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Behaviour of the plathelminth *Symsagittifera roscoffensis* under different light conditions and the consequences on the symbiotic algae *Tetraselmis convolutae*

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Abstract

Symsagittifera roscoffensis is a plathelminth living in symbiosis with the green algae *Tetraselmis convolutae*. Host and symbiont are a model system for the study of endosymbiosis, so far mainly focused on their biochemical interactions. *S. roscoffensis* is well known for its positive phototaxis that is hypothesized to optimize the symbiont's light perception for photosynthesis. In this study, we conducted a detailed analysis of phototaxis using light sources of different wavelength and brightness by videotracking. Furthermore, we compared the behavioral data with the electron transfer rate of the photosystem from cultured symbiotic cells. The symbiotic algae is adapted to low light conditions showing a positive electron transfer rate (ETR) already at a photosynthetically active radiation (PAR) of 0.112 µmol m^{-2} s^{-1} (mol photons per square meter and second), and *S. roscoffensis* showed a positive phototactic behaviour for light intensities up to 459.17 µmol m^{-2} s^{-1} which are not optimal regarding the needs of the symbiotic cells and even may harm host and symbiont. Red light can not be detected by the animals and therefore their eyes seem to be not suitable for measuring the exact photosynthetically active radiation to the benefit of the photosymbionts.

Introduction

Oxygen dependent photosynthesis is a substantial pathway for many organisms. It is suggested that the development of oxygen only occurred once in evolution in the cyanobacteria or late Archaean (Cavalier-Smith, 2006). Later the pathway was passed over by endosymbiosis to basal eukaryotes leading to primary, secondary and tertiary plastids of various algal species as well as the plastids of higher plants that evolved from green algae (Johnson, 2011; Taylor, 1973; Trench, 1993). However, there are also many groups of organisms which developed mechanisms to capture photosynthetic products through the formation of symbiotic associations. These include the phyla Porifera (marine and freshwater sponges), Cnidaria (corals, sea anemones and freshwater hydra), Acoelomorpha (marine turbellaria), Mollusca (e.g. giant clams and nudibranchs) and Chordata (marine ascidians) (Serôdio et al., 2014; Trench, 1979; Venn et al., 2008). One special variety of

photosynthesis in the animal kingdom occurs in the sea slug taxon *Sacoglossa*. They feed on algae sequestering the chloroplasts. Some species of slugs are able to keep these chloroplasts alive within their own cells for several month (Händeler et al., 2009; Kremer, 1976; Kremer, 1977). A horizontal gene transfer from the host algae to the slugs was suggested, but this suggestion is still controversially discussed and an area of ongoing research (Bhattacharya et al., 2013; Rumpho et al., 2008; Schwartz et al., 2014; Wägele et al., 2010).

The plathelminth of the order Turbellaria *Symsagittifera roscoffensis* (Graff, 1891), formally known as *Convoluta roscoffensis*, is a simply organized unsegmented worm, living in a symbiosis with the algae *Tetraselmis convolutae* (Norris et al., 1980; Parke and Manton, 1967). It is two to four mm long and contains 20,000 to 70,000 endosymbiotic cells of the algae within its body cavities (Doonan and Goody, 1982). These cells are not forwarded maternally, but must be acquired by each individual during the first days after hatching (Keeble and Gamble, 1907). This is a critical process, as the symbiosis is obligatory for the animals to survive. Thus the worm becomes a photoautotrophic organism consuming nutrients provided by the symbiotic algae (Keeble, 1910; Muscatine et al., 1974). The algae also profit from the symbiosis because the algae reaches a higher rate of carbon fixation, as compared to a free living phytoplankton organism in habitats with tidal changes (Doonan and Gooday, 1982; Gooday, 1970). Doonan and Gooday discussed that the worms may regulate the photosynthesis of their symbionts, but only little is known about the worm's influence. Behavioural and physiological studies are rare but it is known that *S. roscoffensis* shows a positive phototaxis and escapes into the sandy sediment upon vibration (Keeble, 1910). Artificial light experiments with a light gradient suggests an optimization of photosynthesis by choosing the optimal light conditions for the algae (Serôdio et al., 2010). In general, the eyes of turbellarians are without lenses, consisting of a shading pigment cup and nerve cells (Yamasu, 1991). Nevertheless, this type of eyes is capable of assessing the intensity and locating the direction of a light source (Jékely et al., 2008). In order to show whether the turbellarians phototactic behaviour is directed to optimize light perception of the symbionts, we used light of different wavelengths and intensities to trigger phototaxis.

Results

Light curve measurement

As a first step towards understanding the mutualism between *S. roscoffensis* and *T. convolutae* optimal light intensity for photosynthesis in the algae was determined. The electron transfer rate (ETR) of photosystem II was recorded as a function of varying intensities of photosynthetic active radiation (PAR) in the range of 2 to 105 µmol m^{-2} s^{-1} photons using chlorophyll fluorescence (n = 6 repeats, Fig. 1). While an almost linear correlation of ETR with irradiation was observed for photon flux densities below 40 µmol m^{-2} s^{-1}, onset of saturation became obvious for intensities higher than 50 µmol m^{-2} s^{-1}. The obtained data for *T. convolutae* and two closely related algae, *T. striata*, and *T. suecia*, were fitted to an exponential growth curve (Equation 1), for details please see Table 1. Interestingly, the minimal light intensity for an ETR larger than zero calculated from the exponential growth curve solved for PAR (Equation 2) revealed that already at light intensities above 0.112 µmol m^{-2} s^{-1} a positive ETR could be detected for isolated algae, clearly demonstrating a low light adaptation of *T. convolutae*.

