

SHORT COMMUNICATION

Wavelength-specific negatively phototactic responses of the burrowing mayfly larvae *Ephoron virgo*Ádám Mészáros^{1,2}, György Kriska^{1,3,4} and Ádám Egri^{1,4,*}

ABSTRACT

Mayflies are typically negatively phototactic during larval development, whereas the adults possess positive phototaxis. However, no extensive research has been done into the wavelength dependence of phototaxis in any mayfly larvae. We measured the repellency rate of *Ephoron virgo* larvae to light as a function of wavelength in the 368–743 nm spectral range. We established that the magnitude of repellence increased with decreasing wavelength and the maximal responses were elicited by 400 nm violet light. This wavelength dependence of phototaxis is similar to the recently reported spectral sensitivity of positive phototaxis of the twilight-swarming *E. virgo* adults. Negative phototaxis not only facilitates predation evasion: avoidance of the blue-violet spectral range could also promote the larvae to withdraw towards the river midline in the case of a drop in the water level, when the underwater light becomes enriched with shorter wavelengths as a result of the decreasing depth of overhead river water.

KEY WORDS: Visual ecology, Insect vision, Spectral sensitivity, Phototaxis, Nymph

INTRODUCTION

Mayflies are semi-aquatic insects mostly known for their extremely short adult lifespan (Sartori and Brittain, 2015). Their larvae possess compound eyes (Gillott, 2005; Gupta et al., 2000; Kühne et al., 2021), which implies a relatively sophisticated vision. The first mention of the responses of mayfly larvae to light stimuli was provided by Wodsealek (1911), who showed that larvae of *Heptagenia interpunctata* (= *Stenacron interpunctatum*) possessed negative phototaxis. Shortly afterwards, the same was reported for the larvae of *Chiroteneles albomanicatus* (Clemens, 1917) (= *Isonychia bicolor*) and *Ephemer danica* (Percival and Whitehead, 1926). During subsequent decades, larvae of additional mayfly species were shown to be negatively phototactic and active mostly at night in darkness (Bailey, 1981; Chapman and Demory, 1963; Elliott, 1968).

The first phototaxis experiment with mayfly larvae in which the spectral properties of the stimulating light were varied was carried out by Heise (1992). He established that *S. interpunctatum* and *Stenonema vicarium* (= *Maccaffertium vicarium*) nymphs were

repelled by green and red light, but infrared light did not elicit any reaction. In contrast, Barmuta et al. (2001) showed that *Nousia* sp. (Ephemeroptera: Leptophlebiidae), similar to findings for infrared stimulation, did not respond to green and red light (at $1.08\text{--}1.14 \times 10^{15}$ photons $\text{cm}^{-2} \text{ s}^{-1}$), which indicates that not all mayfly larvae are negatively phototactic. Moreover, *Baetis harrisoni* turned out to be positively phototactic in flowing water under laboratory conditions and this finding was confirmed also in the field (Hughes, 1966a). In the latest study on the subject, with the use of underwater light traps emitting blue, green, yellow and red quasi-monochromatic light, Kühne et al. (2021) demonstrated that certain unidentified Ephemeroptera larvae were attracted to green, yellow and red light but not to the shorter wavelength blue light.

Similar to *E. danica*, *Ephoron virgo* (Olivier 1791) mayflies belong to the Ephemeroidea superfamily of Ephemeroptera, the so-called burrowing mayflies. Their larvae live in cavities in the river bottom and feed on filtered organic particles (Kazanci, 2013). During field observations, we observed that their larvae avoid light (Egri et al., 2022), as described for *E. danica* (Percival and Whitehead, 1926).

Contrary to the majority of mayfly larvae, adult mayflies are rather positively phototactic (Fremling, 1960; Pohe et al., 2018; Tojo et al., 2006), including the adults of *E. virgo*, which are well known for their mass swarmings in light-polluted urbanized areas all over Europe (Egri et al., 2017a). Although spectral-specific phototactic behaviour of mayfly adults has been investigated in relatively few cases, studies have shown that light sources with short wavelength-dominated emission spectra are the most attractive for certain mayflies (Durmus et al., 2021; Pohe et al., 2018). In field experiments applying quasi-monochromatic equal-intensity light sources, Mészáros et al. (2021) found that attraction of *E. virgo* adults to light increases with decreasing wavelength; thus, blue and UV light are most attractive for these mayflies. Another recent study suggests that both larvae and adults of *E. virgo* possess at least two kinds of photoreceptor, a UV- and a green-sensitive type (Egri et al., 2022). In the present study, we measured the spectral sensitivity of the phototaxis of *E. virgo* larvae, which resulted in the most detailed measurement of this kind to date on the larvae of a mayfly species.

