Nutritional effects on the visual system of the rotifer *Brachionus plicatilis* sensu stricto (Rotifera: Monogononta)

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Abstract

Rotifers have a light sensor called “eyespot” which is expected to be composed of rhodopsin. Based on the molecular feature of rhodopsin as regenerated with 11-cis-retinal, we hypothesized that phototactic behavior should be affected by the nutritional level of food; especially vitamin A availability. This study intended to address the following questions on the nutritional effects of using baker’s yeast (*Saccharomyces cerevisiae*) and *Nannochloropsis oculata*: how does diet affect the pigmented area and absorbance of the eyespot, and how do these changes characterize phototactic behavior and population growth in the monogonont rotifer *Brachionus plicatilis* sensu stricto. The pigmented area of the eyespot decreased to 14.7 μm^2 with baker’s yeast while it was maintained at the initial size of 82.9 μm^2 with *N. oculata*. Maximum absorbance of the eyespot was observed at a range of 470 to 525 nm in the initial rotifers and it was not significantly changed with diet type and culture day. The value of the maximum absorbance was maintained with *N. oculata*, while it rapidly decreased on day 10 with baker’s yeast. Stronger positive phototaxis with *N. oculata* was observed under lower light intensity (0.1 and 0.5 W m^{-2}) at 470 nm. On the other hand, phototaxis with baker’s yeast became weak and no phototactic reactions were observed under the same lighting condition. From the genomic DNA database of rotifers, 12 putative opsin-relevant genes were identified. These results corroborate the hypothesis that rhodopsin is the visual pigment in the rotifer eyespot. Lack of vitamin A with baker’s yeast should induce reduction of the pigmented area and the sensitivity of the rotifer eyespot resulting in weak phototaxis. The population growth of rotifers showed different patterns related to the food type and light intensity. The lowest population growth (0.33-0.37 day^{-1}) was shown with baker’s yeast diet at 0.5 W m^{-2}. This phenomenon may be significantly related to malnutrition on baker’s yeast which is deficient not only in vitamin A but also fatty

acids, vitamin B_{12} and its derivatives.

Keywords

Rotifera / Nutrient / Eyespot / Phototaxis / Population growth

1. Introduction

In the wild, planktonic metazoans including rotifers exhibit diel vertical migration caused by light stimulation (Gerhardt et al., 2006; Jékely et al., 2008; Martynova and Gordeeva, 2010) and many other factors such as temperature, prey-predator relationship and surface current (Stich and Lampert, 1981, 1984; Hill, 1991). Among these factors, light is considered to be the main stimulus which would guide their positioning in the water column (Barcelo and Calkins, 1979). Monogonont rotifers belonging to the genus *Brachionus* possess a red light sensor called eyespot, and show phototactic and photokinetic reactions associated with the sensitivity of the eyespot toward wavelength and intensity (Clément et al., 1983; Cornillac et al., 1983).

Phototaxis of *Brachionus plicatilis* species complex is simultaneously affected by light wavelength and intensity (Kim et al., 2014). Wavelength induced strong positive phototaxis of rotifers was related to maximum absorbance of the pigmented area of the eyespot (Cornillac et al., 1983; Kim et al., 2014). Moreover, rotifer photokinetic movements were also influenced by the light wavelength and intensity (Clément et al., 1983) and these movement patterns were expected to affect their reproduction (Kim et al., 2014). The rotifer eyespot, cerebral eye consists of two types of pigment-bearing cells: one epithelial cell cup containing accessory pigment and one or more sensory neurons (sensory pigments) with

membranous structure (Cornillac et al., 1983). These pigments i.e., accessory and sensory pigments have the following function: to perceive the direction of light and to elicit any responses, respectively (Clément, 1980; Clément et al., 1983; Cornillac et al., 1983). Due to the synergistic action of these two pigments, the rotifers show light wavelength and intensity-dependent phototaxis (Clément et al., 1983). Red pigments (accessory pigments, Clément, 1980) are expected to consist of rhodopsin similar to other invertebrates (Wolken, 1971; Clément, 1980). Rhodopsin is composed of opsin protein covalently linked to 11-*cis*-retinal which is a derivate of vitamin A (Palczewski et al., 2000; Zhong et al., 2012).

Harris et al. (1977) reported the correlation between visual system performance and nutrient level of food as follows: deprivation of dietary vitamin A causes a reduction of visual sensitivity and photopigment concentration in both vertebrates and invertebrates. In this study the hypothesis that the nutritional value of diet affects the visual system of rotifers is investigated under controlled light conditions. The following questions associated with the nutritional level of two diets, baker's yeast (*Saccharomyces cerevisiae*) and *Nannochloropsis oculata*, are addressed: (1) how the diet affects the area and absorbance of the rotifer eyespot and (2) how changes in these parameters influence behavior (phototaxis) and population growth.