Table 1: Photosynthetic parameters obtained for, *T. convolutae, T. striata,* and *T. suecia* (data are given with mean ± SE)

	T. convoluta	*T. striata*	*T. suecia*
ETR_0 [µmol m^{-2} s^{-1}]	-0.0290 ± 0.2444	0.0745± 0.1722	0.1719 ± 0.2713
ETR_{max} [µmol m^{-2} s^{-1}]	11.38 ± 0.6107	20.3466 ± 1.9507	18.7784 ± 2.2331
k	0.0227 ± 0.0032	0.0104 ± 0.0017	0.0121 ± 0.0026

$$ETR = ETR_0 + ETR_{max} * (1 - e^{(-k*PAR)}) \quad (1)$$

Equation 1: Exponential growth curve with maximum: ETR, ETR_0, ETR_{max}, PAR and k are the electron transfer rate as dependent parameter, electron transfer rate at zero light, maximal electron transfer rate, photosynthetic active radiation as independent parameter, and a constant factor, respectively.

$$PAR = \ln(1-(ETR-ETR_0)/ETR_{max})/(-k) \quad (2)$$

Equation 2: Equation 1 solved for PAR

Measurement of photosynthetic oxygen formation in isolated algae

Oxygen formation was measured using suspensions of algae isolated from the symbionts to gain information about the aptitude of the light sources for photosynthesis (Fig. 2). The oxygen concentration of the medium increased with a rate of 0.19 ± 0.12 µmol O_2/(l s $g_{biomass}$ PAR_{LED}) for blue light and 0.50 ± 0. µmol O_2/(l s $g_{biomass}$ PAR_{LED}) for red light, respectively. A t-test revealed a significantly higher oxygen evolution rate for red as compared to blue light (p = 0.043, n = 5).

Behaviour of *S. roscoffensis* exposed to varying light regimes

To test whether symbiotic *S. roscoffensis* preferred illuminated areas over shaded ones, the animals (n = 57) were exposed to three different light colours each at a photon flux density of 5 µmol m^{-2} s^{-1}. Setup of experiments is depicted in Fig. 3. A significantly positive response to illumination, i.e. a higher allocation time in illuminated vs. shaded areas, was observed for green and blue light but not for red light (Fig. 4). In red light, average stay time was 510 ± 257 s vs. 385 ± 255 s in shaded area (p = 0.08), while the allocation time for green light was 566 ± 200 s vs. 333 ± 201 s in shaded area (p = 0.00005) and 550 ± 182 s in blue light vs. 348 ± 183 s in shaded area (p = 0.0002).

When the allocation times in areas illuminated by different light sources were compared in an ANOVA on ranks, no effect of light colour could be revealed.

When the animals (n = 40) had the choice between all three colours and a shaded sector at the same time, the blue area was the most preferred with a mean stay time of 455 s ± 193 s, followed by 162 s ± 114 s for green, 130 s ± 99 s for red and 131s ± 138 s for shaded sectors, respectively (Fig. 5). Even if the blue light was less intense, it was still preferred over a brighter red sector (n=30) (Fig. 6). The allocation time in the blue sector of the arena was significantly longer as compared to the red part with 600 ± 274 s vs. 239 ± 274 s (p = 0.006).

Behaviour of *S. roscoffensis* within a light gradient

The behaviour of *S. roscoffensis* (n = 18) within a light gradient was tested in petri dishes with 3.2 cm diameter. The arena was divided into two areas of the same size: a highly illuminated part and a part with the gradient itself, the umbra. Allocation times of 18 animals were recorded over 1800 s. The animals preferred the fully illuminated half of the arena with an average allocation time of 1235 s ± 732 s compared to 563 s ± 733 s in the umbra the illuminated area by trend ($p = 0.06$) (Fig. 7).

Over all activity of the turbellarians

All data from the previous experiments were analyzed to characterize the movement behaviour of *S. roscoffensis*. During 71 h observation time in total, the animals showed measurable movement for 34 h. The overall distance of all animals was 135.3 m, resulting in an average speed of 1.1 mm/s (3.9 m/h). With a probability of 99 %, the speed of motions was in the range of 4.5 mm/s (17.55 m/h) or below (Fig. 8).

Discussion

The aim of this study was to investigate the interaction between the host *S. roscoffensis* and symbiont *T. convolutae*, in order to investigate whether the host provides an optimal environment for photosynthetic activity of the algae. Regarding our light sources used for the experiment, the blue and red LEDs were suitable for algal photosynthesis, and an oxygen development was detectable. However, the blue light was exceptionally less efficient and the red light is the optimal colour for the green algae photosystems (Butler, 1978; Emerson and Charlton, 1943). Concerning light intensity, *T. convolutae* reaches its optimal electron transfer rate at relatively low light doses. This was also observed in this study by us for the closely related species *Tetraselmis striata* and *Tetraselmis suecica*. Other common zooxantheallae such as diatoms or dinoflagellates reach their peak activity at an illumination level of 500 to 1500 µmol m^{-2} s^{-1} (Geel et al., 1997; Guarini and Moritz, 2009) or 600 to 800 µmol m^{-2} s^{-1} (Rodríguez-Román and Iglesias-Prieto, 2005), respectively.