MATERIALS AND METHODS

Larvae

Ephoron virgo is protected in Hungary, and therefore permission for the collection of individuals was obtained from the relevant local authority (Government Office for Pest County, Hungary; document no. PE-06/KTF/04563-6/2020 and PE-06/KTF/04563-7/2020). *Ephoron virgo* larvae were collected from the river Ipoly (northern Hungary, 47°53'08.2"N 18°45'48.0"E) on mornings in June and August in 2020 and 2021. Trials were performed with freshly collected individuals. Females and males were not distinguished during the trials.

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Phototaxis experiment

The repellency rate of *E. virgo* larvae (action spectrum of negative phototaxis) to light as a function of wavelength was measured in the 368–743 nm spectral range. The experiment consisted of trials performed in an arena with a light source positioned at one end. The arena was an elongated, extremely shallow Plexiglas aquarium with a size of 45 cm×9 cm×1 cm (length×width×depth) half-filled with dechlorinated tap water. The arena was placed on a black cloth to avoid the ‘dorsal light response’ (Hughes, 1966b) which can occur if the bottom is bright. The light source was a custom-built LED-based one that was able to create quasi-monochromatic light stimuli at 14 different wavelengths with variable light intensity. The stimulus was a homogeneously illuminated circular area (diameter 10.6 cm) and the available wavelengths (±half bandwidth of LED) were 368 nm (±6.8 nm), 389 nm (±5.9 nm), 400 nm (±6.4 nm), 423 nm (±8.1 nm), 436 nm (±8.3 nm), 455 nm (±12.7 nm), 469 nm (±11.1 nm), 497 nm (±14.5 nm), 517 nm (±17.3 nm), 552 nm (±18.2 nm), 596 nm (±7.9 nm), 630 nm (±8.6 nm), 665 nm (±10.4 nm) and 743 nm (±11.0 nm). More details about the lamp are provided by Egri et al. (2024).

Trials were conducted in a darkened room at 25±1°C with larvae dark-adapted for at least 45 min. In each trial, a single larva was placed in the arena centre and was covered with a black opaque cap. Once the desired wavelength and light intensity were set, the larva was released by removing the cap. At the same time, a webcam modified for infrared sensitivity took photographs of the arena from above with a rate of 1 image per second. A 940 nm LED strip provided illumination for the webcam. According to Egri et al. (2022), the larvae do not sense this infrared background illumination. A total of 15 photos were taken to record the movement of the larva.

In the case of all 14 available wavelengths, three different, logarithmically increasing light intensities, 9.48×10^{11} , 9.48×10^{12} and 9.48×10^{13} photons cm⁻² s⁻¹, were used for creating light stimuli. These values were calibrated at the arena centre. Trials were performed in blocks. Each block comprised 15 randomly ordered trials, 14 with a given wavelength at a fixed light intensity and a control trial during which the light source was switched off. The purpose of the dark trials was to test whether the darkness was good enough to prevent any directed movement from the larvae. Because of the limited number of individuals we were allowed to collect, only 20 blocks of trials could be performed. Thus, 20 (blocks)×15 (14 wavelengths+1 control)=300 trials were made. A given individual was used only once in the entire study. Thus, a total number of 300 specimens were tested.

Data evaluation

For each trial, the horizontal position of the larva in the corresponding 15 photos was extracted manually in pixel units and these positions were averaged. By taking the average, all 15 recorded larval positions contributed to the final quantified response of the larva, resulting in minimal loss of information. This mean horizontal position x was then linearly rescaled in such a way that -1, 0 and 1 corresponded, respectively, to the arena terminal where the light source was located, to the arena centre and to the darker arena terminal. For example, $x=0.5$ means that the mean larval position in the 15 s long trial was exactly halfway between the arena centre and the darker arena terminal. Also note that positive and negative x -values mean negative and positive phototactic reactions, respectively. Finally, the response of a given specimen was this x value.

