Hansen 2011). The benthic mixotrophic *M. coatsi* has been shown to achieve a
288 growth rate of 0.22 d^{-1} in complete darkness when supplied the cryptophyte *Rhodomonas* sp., but
289 this species also grows faster in the light (Kim et al. 2019). No data are available for mixotrophic
290 *M. chamaeleon* in complete darkness, but this species has been shown to grow as fast as 0.35 d^{-1} at
291 an irradiance of 4 µmol photons m^{-2} s^{-1} at temperature of 18°C (Moeller and Johnson 2018). Thus,
292 despite the limited data sets on light dependency on growth among *Mesodinium* spp., they point to
293 some significant differences among the *Mesodinium* spp.

294 The *Mesodinium* spp. that only utilize prey from the *Teleaulax/Geminigera/Plagioselmis* clade
295 seem to be dependent on light for growth, while the other benthic mixotrophic and heterotrophic
296 *Mesodinium* spp. that can utilize a larger variety of prey species are able to grow in the dark,
297 although at a reduced growth rate. Light dependence on growth rates of other marine planktonic
298 mixotrophic ciliates are sparse. A study of the mixotrophic *Strombidium rassoulzadegani* revealed
299 growth rates (over three days) of ~0.6 d^{-1} in complete darkness (McManus et al. 2012), and a
300 maximum growth rate of 1.0 d^{-1} at an irradiance of 100 µmol photons m^{-2} s^{-1}. The growth in
301 complete darkness was though dependent upon time in the dark and on the prey species. *S.*
302 *rassoulzadegani* was able to grow at a rate of 0.6 d^{-1} for > 10 days, when fed the dinoflagellate
303 *Prorocentrum cordatum* (=*P. minimum*), while it suffered mortality if fed the cryptophyte
304 *Rhodomonas lens*.

305 *Dependence on light for prey ingestion, photosynthesis and cellular Chl* a
306 Ingestion rates of *M. major* feeding on *T. amphioxeia* were in the range of 8-15 cells $predator^{-1}$ d^{-1}
307 at prey concentration of 2000-8000 cells ml^{-1}, but no significant relationship to irradiance was
308 observed (Fig 2D). Previous studies of a temperate strain of *M. rubrum* have found maximum
309 ingestion rates between 3-4 prey cells $ciliate^{-1}$ d^{-1}, if fed in excess at 15°C (Hansen and Fenchel
310 2006), and no significant difference was obtained at the two studied irradiances (20 and 100 µmol
311 photons m^{-2} s^{-1}). The mixotrophic *M. chamaeleon and M. coatsi* were found to have maximum
312 ingestion rates similar to *M. major*, at ~10-25 prey cells $ciliate^{-1}$ day^{-1}, whereas the heterotrophic *M.*
313 *pulex* ingested up to 30 prey cells $ciliate^{-1}$ day^{-1} at 15 °C (Kim et al. 2019; Moeller and Johnson
314 2018; Tarangkoon and Hansen 2011).

315 Photosynthetic activity increased as a function of irradiance and measured values ranged from 89.2
316 to 323 pg C $cell^{-1}$ h^{-1} at 15°C, at irradiances of 25 - 200 µmol photons m^{-2} s^{-1} and prey
317 concentrations of ~4,000 cells ml^{-1}. The data could be fitted to Michalis-Menten kinetics,
318 suggesting a saturation of photosynthesis > 75 µmol photons m^{-2} s^{-1}. This is, to our knowledge one
319 of the highest photosynthetic rate measured for a ciliate ever. It is only exceeded by the large
320 *Laboea strobila*, in which a photosynthetic rate of 465 pg C $cell^{-1}$ h^{-1} was achieved at an irradiance
321 of 100 µmol photons m^{-2} s^{-1} and a temperature at 12°C on cells picked from natural plankton
322 samples (Stoecker et al. 1989). For comparison, published maximum photosynthetic rates ~88 pg C
323 $cell^{-1}$ h^{-1} have been found for *M. rubrum* at irradiances of >200 µmol photons m^{-2} s^{-1} (Table 1;
324 (Stoecker et al. 1991)). Photosynthetic rates of benthic mixotrophic *M. chamaeleon* has been shown
325 to be much lower, ~6.3 pg C $cell^{-1}$ h^{-1} (Moestrup et al. 2012). Maximum photosynthetic rates are
326 only available for a few other mixotrophic ciliates. In the genus *Strombidium*, *S. basimorphum, S.*
327 *conicum*, *S. rassoulzadegani* and *S. reticulatum* maximum photosynthetic rates of 29, 12, 63 and 31
328 pg C $cell^{-1}$ h^{-1}, respectively, have been reported (Jonsson 1987; Schoener and McManus 2017;
329 Maselli et al. 2020). The high photosynthetic rates of *M. major* may not be surprising. It is a very
330 large ciliate and it contains a larger number of chloroplasts. The maintenance requirements for *M.*
331 *major* could be estimated from a plot of growth versus photosynthetic activity calculated for *M.*