The eyes of plathelminthes are very simple organs, built of only one type of receptor cells half shaded by a pigment cup cell. The receptor cells do not have enlargement of the membrane surface like cilia or microvilli (Nilsson, 2009; Yamasu, 1991). These simple eyes are capable of detecting the direction of a light source (Jékely et al., 2008). Nilsson showed by simulations that eyes of this kind are able to find the direction of a light source in water depth of up to 200 m. Photoreceptors are proposed to originate from a single common ancestor and are all based on the pigment rhodopsin. They detect one specific wavelength according to the rhodopsin's absorption spectrum (Arendt, 2003; Shichida and Matsuyama, 2009). It is very likely, that *C. roscoffensis* only express one type of receptor and therefore is limited in its viable light spectrum. Depending on the water conditions, blue or green light will reach deeper areas in the sea, which makes light of shorter wavelength more important for phototaxis. *C. roscoffensis* lives in a tidal environment in water depth from about 0 to 4 m (Doonan 1982). The average light intensity in summer month in 4 m deep water is at about 5.47 % compared to the surface, but there is a significant difference in the transmission of different wavelength depending on the water type: The observed light transmissions were 67 %/m, 52 %/m blue and 55 %/m for green, blue and red light, respectively in summer in a tidal environment (Lüning and Dring, 1979).

Our experiment revealed that the host's reaction on light stimuli is not adjusted to the symbionts' needs. *S. roscoffensis* did not show a reaction on red light, but on blue and green light, while the symbiotic algae show the highest photosynthetic rate for red light.

Our understanding is that *S. roscoffensis* does not react on red light, because they are not able to detect it and therefore their eyes are not suitable for measuring the exact photosynthetically active radiation to the benefit of the photosymbionts. Nevertheless, perception of wavelengths that are not dominant in a specific depth may be useful for the detection of fluorescent light, e.g. for communication, which have been observed in different fish species (Michiels et al., 2008; Shcherbakov et al., 2013; Shcherbakov et al., 2012).

Considering light intensities, the animals moved in the areas with an illumination higher than optimal for the algae. Possibly, these light intensities may be even harmful for the photosynthetic apparatus, though they may use photoprotectants to avoid oxidative stress (Cruz et al., 2013; Cruz et al., 2015; Peers et al., 2009). However, *S. roscoffensis* is very mobile with speeds of up to 17.5 m/h and would be able to reach water depth providing *T. convolutae* with optimal light by phototaxis. A previously published study by Serôdio et al. suggest an active regulation of the photosynthetic activity (Serôdio et al., 2010). However, in their setup the light is coming from below with the gradient generated by a linear attenuation filter. Therefore, the animals trying to approach the light source will estimate it near the centre of the arena, maybe with a shift from the dark to bright side of the arena. This could in itself explain the result presented by Serôdio et al. without any active photosynthesis regulation. We avoided this problem with our system that is closer to the situation in nature with our light gradient generated with a light source illuminating the arena with an angle of 70 °. In our setup, we could not see any avoiding of high illumination, but further studies are necessary to determine whether the host's behaviour may even harm the photosynthetic symbionts in case of very a high illumination level on bright summer days. The saturation of the ETR was observed from Serôdio et al. for individual animals, with their results in the same range of our measurements with the separated algae.

Avoiding high levels of illumination is known for other symbiotic animals: Negative and positive phototaxis is known in symbiotic sea anemones *(Actiniaria* spec) depending on light intensities, e. g. negative phototaxis at a high illumination of 700 foot candle and positive phototaxis at 250 foot candle, which is about 140 PAR and 50 PAR, respectively (Pearse, 1974). Pearse noted that non symbiotic anemones showed negative or indefinite reactions on light stimuli, which implies a relation between host behaviour and phtotsynthesis of the symbiotic algae, but did not give any data about photosynthesis of the symbiotic algae. According to measurements made by other authors the high illumination is below the photosynthetic optimum of common zooxanthellae (Geel et al., 1997; Guarini and Moritz, 2009; Rodríguez-Román and Iglesias-Prieto, 2005). We speculate that this allows for keeping a safety margin in light exposure for the anemone's symbionts. Such behaviour was not seen in *S. roscoffensis.*

Doonan and Gooday (1982) reported a decreasing population of *S. roscoffensis* in summer months and speculated that this may be the result of high illumination levels, which can be up to 1200 µmol m^{-2} s^{-1} on summer days (Migné et al., 2004). An active regulation of the light exposure suggested by Serôdio et al. would avoid such a decline in the population. With respect the animal's inability to react on red light we observed in this study, we think that the position of Doonan and Gooday is more likely, but further studies are necessary to proof a harmful effect of high illumination levels in summer month.

Material and Methods

Animal and Algae Culture

The specimens of *S. roscoffensis* were obtained from the "Station Biologique de Roscoff" (Roscoff, France) and cultured with artificial seawater provided by the zoological and botanical garden "Wilhelma Stuttgart" (Stuttgart, Germany). Cultures were kept in petry dishes (Schott, Mainz, Germany) of different sizes in a climate chamber at 7 °C and a low level of illumination for 12 h per day, starting at 8 am (photosynthetically active radiation (PAR) 7.9 µmol m^{-2} s^{-1} photons). For the experiments, the animals were transferred to single wells of 24 well plates, and acclimated in a climate chamber at 16 °C for at least one day before the measurement. The illumination was 16.7 µmol m^{-2} s^{-1} photons for 12 h per day, starting at 8 am. Despite the rather short time for acclimatization the animals showed a normal behaviour, which is was expected as high temperature differences of up to 10 °C within a day are common in tidal environments (Morris and Taylor, 1983). All experiments were performed during standard laboratory work time from 9 am to 5 pm.

Symbiotic algae were extracted from naturally deceased worms by pipettes and transferred in 50 ml Erlenmeyer flasks (Schott, Mainz, Germany) with the Seawater Medium (SWES) according the protocol (version 10.2008) of the SAG (Culture Collection of Algae, Göttingen, Germany). For chlorophyll fluorescence measurements of the algae, fine biofilms were grown in 9 cm Petri dishes on 1 % agarose in the SWES solution.