2. Materials and methods

2.1. Area and absorbance of eyespot

This study employed the monogonont rotifer *Brachionus plicatilis* sensu stricto (Makishima strain) which does not undergo mixis (Hagiwara et al., 2007). The culture medium (22 practical salinity unit, psu) was made by diluting natural seawater with Milli-Q

water (Millipore 0.22 μm) followed by GF/C filtration and sterilization (at 121°C for 20 min). The rotifers were stock-cultured at 25ºC in total darkness with daily feeding of *Nannochloropsis oculata* at 7×10^6 cells mL^{-1}. *N. oculata* was cultured in modified Erd-Schreiber medium (Hagiwara et al., 1994) under continuous light with gentle aeration. Prior to feeding *N. oculata* were centrifuged at 3968×g for 10 min and re-suspended in the rotifer culture medium.

Rotifers for feeding trials were started from parthenogenetic eggs collected from amictic females of the stock culture. To obtain these eggs, we pipetted out 500 rotifers carrying female eggs and transferred them into a 30-mL screw-capped bottle containing 10-mL of the same saline water as the stock culture and then agitated them to shake off the eggs. Separated eggs were collected with a Pasteur pipette and incubated in a laboratory dish (90 mm ø) in 40-mL stock medium (22 psu of saline water). Hatchlings (< 3 h) from those eggs were inoculated into a 100 mL of glass flask containing 100 mL of 22 psu sterilized saline water at 1 ind mL^{-1} and cultured for 30 days in triplicates. The cultures were fed with two types of foods: *N. oculata* (at 7×10^6 cells mL^{-1}) and baker's yeast *Saccharomyces cerevisiae* (Oriental yeast Co. Itd., Japan, at 2.5×10^6 cells mL^{-1}) every 12 hours to provide the same dry weight of both foods. Weak aeration (at 10 mL min^{-1}) was provided only in rotifer cultures with baker's yeast to prevent precipitation and maintain dissolved oxygen concentration. Three rotifers from each culture were sampled every 10 days to determine the area and absorbance of the eyespot. The animals used for measuring the pigmented area with digital imaging software (Axio Vision Rel. 4.8, ZEISS) were fixed in formalin. The pigmented area calculation was based on an approximated elliptical shape corresponding to the length of the minor and major axis. Three other specimens from each culture were prepared for estimating the absorbance of the pigmented area. Prior to this procedure, each specimen was transferred onto a slide glass and then trapped under a cover glass without anesthesia.

Light absorbance was measured using the microspectrophotometer system composed of spectrophotometer 308 PV (Craic Technologies, USA) and BX 61 (Olympus, Japan) compound microscope and calculated according to the equation:

$$\text{Absorbance} = \text{Log}\ (I_0/I)$$

I_0: the light intensity of radiant energy striking sample

I: the light intensity of energy emerging from sample

2.2. Phototaxis

On the last day of the 30-day culture period, the phototaxis of rotifers on different feeding regimes was investigated in 20 females that were randomly selected from the same cultures as described in the previous section of this study. Selected individuals were immediately inoculated into the middle compartment of the experimental container (15×3×3 cm) which was divided into three compartments by two sliding partitions (Fig. 1a). The container was constructed manually using reflective black plastic plank (0.3 mm of thickness); it contained 20 mL of the stock culture medium (22 psu) resulting < 4 mm of water depth to limit vertical movements of rotifers. Inoculated rotifers experienced dark adaptation for 5 min and then were illuminated with two different light emitting diodes (LEDs: blue with a peak at 470 nm and red at 660 nm; IS-mini, CCS Inc., Japan) one at a time from the side for 15 min without partitions removed (Fig. 1b). The light intensity was adjusted to 0.1, 0.5 and 15.0 W m^{-2} using a light meter (LI-1400, LI-COR Inc., Japan). After irradiation, the partitions were replaced (Fig. 1c) and the number of rotifers in each compartment was counted under a stereomicroscope (SZX-ILLD2-100, Olympus, Japan). For the controls, these steps were performed in complete darkness followed by immediate replacement of the partitions under weak room light (< 0.07 W m^{-2}). The same batch of rotifers under each feeding regime was

used to investigate the wavelength (blue and red) effects on rotifer phototaxis related to nutrients. The mean value of five replicates was used to calculate rotifer distribution patterns.

2.3. Population growth

Collection of parthenogenetic eggs and subsequent 11-day culture of hatchlings from these eggs were performed by the same methods as described in the first section (area and absorbance of eyespot). Light condition of triplicate feeding trials was adjusted to yield continuous illumination with blue (with a peak at 470 nm) and red (at 660 nm) lights at 0.5 and 6.0 W m^{-2}. Controls were kept in complete darkness. The number of rotifers in 1 mL of culture sample was counted daily in triplicate. The means of triplicate observations were used for calculating the population growth (*r*) of rotifers on different feeding regimes by the equation.

$$\text{Population growth } (r) = \ln (N_t/N_0) / t$$

N_0: Initial density of rotifers, N_t: The number of individuals on day t, t: culture days

2.4. Retrieval and annotation of opsin-relevant genes

To obtain putative opsin-relevant gene information, we searched the *B. koreanus* (formerly known as *B. ibericus*; Hwang et al., 2013, 2014) genomic DNA database constructed by Lee et al. (2011). The assembled contigs coding for proteins obtained in this study were subjected to BLASTx analysis in the GenBank non-redundant (NR; including all GenBank, EMBL, DDBJ, and PDB sequence except EST, STS, GSS, or HTGS) amino acid sequence

database. All the gene information has been submitted to the GenBank database, and the accession number of each gene is appended in Table 2.