332 *major* to be ~70 pg C cell^{-1} h^{-1} (Fig 3A; Table 1). The current literature does not provide this
333 information for any other ciliates.

334 If we compare the carbon obtained from photosynthetic activity and from ingestion of prey, the
335 photosynthetic activity account for ten times more carbon than prey ingestion at high irradiance (75-
336 200 µmol photons m^{-2} s^{-1}) in *M. major* (Fig 3B). At lower irradiances the contribution from prey
337 ingestion increased to account for up to 44 % at an irradiance of 25 µmol photons m^{-2} s^{-1}. For
338 comparison, *M. rubrum* obtains a similar contribution of carbon from photosynthesis ~95% and ~80
339 % at irradiances of 100 and 20 µmol m^{-2} s^{-1}, respectively (Smith and Hansen 2007). The benthic *M.*
340 *chamaeleon* on the other hand gets less of its carbon from photosynthesis (0-70 %; Moeller and
341 Johnson 2018).

342 Cellular Chl *a* levels decreased from 130 to 80 pg Chl *a* cell^{-1} as the irradiances increased from 25
343 µmol photons to 200 µmol photons m^{-2}s^{-1} in *M. major* (Fig. 2C). Similar observations have been
344 found in an Antarctic strain of *M. rubrum* (clade A). Here, the cellular levels of Chl *a* decreased
345 from 60 to 30 pg Chl *a* cell^{-1} when irradiances increased from 2.5 to 55 µmol photons m^{-2} s^{-1},
346 respectively (Johnson and Stoecker 2005). Further detailed studies of the photosynthetic apparatus
347 are however necessary to elucidate if *M, major* is actually capable of photoacclimation, like what
348 has been shown for *M. rubrum* (clade A) (Moeller et al. 2011). The light dependency of
349 photosynthetic activity is likewise illustrated by the Chl *a*-specific photosynthetic capacity. Here we
350 show that *M. major* is able to preserve the capacity of the chloroplasts to the same extent as *T.*
351 *amphioxeia* (Fig. 4A). The same ability has been shown for *M. rubrum* (Johnson et al. 2006).
352 Whereas the mixotrophic *Strombidium rassoulzadegani* was found be able to maintain ~50 % of
353 Chl *a*-specific photosynthetic capacity compared to its prey (McManus et al. 2012).

354 It was impossible to count the total the number of chloroplasts inside the large *M. major* cells
355 directly. However, quantification of the number chloroplasts in one-half of the cell was obtained,
356 and assuming that the cell contains the double amount, estimates of ~40-80 chloroplasts cell^{-1} were
357 found. If instead the photosynthetic activity was used to estimate the number of chloroplasts inside
358 *M. major*, the results indicate that the cells would have > 120 cryptophytes at an irradiance of 100
359 µmol photons m^{-2} s^{-1} (Fig. 4B). However, such higher numbers did not match at all the number of
360 chloroplasts counted with a microscope. Similar results were found using Chl *a* cell^{-1}. A possible
361 reason for this mismatch could be that the cryptophyte chloroplasts enlarge inside the cell, as
362 reported in *M. rubrum* (Hansen and Fenchel 2006).