Light sources for illumination experiments

The intensities of the different light sources (PAR) were measured with a quantum sensor "QS" (Delta-T Devices, Cambridge, England). LEDs of different colours were used to provide different wavelengths: Red (LED CQY 40 L, Osram, Munich, Germany, λ_{max} = 660 nm; spectral line half width $\Delta\lambda_{1/2}$ = 40nm), Green (LED CQY 72 L, Osram, Munich, Germany, λ_{max} = 560 nm; spectral line half width $\Delta\lambda_{1/2}$ = 20nm) and Blue (LED L-53MBDL, Kingbright Elec., Taipei, Taiwan, λ_{max} = 466 nm; spectral line half width $\Delta\lambda_{1/2}$ = 60nm). Each LED was run with a battery of its nominal voltage and controlled by a 1 kΩ potentiometer for adjusting an intensity of 5 PAR. LEDs were mounted in an interior reflector to provide a homogenously illuminated field that allowed the positioning with a well defined border between illuminated and shaded parts of the arena (SMZ1089, Signal-Construct, Niefern, Germany) Due to scattered light, the average brightness in the shaded sector reaches 63% of the average brightness in the illuminated sector, which was a good compromise for getting enough light for video recording and a behavioural responses of the animals. The response of the animals to different light intensities was tested in a light gradient. An adjustable light source from a binocular loupe (GSZ loupe, Zeiss Jena, Germany) was fixed with an angle of 70 ° in 20 cm height and a piece of aluminium foil on the upper half of the lamp for shading. The brightness in this gradient started at 24.92 PAR, peak value was 459.17 PAR within a Petri dish of 37 mm diameter.

Fluorescence measurements and oxygen evolution in response to different light regimes

The electron transfer rate (ETR) of photosystem II of isolated algae was measured at varying actinic light intensities using a PAM 2000 fluorimeter with the default settings "run 9, actinic light intensity series" (Heinz Walz, Effeltrich, Germany). The sensor had a distance of 1 cm to the surface of the algal biofilm.

Oxygen measurement for measuring the reaction of algae on different wavelength

Oxygen evolution by suspensions of algae isolated from the symbionts was measured with a Clark type electrode (Type S1, Hansatech, Northfolk, England). The rate of photosynthesis following illumination by blue and red diodes, respectively, was determined as the increase of the oxygen concentration in the suspension per dry weight of algal biomass, measured after drying the algae two days in a "SpeedVac" concentrator (Savant, Fisher Scientific, Schwerte, Germany). Five replicates, 0.5 ml each, of algal suspension were tested at a photon flux density of 25 µmol m^{-2} s^{-1} (blue) and 38 µmol m^{-2} s^{-1} (red).

Videotracking of *S. roscoffensis*

The behaviour was recorded with a standard camcorder (Sony DCR-PC 109 E; Sony, Tokyo, Japan) using the software Virtualdub v1.8.6 (www.virtualdub.org) (Fig. 3). The video frame rate for recording and analysis was one picture per second. The location coordinates of *S. roscoffensis* were evaluated as the allocation time in different sectors of the arena by the program BioMotionTrack D.S. (Shcherbakov et al., 2010) and stored into an Access (Microsoft, Redmond, USA) database. The response to the different wavelengths was tested for 900 s at an illumination of 5 PAR in petri dishes with a diameter of 15.5 mm and a water high of 5 mm sea water. The measurements within the light gradient were performed in Petri dishes 37 mm diameter and 5 mm water high for 1800 s. The behaviour on different wavelength was tested in a series of experiments by comparing the length of stay in within the illuminated and not illuminated half of the arena. In a second series, experiments were performed with sectors representing each of the three wavelengths plus a fourth not illuminated, shaded sector.

For testing the effect of light intensity, the blue light source was adjusted to a photon flux density of 5 µmol m^{-2} s^{-1}, while the red LED (660 nm) was operated at 44 µmol m^{-2} s^{-1}. The total irradiance for the blue LED was 1.3 mW m^{-2} and for the red light source 7.6 mW m^{-2}. The conversion from photon flux density to radiation power was calculated for the dominating wavelength (Schröder and Treiber, 2007). During the behavioural experiments, the light sources were used in random order. To characterize motivation for active orientation behaviour animals' speed of motions was additionally calculated.

Statistics

Statistical analysis of allocation time data was performed using either the Exact Wilcoxon signed rank test with continuity correction for pairwise analysis or an ANOVA on ranks. Statistical significance of differences in the oxygen evolution was tested using the t-test. Significance was assumed for $p < 5$ in all statistic tests. The date for electron transfer rates were fitted with an exponential growth function with maximum. All statistics were performed with Sigmastat. Values are given as means ± SD.