2.5. Statistical analysis

Nutritional effects on the absorbance of the pigmented area and on the phototaxis of rotifers were analyzed by 2-way repeated measures ANOVA followed by Tukey-Kramer *post hoc* test. Moreover, we also used Tukey-Kramer *post hoc* test after repeated measures ANOVA test to estimate the nutritional effect on the pigmented area. Differences in the population growth rate (*r*) associated with three factors i.e., food species, light wavelength and intensity were tested by 3-way ANOVA. All of the statistical analyses were carried out using Statview version 5.0 software (SAS Institute, Inc., USA).

3. Results

3.1. Area and absorbance of eyespot

In the rotifers fed on *Nannochloropsis oculata*, the pigmented area of the eyespot showed no significant changes in 30 days. It stayed in the 72 to 88 μm^2 range. With baker's yeast, on the other hand, the area steeply decreased on day 10 and was reduced to 16 μm^2 on the last day of culture (day 30, Tukey-Kramer *post hoc* test, $P<0.05$, Fig. 2).

The initial absorbance of the pigmented area (Fig. 3a) peaked in the 470 to 525 nm range and the value (i.e. wavelength with maximum absorbance of pigmented area, λ_{max}) was 16.5 to 17.6-fold higher than at 660 nm. On day 10 (Fig. 3b), λ_{max} was at around 460 nm in both feeding trials, however, the value was 2.2-fold higher with *N. oculata* (1.1) than with baker's

yeast (0.5). On day 20 (Fig. 3c), λ_{max} was at around 470 nm in both feeding trials. The value was about 4-fold higher with *N. oculata* (1.2) compared to baker's yeast (0.3). On day 30 (Fig. 3d), λ_{max} ranged from 480 to 490 nm in both feeding trials; it was 5.5-fold higher with *N. oculata* (1.1) than with baker's yeast (0.2).

3.2. Phototaxis

In complete darkness (Fig. 4), the rotifers under both feeding conditions generally remained in the middle compartment (cf. Fig. 1, compartment II). Under 0.1 W m^{-2} of blue light (Fig. 4), the rotifers fed on baker's yeast were more abundant in compartment I and II than III, but those with *N. oculata* mainly localized in compartment I which is the illuminated side (Tukey-Kramer *post hoc* test, $P<0.05$). Under 0.1 W m^{-2} of red light (Fig. 4), the rotifers fed on baker's yeast were more abundant in compartment II, but there were no significant differences in rotifer distribution among three compartments with *N. oculata* feeding. Under 0.5 W m^{-2} of blue light, the rotifers fed on baker's yeast congregated in compartment I and II, while those fed *N. oculata* were found mainly in compartment I (Tukey-Kramer *post hoc* test, $P<0.05$). Under the same intensity of red light (Fig. 4), the distribution of rotifers showed no differences from the horizontal pattern noted under complete darkness (control) in both feeding trials. The highest intensity (15.0 W m^{-2}) of both wavelengths induced positive phototaxis in both feeding trials and the highest proportion of animals was observed in the compartment I (Tukey-Kramer *post hoc* test, $P<0.05$).

3.3. Population growth

The rotifers fed *N. oculata* for 11 days showed higher population growth rate than those

fed baker's yeast in the control and all light-treated groups (3-way ANOVA, F=184.758, $P<0.01$, Table 1). Light intensity also affects the population growth of rotifers and highest population growth rate was observed at 6.0 W m^{-2} (3-way ANOVA, F=5.479, $P<0.05$). Light wavelength, on the other hand, did not affect the population growth of cultured rotifers.

3.4. *In silico* identification of opsin-relevant genes in rotifers

To support the observed results on the response of rotifer rhodopsin to nutritional changes, the available rotifer genome database was searched for opsin-relevant genes. Twelve putative opsin-relevant genes that showed high similarity with opsin genes characterized in other animal taxa (Table 2) were identified.