Calculation of the action spectrum of phototaxis was almost completely identical to the method of Egri et al. (2020) and Kecskeméti et al. (2021). For a given wavelength, all x -values were plotted against stimulus light intensity and the Naka–Ruhston function (James et al., 2016) was fitted on the points, which takes a sigmoid shape when the light intensity is in logarithmic scale:

$$x(I) = x_{\max} \frac{I^n}{I^n + k^n}. \quad (1)$$

Here, I is the light intensity of the stimulus, $x_{\max}$ is the maximal elicited response, k is the stimulus light intensity at $x_{\max}/2$, and n is related to the slope of the function. This formula was appropriate because under darkness/control conditions when $x(I=0)=0$, we indeed expect that the mean larval position is at the arena centre. By expecting repellence for all wavelengths, which was verified after the experiments, and taking into account that the larvae could not leave the arena, the constraint $x_{\max}=1$ simplifies Eqn 1. Hence, two parameters, k and n , were left to be fitted for each wavelength.

When all of the 14 sigmoid exposure–response curves were fitted, the spectral sensitivity of phototaxis was determined by calculating, for each wavelength, the reciprocal of the stimulus intensity required to evoke a critical response magnitude. For the critical response magnitude, $x_{\text{crit}}=0.4728$ was chosen, being the median of all 300 mean larval positions that were registered in the trials.

To test how sensitive the shape of this action spectrum of phototaxis is to the x_{crit} critical response value, this calculation was carried out for 20 additional critical response values in the $x_{\text{crit}} \pm 20\%$ range. In other words, the whole calculation was repeated 20 times with perturbed critical response values distributed evenly around x_{crit} . Finally, the resulting 21 action spectra were plotted in a box plot after being normalized with the median of curve points at 400 nm.

Statistics

Because the x -values for the control trials were not normally distributed, one-sample Wilcoxon signed rank test was performed for checking whether they really scatter around zero. For each wavelength, to test whether a given light stimulus elicited responses significantly differing from the control responses, Dunn’s non-parametric many-to-one comparison test with Bonferroni correction was performed (PMCMRplus R library). Statistical tests were made with the R statistical package v4.2.1 (<http://www.R-project.org/>).

RESULTS AND DISCUSSION

For all wavelengths the larval phototactic responses increased with increasing stimulus light intensity (Fig. 1A–N). Accordingly, *E. virgo* larvae were repelled by light at all wavelengths. According to Dunn’s non-parametric many-to-one comparisons, as stimulus intensity increased, the distribution of x -values significantly differed from that of the dark trials (Fig. 1O). This was true for all wavelengths except 517 nm and 743 nm (Fig. 1I and N). The case of 743 nm can be explained by the practically zero infrared sensitivity of the compound eyes (Egri et al., 2022). The fact that 517 nm light could not elicit a significant reaction from the larvae might originate from the relatively low number of animals tested. As (i) the emission spectrum of the neighbouring LEDs markedly overlapped each other, (ii) the spectral sensitivity of visual pigments is usually wider than the spacing of the peak wavelength of our LEDs and (iii) the neighbouring wavelengths elicited significant negatively phototactic reactions for increased stimulus intensities, it is reasonable to assume that 517 nm light was also