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Figures:

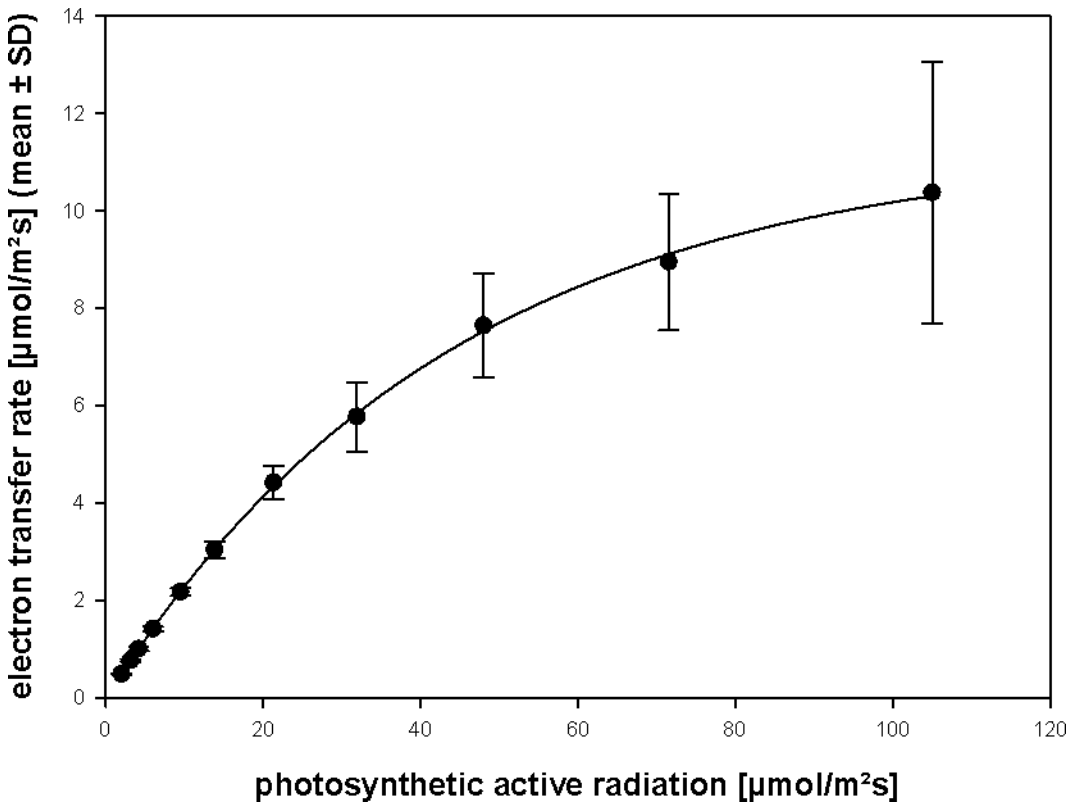

Fig. 1: Electron transfer rate of *T. convolutae* in µmol m^{-2} s^{-1} (mean ± SD) vs. photosynthetic active radiation in µmol m^{-2} s^{-1} photons (n=6).

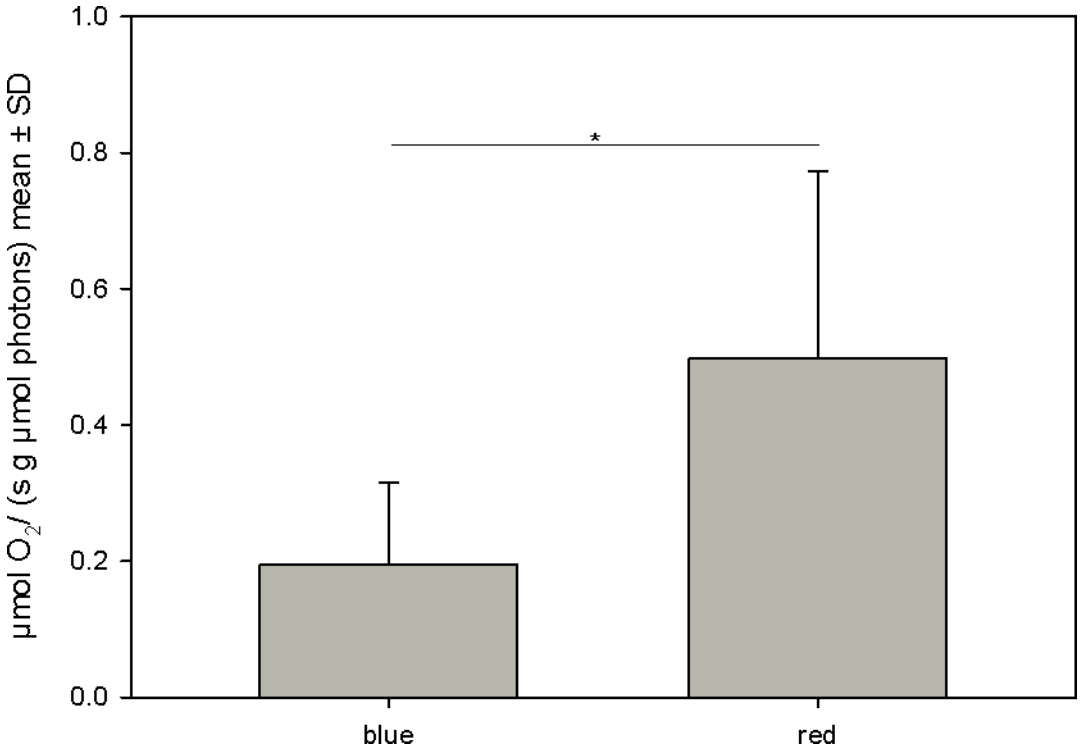

Fig. 2: The measured oxygen generation of free living *T. convolutae* per s, biomass and illumination in µmol O_2/(l s $g_{biomass}$ PAR_{LED}) (mean ± SD) for red and blue light. The difference is significant (p = 0.043 with t-Test, n = 5). Significant differences are marked with an asterisk.