4. Discussion

In this report the effect that the nutritional value of food has on the visual system and population growth of the monogonont rotifer *Brachionus plicatilis* s. s. was investigated. The red pigmented area of the eyespot perceives light intensity and wavelength (Clément et al., 1983; Cornillac et al., 1983). In the present study, the pigmented area of rotifers fed on *Nannochloropsis oculata* maintained the function of light sensor with no changes in both area (Fig. 2) and absorbance range (Fig. 3) when compared to the initial population of rotifers hatched from parthenogenetic eggs. On the other hand, in rotifers fed on baker's yeast the pigmented area diminished (Fig. 2) and the absorbance of the pigment decreased (Fig. 3) over time. The same phenomenon was observed in the freshwater rotifer *Asplanchna*; the red pigment in the eyespot diminished under a carotenoid-deficient condition over several generations (Clément and Wurdak, 1984). Wolken (1971) and Clément (1980) suggested

that the visual pigment of rotifers would be strongly associated with rhodopsin. This suggestion is supported by the result obtained on the light absorbance of the initial rotifers; the wavelength with maximum value (Fig. 3; 470-525 nm) falls entirely into the range of rhodopsin (450-550 nm, Cronin and Marshall, 1989). Moreover, genome-wide analysis revealed that the rotifer *B. plicatilis* species complex possesses opsin-relevant genes in its genome (Table 2), suggesting that rhodopsin is the visual pigment of rotifers as noted in the previous reports. The potential function of opsin genes should be tested in rotifers. Regeneration of the light-absorbing molecule rhodopsin only occurs when retina is attached to the retinal pigmented epithelial cell. The latter converts *trans*-retinol to 11-*cis*-retinal (vitamin A aldehyde). A deficiency of vitamin A inhibits the reformation of rhodopsin (Wolf, 2001). The microalgae, *Nannochloropsis* sp. contains pro-vitamin A carotenoids, especially 0.29 ± 0.04 mg g^{-1} of β-carotene and < 0.25 μg g^{-1} of vitamin A when the microalga is cultured under continuous fluorescent light (Brown et al., 1999), while baker's yeast does not contain these nutrients (Hamre et al., 2008). Therefore, the dietary deficiency of vitamin A and its derivatives in a baker's yeast diet should lead to a malfunction of the rhodopsin regeneration system in the rotifer *B. plicatilis* s. s.

Rhodopsin as a visual pigment of rotifers is generally necessary to sense low intensity light in rod cells (Khorana, 1992). The positive phototaxis of rotifers was stronger under relatively weak light intensity (0.5 W m^{-2}) at 470 nm (blue), which is the wavelength at which the pigmented area absorbs maximally, and under higher light intensity (15.0 W m^{-2}) at 660 nm (red) which is the light wavelength with minimum absorbance (Kim et al., 2014). Therefore, three light intensities (i.e., 0.1, 0.5 and 15.0 W m^{-2}) were tested to compare phototaxis patterns at two wavelengths in rotifer subjected to two feeding regimes that resulted in different absorbance of eyespot. Rotifers cultured with *N. oculata* showed the same pattern as before by maintaining the function of eyespot (Fig. 4). One the other hand,

the rotifers cultured with baker's yeast showed different patterns, and positive phototaxis was observed only with the highest intensity (15.0 W m^{-2}) at both wavelengths (i.e., 470 and 660 nm) caused by the blunting of eyespot sensitivity. Consequently, vitamin A deficiency in the yeast diet affects the rotifer light sensing system by decreasing the size and absorbance of the pigmented area. These changes, in turn, affect the phototactic behavior of the rotifers.

The results of this study reveal that the sensitivity of the eyespot of rotifers is determined by the nutritional value of their food and subsequently it influences their phototactic behavior. In the wild, the vertical distribution of zooplankton is functionally related to their phototactic behavior (Barcelo and Calkins, 1979; Stewart and George, 1987). Vertical distribution is clearly observed in the littoral area, while it is less conspicuous in limnetic area (Jose de Paggi, 1995). The results of this study may clarify the reason for this pattern. These two areas differ in nutritional condition; relatively higher nutrients were detected in littoral area (Victor et al., 1997). Dissolved nutrients in hydrosphere determine the qualitative and quantitative value of phytoplankton which is the food source for rotifers (DiTullio et al., 1993; Balode et al., 1998) and food quality is expected to be higher in the littoral area. Based on these factors, it can be concluded that the clear pattern of vertical distribution is due to the higher sensitivity of the eyespot produced through feeding upon qualitatively or quantitatively good phytoplankton.

Rotifers cultured with *N. oculata* showed higher population growth rate compared to those with baker's yeast (Table 1). It is known that baker's yeast is nutritionally deficient for rotifers and cannot support population growth in axenic culture (Hirayama et al., 1989). The lower population growth of rotifers fed baker's yeast is caused by the deficiency of vitamin A (Satuito and Hirayama, 1986), vitamin B_{12} (Hirayama and Funamoto, 1983) and fatty acids (Satuito and Hirayama, 1991). Other factors, such as growth promoting substances (Gallardo et al., 1997; Ohmori et al., 2011) and stress resistance (Gallardo et al.,

1999; Kaneko et al., 2011) play a role in regulating rotifer population dynamics (Hagiwara et al., 2001). In this study, rotifers cultured with different feeding regimes were illuminated with two different light intensities (0.5 and 6.0 W m^{-2}). These light intensities were selected based on the results of a previous study (Kim et al., 2014): 0.5 W m^{-2} induced variation in population growth rate but 6.0 W m^{-2} did not modulate population growth. The population growth of illuminated rotifers subjected to two different feeding regimes (Table 1) was affected by light intensity and showed higher growth rate at 6.0 W m^{-2} than at 0.5 W m^{-2}, but was not affected by light wavelength under both light intensities in contradiction to the results of the previous study (Kim et al., 2014). This inconsistency may be partly due to the different nutritional condition of *N. oculata* fed to rotifers including ancestral generations (Hagiwara and Hino, 1990).