363 **Responses of *Mesodinium major* to prey deprivation and refeeding**
364 The studied strain of *Mesodinium major* coped very well with prey starvation. Within 5 days of
365 prey starvation, the Chl *a*-specific photosynthetic capacity decreased by 50%, but thereafter
366 remained constant throughout 51 days of prey starvation. In that period, the culture went through
367 five cell divisions. Similar results have been found for several strains of *M. rubrum*. This ciliate
368 likewise is able to starve for long periods when subjected to prey starvation, and it is
369 photosynthetically active for > two months (Table 1; (Johnson and Stoecker 2005; Myung et al.
370 2013; Smith and Hansen 2007). The ability to survive without prey for this amount of time gives
371 organisms great advantages to other similar organisms, and could explain the annual presence that
372 we find in many coastal areas (like in Helsingør harbor (personal observations) and at Helgoland
373 (Yang et al. 2014). For comparison, the heterotrophic *M. pulex* and mixotrophic *M.*
374 *chamaeleon/coatsi* are only able to starve for up to two weeks, and maximally divide once when
375 subjected to prey starvation (Tarangkoon and Hansen 2011; Moeller and Johnson 2018). Starvation
376 experiments with other mixotrophic ciliates are sparse. In the case of the ciliate, *S. capitatum*, little

377 tolerance to prey deprivation was observed. After only 40 hours, the cell populations were declining
378 quickly in numbers (Stoecker and Silver 1990).

379 *M. major* was able to recover quickly from 22 days of prey starvation. After only 6 days of
380 refeeding, photosynthetic activities were back to the levels found in well-fed cultures. Cellular Chl
381 *a* increased from 58 to 122 pg cell^{-1} after 8 days of exposure to prey cells (Fig. 8B). This is
382 surprising since we did not find a decrease in the cellular Chl *a* of the ciliates during starvation. *M.*
383 *major* quickly started to ingest prey cells when refed, and ingestion rates were very high (~ 50,000
384 cells ml^{-1}; > 150 prey predator^{-1} day^{-1}) after some days of refeeding (fig 7B). After six days of
385 refeeding both prey treatments had depleted all available prey (Fig. 7A). *M. rubrum* has previously
386 been subjected to a similar starvation exposure, and here it took *M. rubrum* 10-13 days to deplete all
387 available prey (Kim et al. 2017). Similar, *M. rubrum* was found to ingest ~1.3 prey predator^{-1} h^{-1},
388 after being starved for 14 days, but the high ingestion rate was only found within the first hours
389 after refeeding, where after ingestion rates rapidly decreased (Gustafson et al. 2000). For
390 comparison, *M. major* could maintain ingestion rates of 7.0 prey cells predator^{-1} h^{-1} for > 24 hours.
391 These results suggest a difference between *M. major* and *M. rubrum*, that could be explained by
392 self-shading of the chloroplasts, as a consequence of the high amount of prey found within *M.*
393 *major*. Self-shading may also explain why no difference was found between the two treatments of
394 30 and 100 prey predator^{-1} apart from the higher ingestion rate.

395 **Control of retained chloroplasts and nuclei during prey starvation**
396 A positive correlation between the centered prey nucleus (CPN) and photosynthetic performance in
397 *M. major* was found during the starvation experiment, similar to the results obtained on *M. rubrum*
398 (clade F) (Kim et al. 2017). For both *M. major* and *M. rubrum* strains studied a decrease in the
399 percentage of cells with a CPN from 55 to 10% of cells results in a 50 % reduction of the
400 photosynthetic activity. We therefore suggest that the relationship between CPN and photosynthetic
401 activity in *M. major* is very similar to that in *M. rubrum*.