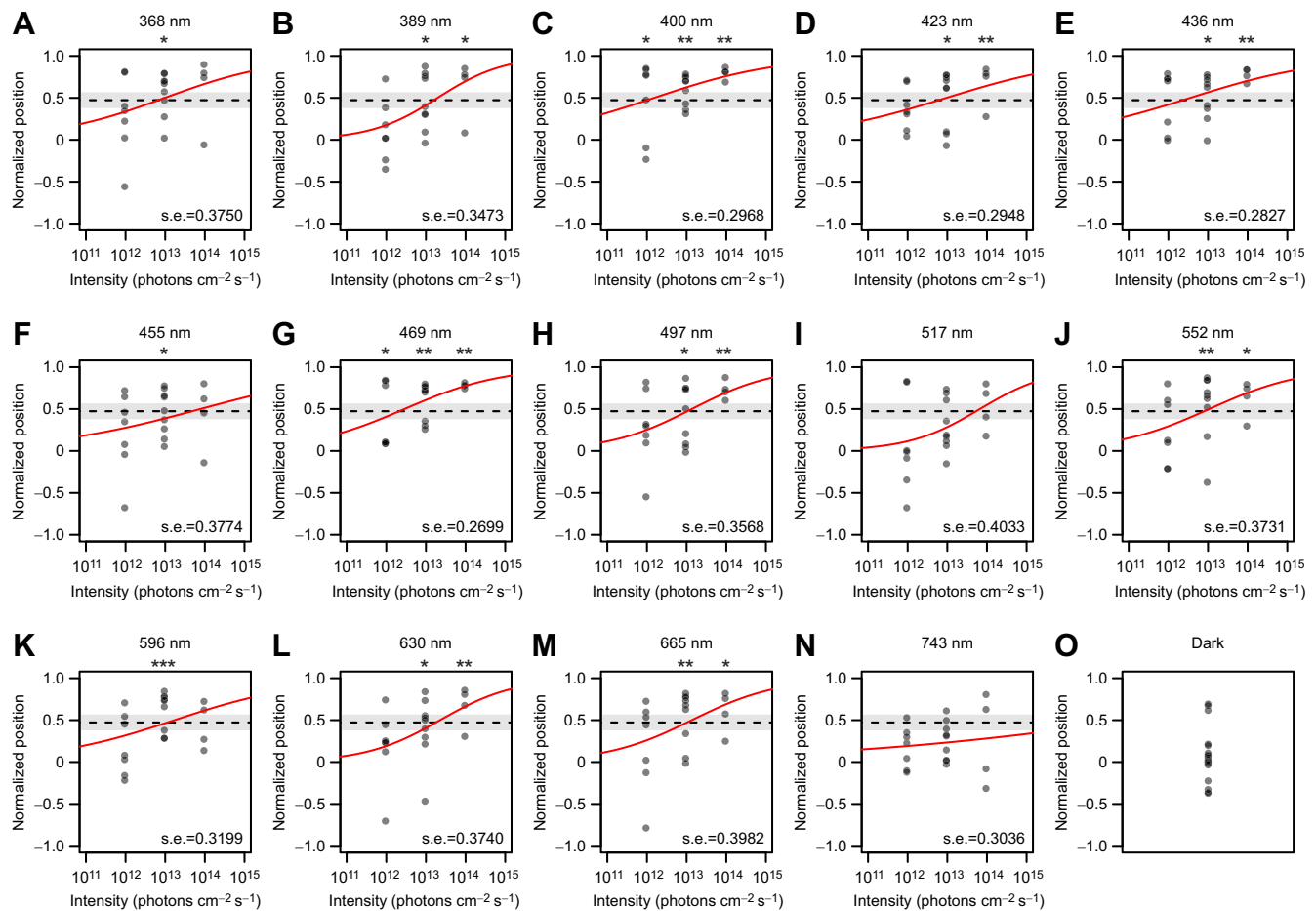


Fig. 1. Mean position of *Ephoron virgo* larvae in the trials as a function of photon flux. (A–N) Larval responses to the different wavelengths and intensities ($N=7$, 9 and 4 trials). (O) Responses obtained for the 20 control trials performed in darkness. Positive and negative values correspond to repellence and attraction, respectively. Red curves are the fitted Naka–Ruhston functions, and s.e. is the residual standard error of the fit. Dashed horizontal lines represent the critical response magnitude $x_{crit}=0.4728$. Grey regions around x_{crit} indicate the range in which 21 evenly spaced critical response values were used for calculating separate phototaxis action spectrum curves. Asterisks indicate the distribution of responses significantly differing from the distribution of control responses according to Dunn's non-parametric many-to-one comparison tests performed with Bonferroni correction (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

repellent for *E. virgo* larvae. According to the one-sample Wilcoxon signed rank test, the distribution of larval responses in the darkness did not differ significantly from zero ($V=134$, $P=0.2943$). Consequently, the Naka–Ruhston function (Eqn 1) being practically zero for very low hypothetical stimulus intensities was appropriate to be fitted to the data.