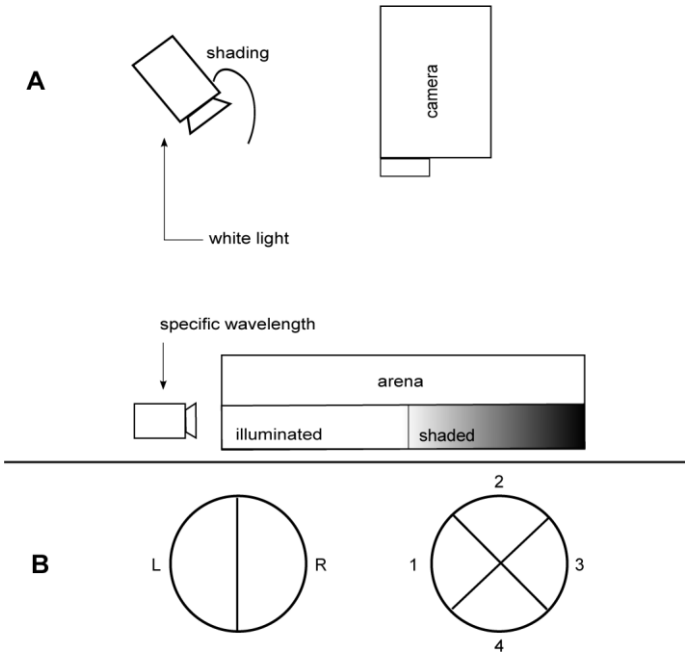

Fig. 3: A: Sketch of the experimental setup's side view with the arena, light source, and video camera. The brightness is indicated below the arena.

B: Sketch of the arena partitioning with two or four sectors. Possible lamp positions are marked with letters (L and R for left and right, respectively) or numbers (1 to 4) for the two and four sector setups, respectively. The two sector version was used for experiments with one or two light sources (results shown in Fig. 5, 6 and 7), the four sector version was used for experiments with 3 light sources and a shaded control sector (results shown in Fig. 6).

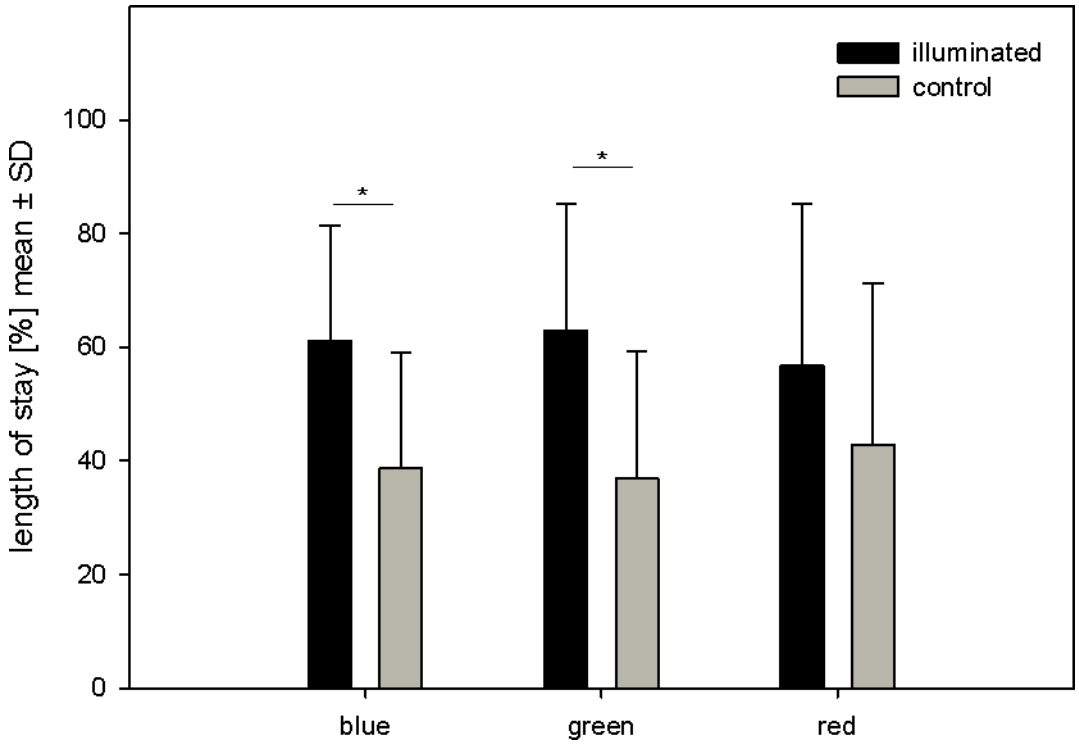

Fig. 4: Behaviour of *S. roscoffensis* (n=57) on a blue, green and red light (5 PAR each): allocation time in illuminated and shaded half of the arena (%) (mean ± SD), 900 s investigation time per colour and animal. Only the allocation preference into the direction of blue and green light is significant. Significant differences are marked with an asterisk.