402 Despite very high ingestion rates in *M. major* when fed at high prey concentrations, no difference in
403 the percentage of acquired CPNs within the two prey treatments was observed (Fig. 8C). Unlike *M.*
404 *rubrum*, the culture of *M. major* only managed to obtain approximately 50 % of cells with CPN,
405 whereas *M. rubrum* obtained 90 % in the same time interval (4 days) (Kim et al. 2017). This could
406 suggest that *M. rubrum* has a faster response in acquisition of the CPNs than *M. major*. Such a
407 difference may also explain why we rarely found extra prey nuclei (EPNs) in the *M. major* cells.
408 We can only speculate if the missing observations of EPNs in *M. major* is caused by *M. major* not
409 retaining them or if it is an effect of their size, shadowing in the epifluorescence microscope.

410 **CONCLUSION**
411 *Mesodinium major* functions quite similarly to *M. rubrum*. However, it needs more light to sustain
412 and to grow. Chl *a*-specific photosynthetic capacity was similar or higher to that of *Teleaulax*
413 *amphioxeia* at all irradiances. Photosynthesis is the primary carbon source in *M. major*, similar to
414 what has been shown for *M. rubrum*. *M. major* is well adapted to prey starvation and tolerates 51
415 days of prey deprivation without any mortality. Refeeding after prey starvation leads high ingestion
416 rates for short time. Thus, *M. major* differs from *M. rubrum* by having a higher demand of light, a
417 photosynthetic activity, which is up to four times higher than *M. rubrum*, and a maximum ingestion
418 rate, which is tenfold higher than *M. rubrum*.

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525

526 **FIGURE LEGEND**

527 **Fig. 1**. Epifluorescence micrographs of *Mesodinium major*. A-C Micrographs from disk-spinning
528 unit revealing arrangement of the chloroplasts in *M. major*, **A.** in the form of a 3D-reconstructed
529 cell, **B.** seen from the top, and **C.** seen from the side. D-E *M. major* stained with Hoechst 33258 and
530 CellMask Green in combination, **D.** Starved *M. major* containing two ciliate macronuclei and one
531 ciliate micronucleus, **E.** Well-fed *M. major* containing centered prey nucleus (CPN), **F**. Overview
532 of *M. major* and the numerous chloroplasts. The scale bar in D is 20 µm and applies to all panels.

533 **Fig. 2.** Experiment 1. Effects of irradiance. **A.** Growth rates as a function of irradiance. The curve
534 was numerically fitted to Michaelis-Menten kinetics. Y = 0.45*(X-21.5)/(19.99+(X-21.5)), R^2 =
535 0.84. Data points represent means ±SE (n = 3). **B.** Photosynthetic activity as a function of
536 irradiance. The data was fitted to Michaelis-Menten kinetics. Y = 530.7*X/(48.9+X), R^2 = 0.76.
537 Data points represent means ±SE (n = 3). **C.** Chlorophyll *a* as a function of irradiance. The data
538 could neither be fitted linear line nor exponential decay kinetics; dotted line represents drawn trend
539 line. **D.** Ingestion rate as a function of irradiance. The data was fitted to a linear line, R^2 = 0.0024.
540 The line is not significant from zero, P = 0.9267.

541 **Fig. 3.** Experiment 1. Effect of irradiance. **A.** Growth as a function of photosynthetic activity. The
542 curve was numerically fitted to Michaelis-Menten kinetics. Y = 0.508*(X-70)/(70.49+(X-70)), R^2 =
543 0.90. **B.** Daily carbon obtained from photosynthetic activity (converted into daily uptake) and from
544 ingestion of prey (ingestion rate d^{-1} * 38 pgC (carbon in *T. amphioxeia* from Smith and Hansen
545 (2007))), as a function of irradiance.

546 **Fig. 4.** Experiment 1. Effects of irradiance. **A.** Chlorophyll *a*-specific photosynthetic capacity for
547 *M. major* and *T. amphioxeia* as a function of light. **B.** Estimated number of chloroplasts derived
548 from measured photosynthetic activity and Chl *a* of *M. major* and *T. amphioxeia*.