The rate at which *E. virgo* larvae were repelled by light generally increased as the wavelength decreased (Fig. 2). The negative phototaxis was negligible for long wavelengths above 500 nm, but started to increase below 500 nm and became most expressed around 400 nm. The high variability in the action spectrum of phototaxis (Fig. 2) was certainly caused by the relatively low number of total larvae tested. Nevertheless, the general trend in the repellence as a function of wavelength was visible. This trend remained the same when trials performed with not significantly repulsive wavelengths (e.g. 517 nm) were excluded from the calculation of the action spectrum of phototaxis. For comparison, compound eye spectral sensitivity of *E. virgo* larvae (Egri et al., 2022) and the attraction rate of adults to light as a function of wavelength (Mészáros et al., 2021) are also shown in Fig. 2. The wavelength dependence of the magnitude of phototaxis is qualitatively similar for larvae and adults, with the only remarkable

difference that adults are attracted to light (Mészáros et al., 2021). We measured the strongest light avoidance of larvae at wavelengths between the two sensitivity peaks of the bimodal spectral sensitivity of the compound eyes; however, the strongest repellence was obtained close to the UV sensitivity peak of larvae (Fig. 2). This behaviour could (i) be simply driven by a short-wavelength receptor or (ii) indicate the presence of an opponency mechanism, in which short- and long-wavelength photoreceptor inputs are compared. This should be tested in a future study.

Negative phototaxis is a typical behaviour of bottom-dwelling invertebrates allowing the avoidance of predation (Peckarsky, 1982). Although there are exceptions, most mayfly larvae also avoid light and the ecological significance of this behaviour might not only be predation related. Light absorption in rivers where *E. virgo* larvae develop increases with decreasing wavelength. This means that the optical environment of the larvae typically lacks blue and UV light, because these components get filtered out first (Egri et al., 2022). The greater the depth of the observer, the more long wavelength shifted the spectral composition of the visual environment, and the light levels decrease with increasing depth. In such a spectral and intensity gradient, a larva possessing negative phototaxis

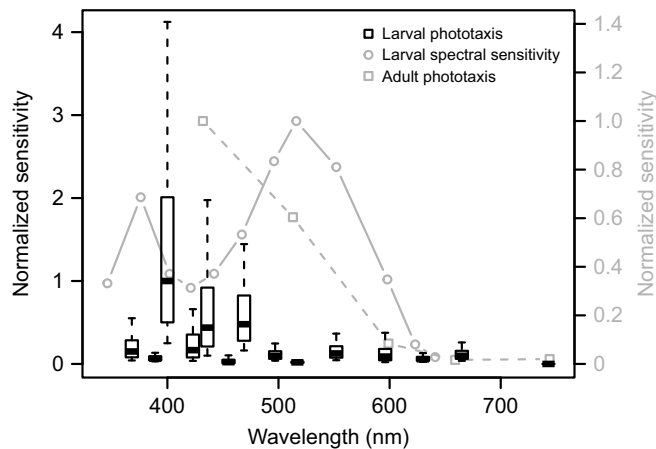


Fig. 2. Comparison of spectral sensitivity of larval phototaxis with that of adults and larval eye spectral sensitivity. Box plots (median, upper and lower quartiles and $1.5\times$ interquartile range) of 21 calculated action spectra of negative phototaxis of *E. virgo* larvae for each tested wavelength in the present study. Each box consists of 21 points, because the critical response magnitude was perturbed 20 times around x_{crit} . Grey circles indicate the compound eye spectral sensitivity of *E. virgo* larvae (Egri et al., 2022). Grey squares are the attraction rate of *E. virgo* adults to light as a function of wavelength (Mészáros et al., 2021).

characterized by our results should feel more and more comfortable when moving deeper. Nevertheless, *E. virgo* larvae prefer the littoral zone not deeper than approximately 6 m (Marković et al., 2017). Because a significant portion of the larval diet is benthic algae (Kureck et al., 2014; Marković et al., 2017), we speculate that the behaviour of the larvae is both light and food related. As benthic algal production linearly scales with light intensity (Duffer and Dorris, 1966; Gallegos et al., 1977), food may remain restricted to the littoral zone. Thus, *E. virgo* might seek an optimal position where food is still available, but the water surface is not too close. This behaviour could provide a safety distance from the shore in the case of a sudden water level drop. Here, we propose that the increase in light intensity and the spectral shift towards shorter wavelengths might be warning indicators of a water level decrease, which could trigger the migration of larvae towards the river midline.