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1

Effects of light wavelength, intensity and diet type on the population growth of rotifers. N and S indicate *Nannochloropsis oculata* and baker's yeast (*Saccharomyces cerevisiae*) which are the diets for the rotifer *Brachionus plicatilis* sensu stricto, respectively.

Light intensity ($W\ m^{-2}$)*		Darkness	0.5		6.0	
Light wavelength (nm)			470	660	470	660
Food*	N	0.62±0.02	0.59±0.03	0.59±0.00	0.60±0.02	0.60±0.01
	S	0.39±0.08	0.37±0.04	0.33±0.08	0.46±0.04	0.36±0.08

Population growth (r) = ln (N_t/N_0) / t. Values are means±SD. Factors with asterisks had significant effects on population growth of rotifers (*, 3-way ANOVA, $P<0.05$, n=3-6).

Table 2

Putative opsin-relevant genes identified in the genome database of *Brachionus koreanus*. The values of three parameters (i.e., E-value, identities and positives) were analyzed by *in silico* BLASTx search in the NCBI database.

Gene	Length (bp)	Accession No.	Species (GenBank No.)	E-value	Identities (%)	Positives (%)
Blue-sensitive opsin-like	267	**KF885941**	*Latimeria chalumnae* (XP_006001498)	7E-10	41	58
C-opsin	882	**KF885939**	*Tribolium castaneum* (NP_001138950)	4E-38	33	54
Ciliary opsin	216	**KF885940**	*Platynereis dumerilii* (AAV63834)	2E-07	33	58
Ciliary opsin	624	**KF885942**	*Terebratalia transversa* (ADZ24786)	1E-31	36	57
GQ-rhodopsin	267	**KF885938**	*Daphnia pulex* (EFX63569)	8E-09	36	58
Melanopsin	747	**KF885936**	*Crassostrea gigas* (EKC19391)	7E-35	32	54
Melanopsin	684	**KF885946**	*Lottia gigantean* (ESO95853)	9E-27	32	47
Melanopsin	276	**KF885945**	*Myotis brandtii* (EPQ10710)	2E-11	36	58
Opsin	273	**KF885944**	*Schmidtea polychroa* (AFB74475)	1E-12	40	59
Opsin (encephalopsin, panopsin)	207	**KF885937**	*Danio rerio* (CAX13063)	7E-10	43	64
Peropsin	792	**KF885943**	*Hasarius adansoni* (BAJ22674)	3E-34	31	50
Rhabdomeric opsin	1,101	**KF885935**	*Platynereis dumerilii* (AGL94565)	2E-53	31	53

Figure captions

Fig. 1. Experimental procedure for the rotifer phototaxis. (A), dark adaptation for 5 minutes (B), light irradiation for 15 minutes without two partitions (C), rotifer count after the replacement of those partitions.

Fig. 2. Area variation of rotifer eyespot under different feeding conditions. Two lines indicate morphometric changes of the pigmented area in rotifer eyespot treated with -▲- *Saccharomyces cerevisiae* and with -●- *Nannochloropsis oculata* against culture days. Plot and bar indicate the mean value and standard deviation, respectively. Different alphabetical letters on the plots represent significant differences (a>b, Tukey-Kramer *post hoc* test, $P<0.05$, $n=3$).

Fig. 3. Absorbance variation of rotifer eyespot under different feeding conditions. The absorbance of rotifer hatchlings from parthenogenetic eggs is described in (A). The pattern of changing absorbance treated with different two different diet (▪▪▪▪ *Saccharomyces cerevisiae*, ▬▬ *Nannochloropsis oculata*) is expressed by three culture days, day 10 (B), day 20 (C) and day 30 (D) of culture days.

Fig. 4. Phototaxis of rotifers under two different feeding conditions. The rotifers showed different patterns of phototaxis associated with light wavelengths (blue with a peak at 470 nm and red at 660 nm) and intensities (0.1, 0.5 and 15 W m^{-2}). S and N represent the different food types such as baker's yeast (*Saccharomyces cerevisiae*) and *Nannochloropsis oculata*, respectively. Columns indicate the proportion of rotifer individuals in each compartments, I (□), II (⊞) and III (■, cf. fig.1). Significant differences were exhibited by different alphabetical letters (a>b>c, Tukey-Kramer *post hoc* test, $P<0.05$, $n=5$).