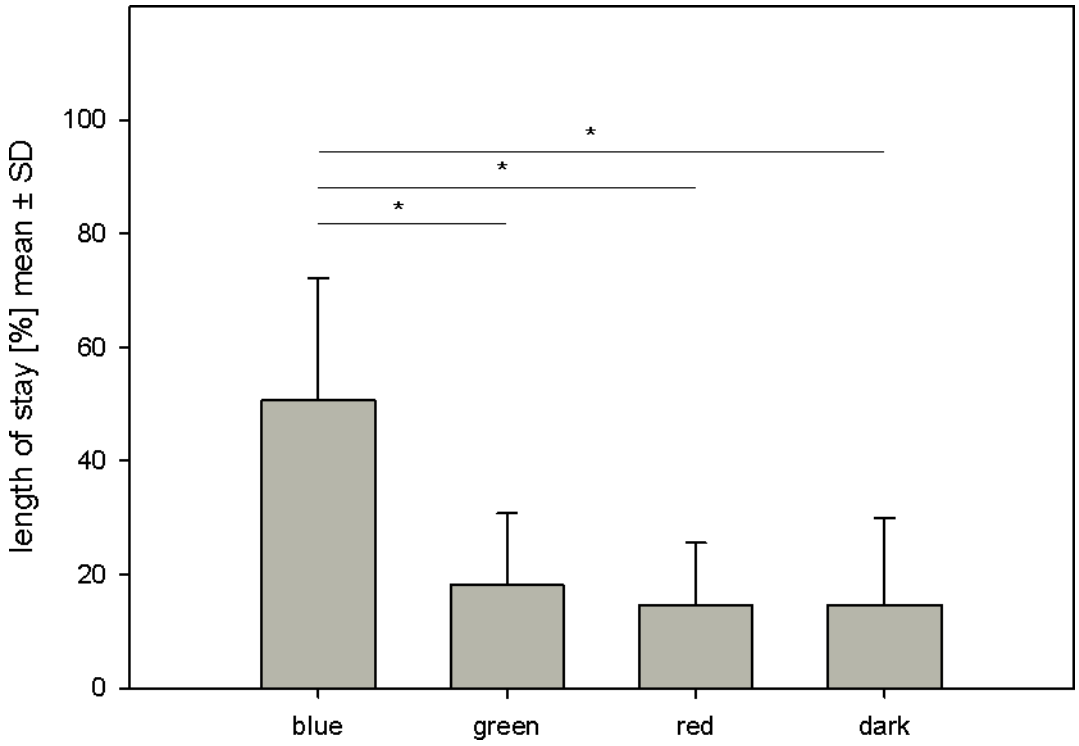

Fig. 5: Allocation time of *S. roscoffensis* (n=40) in three arena sectors illuminated by three different light sources compared to a shaded control sector. Observation time was 900 s. The influence of the illumination on the allocation time in the four sectors is significant ($p < 0.001$, ANOVA on ranks). The allocation time in the blue sector is significantly higher then in all other sectors ($p < 0.05$, Tukey Test), while there is no significant difference between the green, red and shaded sectors. Significant differences for a specific pair of sectors are marked with horizontal lines and asterisks.

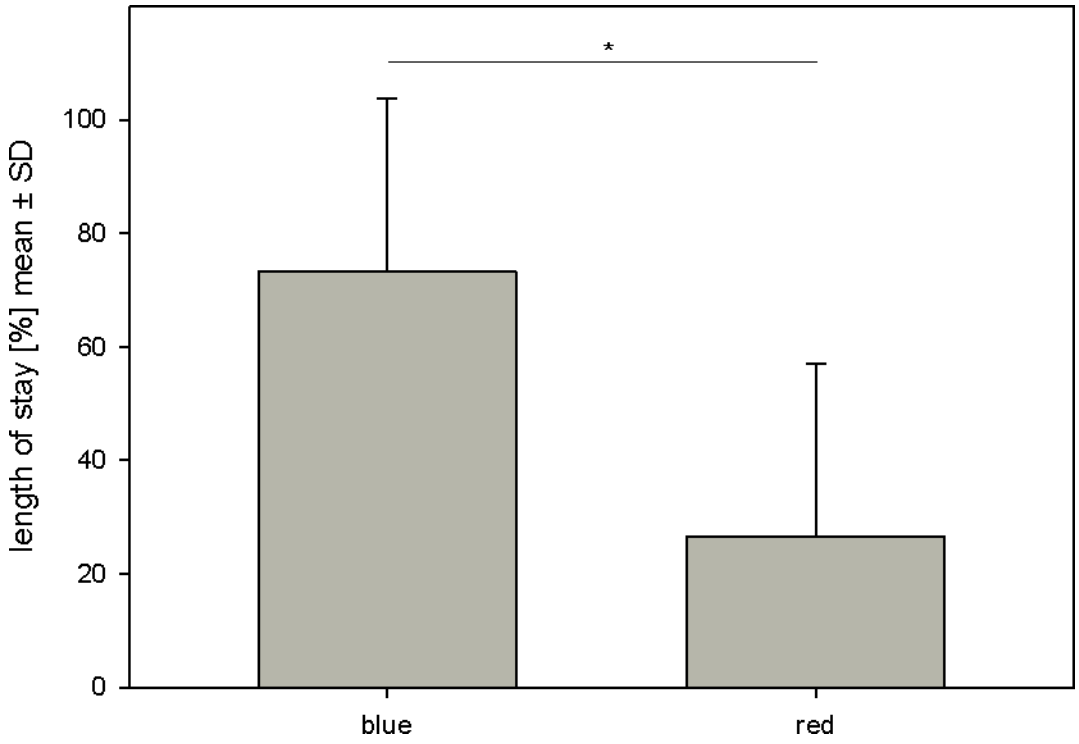

Fig. 6: Allocation time of *S. roscoffensis* (n=30) in two arena sectors illuminated by blue light (photon density 5 µmol/m²s) and red light (44 µmol/m²s), respectively. Observation time was 900 s. The length of stay in the blue illuminated half of the arena was significantly longer ($p = 0.006$ in Wilcoxon signed rank test with continuity correction). Significant differences are marked with an asterisk.