Mészáros et al. (2021) showed that attraction of *E. virgo* adults to light is highest for short wavelengths, which is a strikingly similar wavelength dependence to the one we obtained for the negative phototaxis of the larvae. Thus, our results suggest that the spectral sensitivity of phototaxis in *E. virgo* during their lifespan has a qualitatively constant shape as a function of wavelength, but its directionality around the larva-to-adult transition changes from avoidance to attraction. When does the phototaxis transform from negative to positive? Just before emergence, the larvae leave the river bottom and swim to the surface. Firstly, this is facilitated by the inflation of the abdomen with air (Lancaster and Downes, 2013). Secondly, we suggest that attraction to light could also guide the larvae upward to the surface. A larva just departing from the bottom must be a newly developed adult still wrapped in its larval skin. The fact that *E. virgo* adults are positively phototactic (Egri et al., 2017a) supports the assumption that during the final minutes of the larval stage, positive phototaxis could play a significant role in emergence at the surface.

It is known that exogenous factors can alter the phototactic behaviour of certain pond-dwelling mayfly larvae. Under anoxia, originally negatively phototactic *Cloeon dipterum* larvae become

positively phototactic (Nagell, 1977), and the same has been reported for the larvae of *Leptophlebia vespertina* (Brittain et al., 1981). Under natural conditions, this occurs when the oxygen concentration diminishes in a pond covered with ice, and the mayflies tend to congregate around the shore just under the ice layer, where the oxygenated meltwater accumulates and the oxygen demand is lower than in the 3–4°C warmer deep regions. As *E. virgo* larvae inhabit rivers, oxygenation is typically facilitated by the natural flow of water. During winter, when there is chance for the formation of ice coverage, not only ponds but also rivers can also undergo oxygen depletion (Whitfield and McNaughton, 1986), although the eggs of *E. virgo* are in diapause in winter and hatch in spring (Cid et al., 2008). In our experiments, the arena contained still water, but it was extremely shallow (depth=0.5 cm) compared with the water surface area (405 cm²), which increased the chance of good oxygenation. It would be an intriguing experiment to test whether low oxygen levels induce a reversal in the direction of phototaxis in *E. virgo* larvae.

The present study on larvae and our preceding studies on the adults and larvae of *E. virgo* (Egri et al., 2022; Mészáros et al., 2021) revealed details about the visual ecology of this species. However, the most intriguing question remains largely unanswered. What triggers the synchronous emergence of enormous numbers of adults in a well-defined short time frame of twilight? Temperature and light intensity might be significant factors in initiating swarming (Sartori and Brittain, 2015). A smooth decrease in light intensity could indeed be a reliable indicator of twilight which could be monitored by the larvae. Larvae of a heptageniid mayfly species, *Stenonema modestum* (= *Maccaffertium modestum*), were shown to be able to discriminate between rapid light intensity changes caused by cloud movement from long-term intensity changes caused by the setting or rising sun (Schloss and Haney, 2006). Future research should also test whether the spectral composition of the twilight sky plays a role in the initiation of emergence in twilight-swarming mayflies.

Because *E. danica* larvae are also negatively phototactic (Percival and Whitehead, 1926) and typically swarm around twilight (Egri et al., 2017b), the wavelength dependence of light avoidance might be similar in *E. danica* to that in *E. virgo*. This might also be the case for other twilight- and mass-swarming burrowing mayfly adults (Fremling, 1960; O'Donnell and Jockusch, 2010; Stepanian et al., 2020; Tojo et al., 2006), the behaviour of which is strikingly similar to that of *E. virgo* (Száz et al., 2015). These mass swarmings often lead to the mass mortality of egg-carrying females near artificial light sources. Larvae of burrowing mayflies are excellent bioindicators of good water quality (Kureck and Fontes, 1996); they not only remove substances from water by feeding on filtered organic particles (Kazanci, 2013) but also contribute to the bioirrigation and bioturbation of the bottom (Clemente et al., 2019). As they play a vital role in riverine ecosystems, it would be important to take into account the response of larvae to light when designing ecologically friendly artificial lighting near their habitats. Our results suggest that applying long wavelength-dominated light sources would minimize the ecological impact not only on *E. virgo* adults (Mészáros et al., 2021) but also on larvae.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.K., Á.E.; Methodology: Á.E.; Software: Á.M., Á.E.; Investigation: Á.M., Á.E.; Resources: G.K.; Writing - original draft: Á.M., Á.E.; Writing - review & editing: G.K., Á.E.; Visualization: Á.M., Á.E.; Supervision: G.K., Á.E.; Funding acquisition: Á.E.

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Data availability

All relevant data can be found within the article. R source code used for data analysis is available upon reasonable request from the corresponding author.

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