549 **Fig. 5.** Experiment 2. Prey deprivation. **A.** Time course of numbers of cells during incubation of 51
550 days. Arrowheads indicate cell divisions of *Mesodinium major*. Dashed line indicates a mixture of
551 the four cultures and resuspension in the 3 replicates. **B.** Time course of percentage of cells with
552 centered prey nucleus during incubation. The data was fitted to "One phase decay kinetics". Y =
553 (54.3-7.68)*exp(-0.16*X)+7.68, R^2 = 0.9113. **C.** Time course of photosynthetic activity during
554 incubation. The data was fitted to "One phase decay kinetics". Y = (124.8-67.14)*exp(-
555 0.20*X)+67.14, R^2 = 0.471. **D.** Time course of amount of Chlorophyll *a* during incubation. The
556 data was fitted to a linear line, R^2 = 0.0058. The line is not significant from zero, P = 0.8336.

557 **Fig. 6.** Experiment 2. Prey deprivation. **A.** Time course of Chlorophyll *a*-specific photosynthetic
558 capacity. The data was fitted to "One phase decay kinetics". Y = (0.144-0.073)*exp(-0.08*X)+
559 0.073, R^2 = 0.659. **B.** Percentage of cells with centered prey nucleus (CPN) as a function of
560 photosynthetic activity. The data was fitted to exponential growth kinetics. Y = 1.388*exp(0.03*X),
561 R^2 = 0.784. Please note that percentage of cells with CPN only goes up to 55%, and that
562 photosynthetic activity was measured > 200 pgC $cell^{-1}$ h^{-1} in exp. 1 at the same irradiance.

563 **Fig. 7.** Experiment 3. Refeeding *Mesodinium major*. *M. major* was refed at two different prey ratios
564 1:30 and 1:100 (predator:prey). **A.** Time course of number of cells of *M. major* (solid lines, left y-
565 axis) and *Teleaulax amphioxeia* (dashed lines, right y-axis) during incubation. **B.** Time course of
566 ingestion rate during incubation.

567 **Fig. 8.** Experiment 3, Refeeding of *Mesodinium major* at two different ratios 1:30 and 1:100
568 (predator:prey). **A.** Time course of photosynthetic activity during incubation. **B.** Time course of
569 Chlorophyll *a* during incubation. **C.** Time course of percentage of cells with centered prey nucleus
570 during incubation.

571

572 **SUPPORTING INFORMAION**
573 **Identification of the small subunit ribosomal RNA gene and internal transcribed spacer 1,**
574 **partial sequence of *Mesodinium major***
575 **Methods**
576 *DNA extraction, PCR and Sequencing*
577 From the *Mesodinium major* culture, single cells were washed in clean medium and transferred to
578 0.2 ml PCR tubes containing 100µl water and 10% (w/v) Chelex 100 (Sigma-Aldrich #C7901). For
579 DNA extraction the PCR tubes were vortexed for 5 s, spun down in a microcentrifuge for 10 s, and
580 subsequently incubated at 95 ºC for 20 min (Richlen and Barber 2005). After incubation, the tubes
581 were centrifuged for 10 s and stored at 4 ºC until use in PCR reactions.
582 2 µl of the DNA extract was used as template in the subsequent PCR reactions. The following
583 primer pairs were used: 4617F – Meso580R; Meso245 – UNIDEUK1416R; Meso580F –
584 Meso1480R, Meso1200F - Meso28S_R; ITS1 – Dir-2CR (see table S1). PCR reactions were done
585 in 25 µl reaction containing, 1,5 mM $MgCl_2$, 0,8 mM dNTPs [VWR #733-1363], 0,5 units
586 polymerase [VWR #733-1301], 0,4 µM primers using the following reaction settings: 2 min at 95
587 ºC, followed by 40 cycles: 95 ºC for 30 s; 56 ºC for 30 s; 72 ºC for 50 s; and finally 5 min at 72 ºC.
588 PCR products were tested on a 2% agarose gel sent to Macrogen (Macrogen Europe, Amsterdam,
589 NL) for purification and sequencing in both directions. Sequence analysis (trimming, assembly,
590 BLAST) was done with Geneious version 2020.2.2.
591
592 **Results**
593 *Mesodinium major* (MW560711**)** aligned with a sequence already described in literature
594 (JN412737) (Garcia-Cuetos et al. 2012).

595