Fig. 1

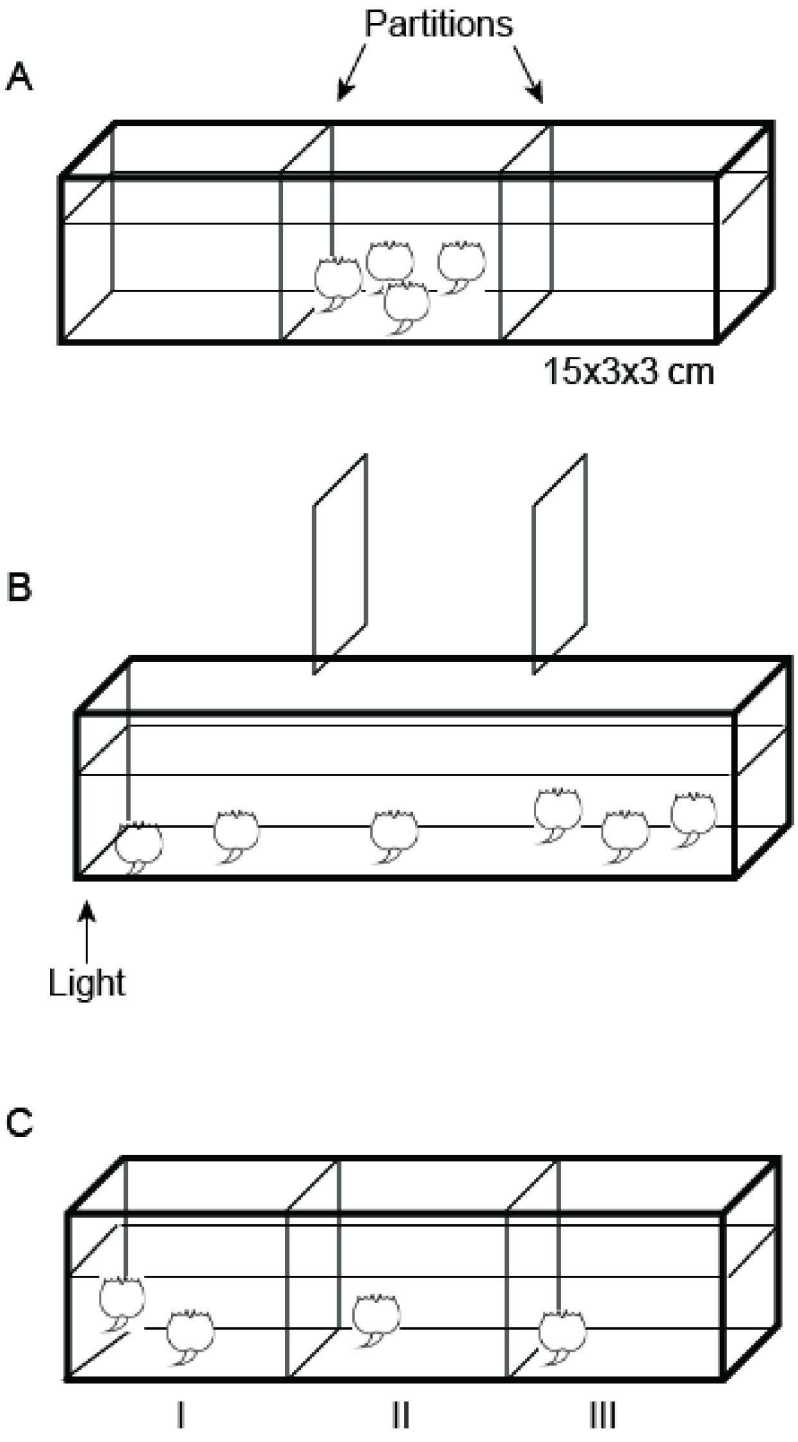


Fig. 2

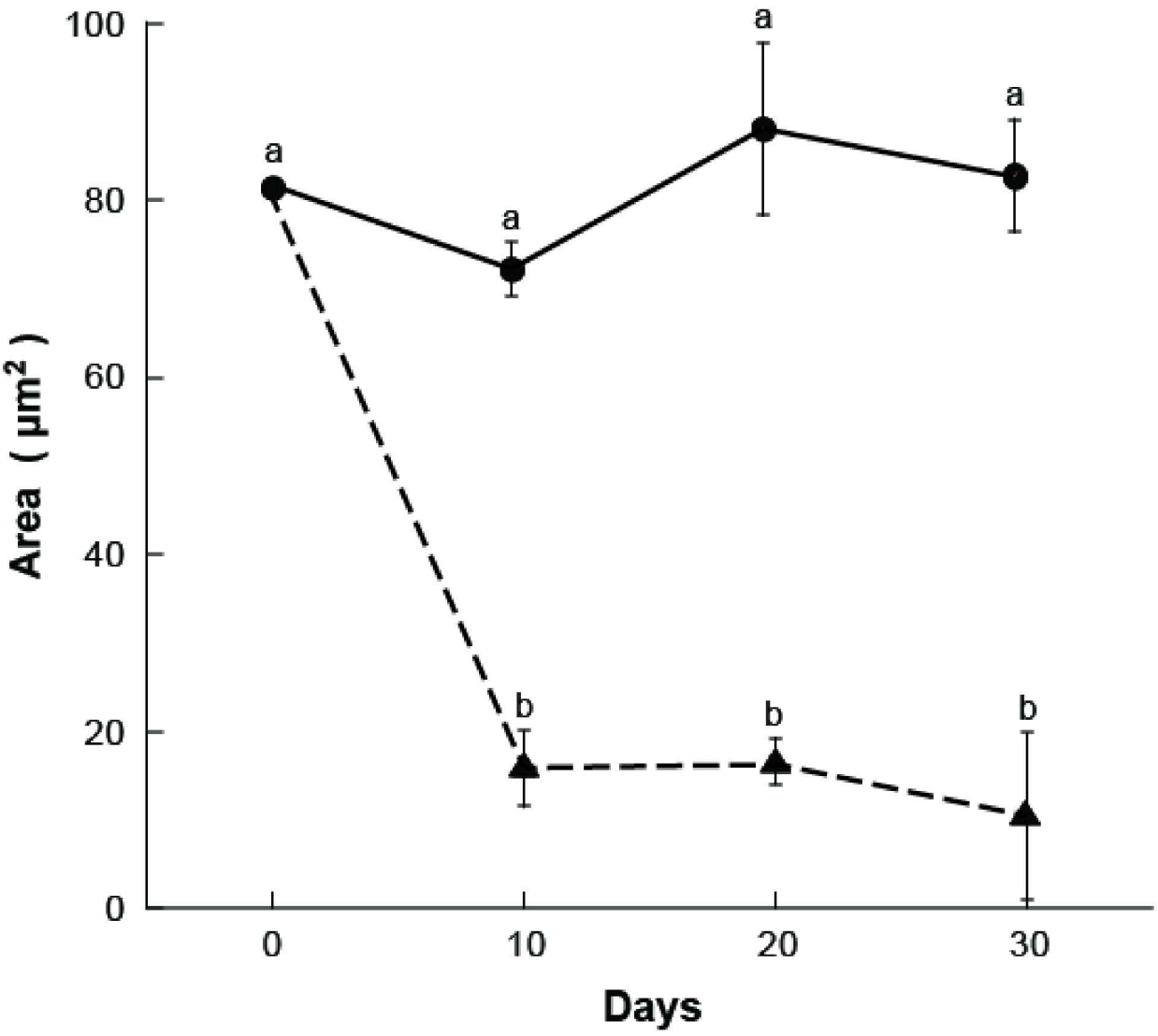


Fig. 3

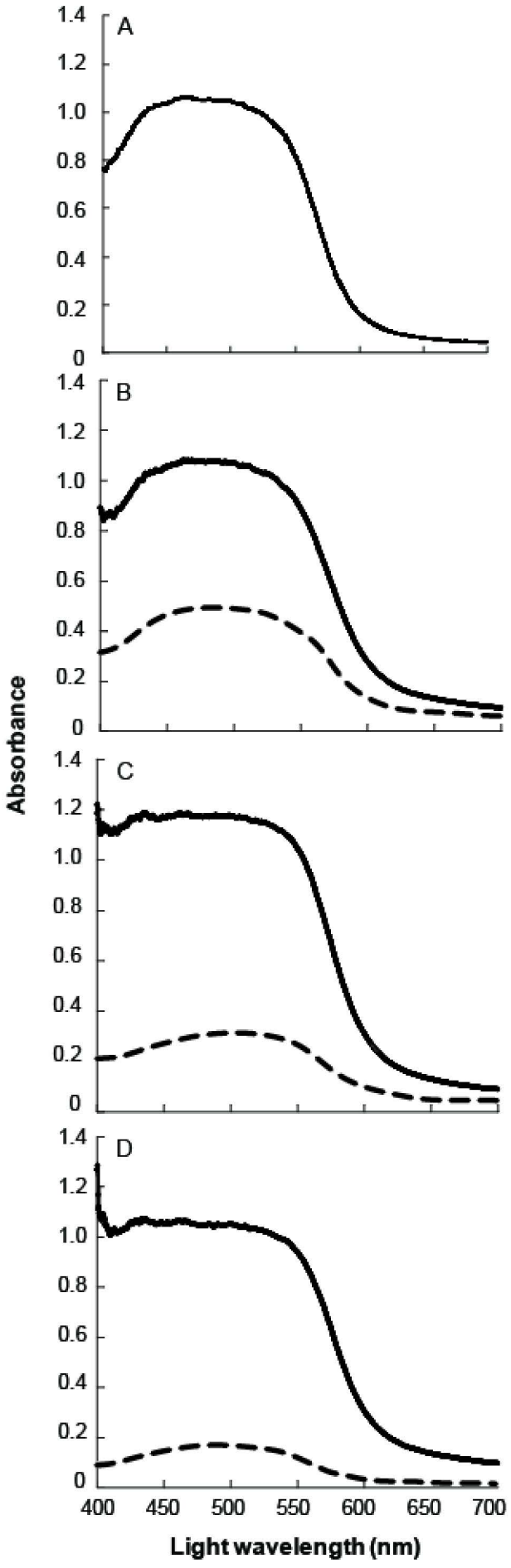
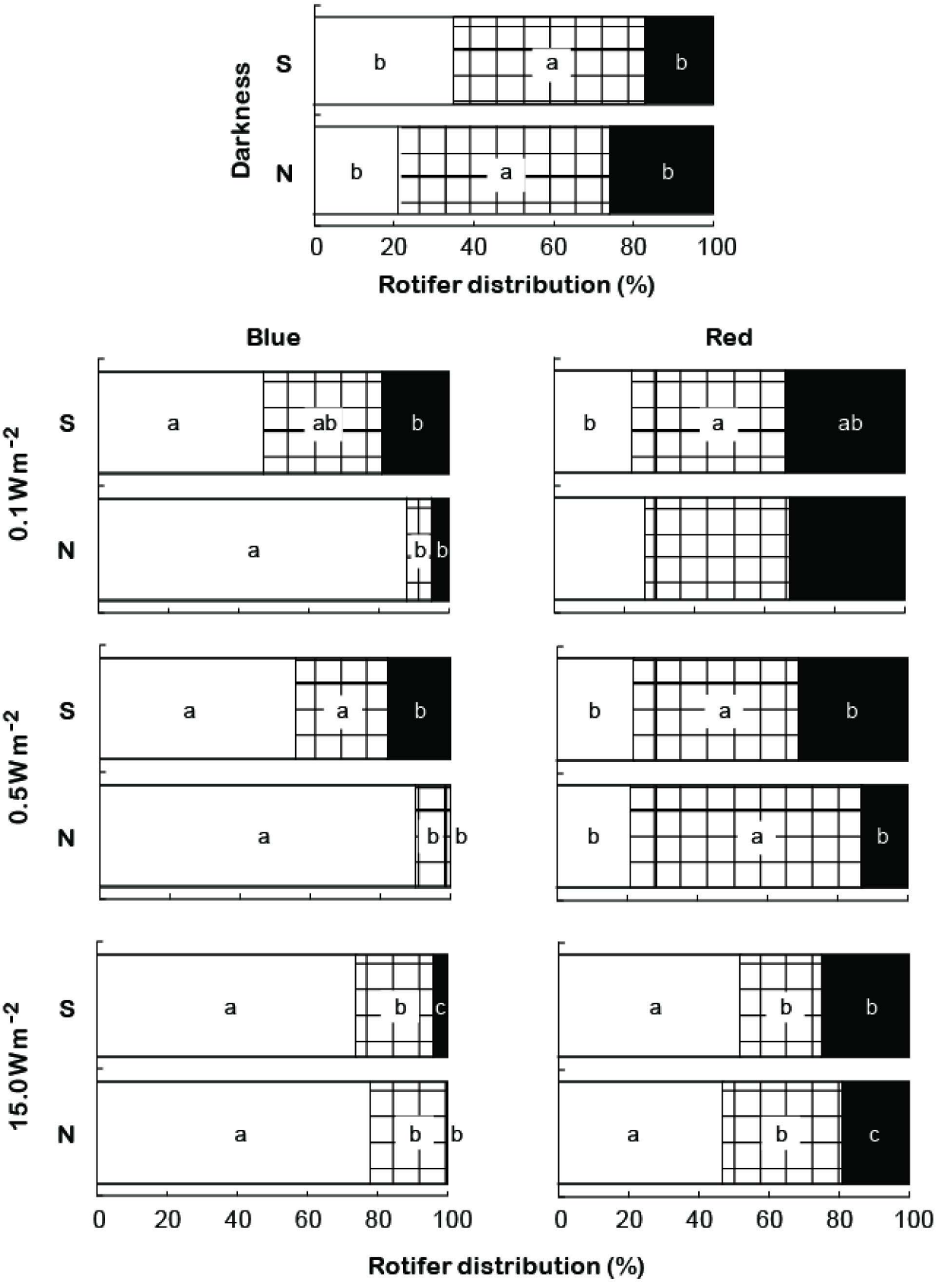

A
B
C
D
Absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
400
450
500
550
600
650
700
Light wavelength (nm)

Fig. 4


Darkness
S
N
b
a
b
b
a
b
0
20
40
60
80
100
Rotifer distribution (%)
Blue
Red
0.1Wm-2
0.5Wm-2
15.0Wm-2
S
N
a
ab
b
b
a
ab
a
b
b
b
a
b
a
a
b
b
b
a
b
a
b
b
a
b
c
a
b
b
a
b
b
a
b
c
0
20
40
60
80
100
Rotifer distribution (%)