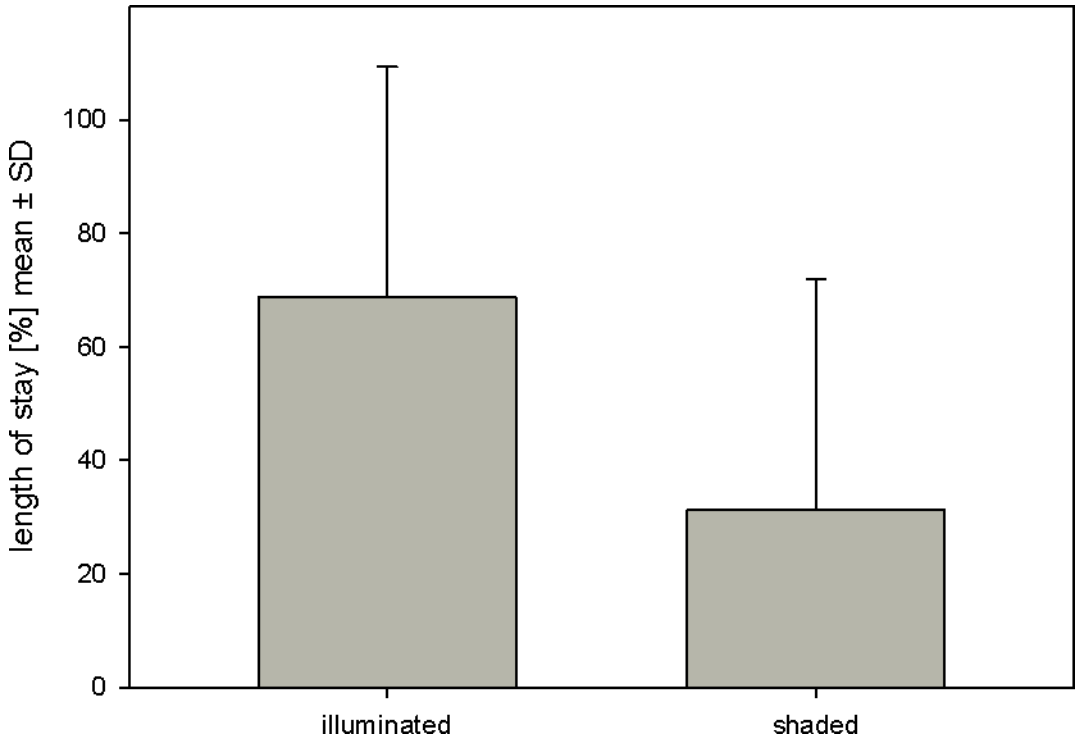

Fig. 7: Allocation preference of *S. roscoffensis* (n=18) in a light gradient, allocation time in the full illuminated half of the arena vs. the shaded half of the arena. Observation time was 1800s. The length of stay in the illuminated half of the arena is by tendency higher (p = 0.06 Exact Wilcoxon signed rank test).

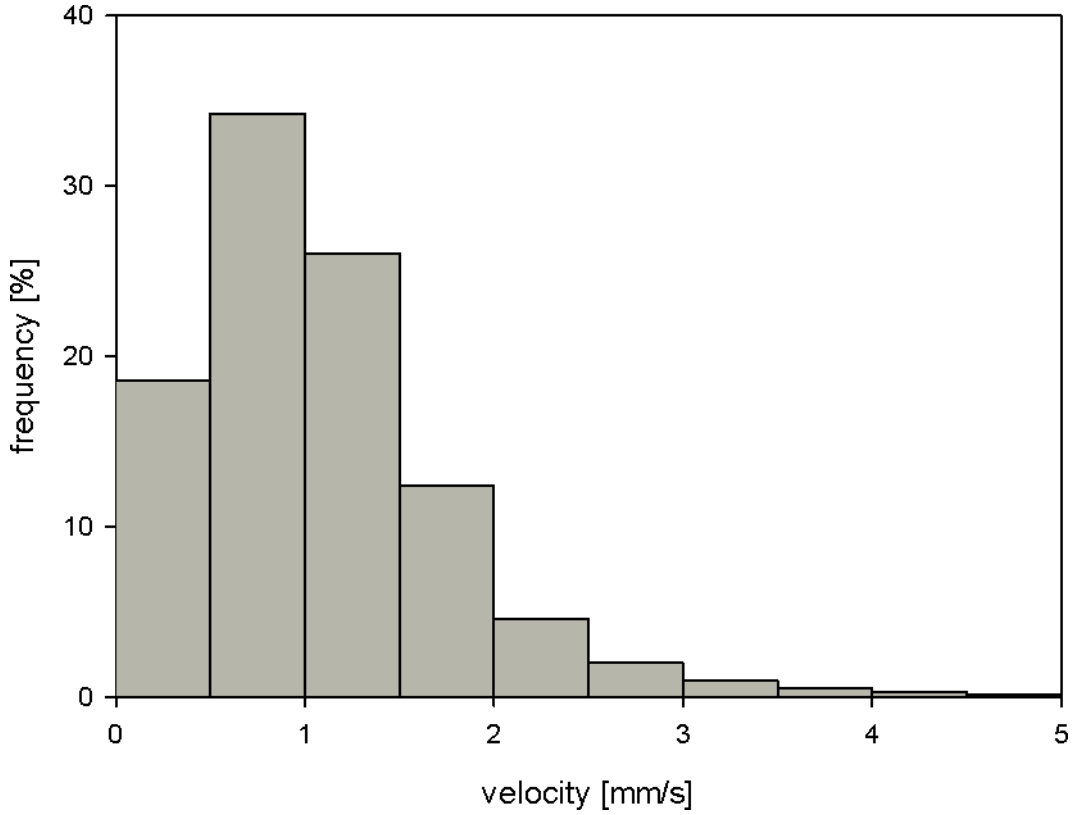

Fig. 8: Frequency distribution (%) of moving animals' speed of motions [mm/s] in steps of 0.5 mm/s. In 99 % the speed of motions is in the range up to 4.5 mm/s.