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Evidence of visual learning and wavelength differential responses in three podocopids (Crustacea: Ostracoda)

Elena Bellavere[1*], Francesc Mesquita-Joanes[2], Donato Romano[3,4] and Giampaolo Rossetti[1]

Abstract

Background Ostracods are small bivalved crustaceans that inhabit a wide range of aquatic environments, from fully marine to brackish and freshwater systems. All non-marine species belong to the order Podocopida. The sensory biology and behavioral capacities of ostracods remain poorly understood, even though these traits play pivotal roles in habitat exploration, food searching, mate recognition, and predator avoidance. In particular, studies addressing vision and learning ability are still scarce. Although visual structures in ostracods are often reduced or simplified, they exhibit considerable variation across taxa. In this study, we investigated the photoreceptive capabilities of three podocopid species, *Vizcainocypria viator*, *Loxoconcha elliptica*, and *Xestoleberis nitida*, representing different families. Using miniaturized, three-chamber lab-on-a-chip arenas, we examined both innate and experience-dependent light-guided behaviors, testing whether differential wavelength sensitivity and learned associations are present, if these traits are species-specific, and which locomotory responses are associated with them.

Results Our results provide evidence for a simple form of long-term associative memory in ostracods, together with species-specific responses to conditioning type, light wavelength, and possibly light intensity. These responses were not restricted to spatial preferences among arena chambers, but also affected locomotory behavior.

Conclusions These findings highlight ostracods as valuable model organisms for studying fundamental biological processes such as sensory integration, behavioral plasticity, and learning. More broadly, this research advances our understanding of sensory evolution in crustaceans and sheds new light on the behavioral complexity of small aquatic invertebrates.

Keywords Miniaturized arenas, Visual ecology, Associative learning, Locomotory behavior

Background

Ostracods are small, bivalved crustaceans and constitute the most abundant arthropod group in the fossil record [1]. Living ostracods fall into two subclasses: the exclusively marine Myodocopa and the Podocopa, which inhabit a wide range of marine and non-marine environments, including brackish, freshwater, and semi-terrestrial habitats [2, 3]. As members of the Oligostraca, one of the earliest lineages to diverge within Eucrustacea, ostracods occupy a key phylogenetic position for understanding crustacean sensory evolution [4, 5].

*Correspondence:
Elena Bellavere
elena.bellavere@unipr.it
[1] Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area Delle Scienze 11A, 43124 Parma, Italy
[2] Institut Cavanilles de Biodiversitat I Biologia Evolutiva, Universitat de València, C/Catedràtic José, Beltrán Martinez 2, 46980 Paterna, Valencia, Spain
[3] The BioRobotics Institute, Sant'Anna School of Advanced Studies, Viale Rinaldo Piaggio 34, 56025 Pontedera, Pisa, Italy
[4] Department of Excellence in Robotics & A.I, Sant'Anna School of Advanced Studies, 56127 Pisa, Italy

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Within arthropods, crustaceans display remarkable diversity in photoreceptive systems [6–8]. Podocopid ostracods possess a tripartite simple eye (the so-called naupliar eye) that operates with either simple mirror optics or a mirror–lens system, depending on the subtype [9–11]. Adaptation to diverse ecological niches may have influenced the evolution of the ostracod naupliar eye, as suggested for copepods [12]. Although eye subtype complexity and sightedness are often reported to decrease with depth (e.g., in the genus *Xestoleberis*, [13]), this relationship appears inconsistent, and recent analyses reveal weak and variable associations between eye structure, sightedness, and depth in podocopids [14], suggesting a more complex link between photoreception and habitat.

At the molecular level, phototransduction in arthropods is mediated by rhabdomeric opsins [7, 15], typically classified across Pancrustacea into long- (LWS), medium- (MWS), and short-wavelength–sensitive (SWS) families [8, 16]. However, visual opsin repertoires in non-marine podocopid ostracods remain largely unknown. MWS opsin genes have been identified in marine myodocopids [8, 16, 17], whereas only a few LWS opsins have been reported for non-marine podocopids, notably in *Heterocypris incongruens* (Ramdohr, 1808) and *Heterocypris* sp. [8, 16]. Behavioral assays in *H. incongruens* revealed responses to green and yellow light, consistent with a long-wavelength bias [18]. Whether this reflects a restricted spectral range or incomplete molecular knowledge remains unresolved.

Light availability in aquatic environments is itself highly variable. Spectral composition changes predictably with depth, long wave light (red, orange) attenuating in shallower waters, blue and green reaching deeper environments, but also depending on the concentration and composition of dissolved and suspended particulate matter [19]. Clear waters preferentially transmit blue–green wavelengths, whereas waters enriched in chlorophyll and colored dissolved organic matter selectively attenuate shorter wavelengths, resulting in a relative dominance of green–yellow light [20, 21]. Podocopid ostracods occur across shallow and deep habitats, and genera such as *Cypria* can inhabit multiple habitat types [22]. This ecological heterogeneity, together with seasonal and interspecific variation in behavior, renders a simple opsin– "habitat color" match untenable. In shallow temporary ponds, the preferred habitat of *H. incongruens*, short optical paths, high bottom reflectance, and particle-induced scattering weaken wavelength-specific attenuation, potentially favoring luminance cues over fine hue discrimination [20, 21, 23].

Understanding how spectral sensitivity translates into behavior also requires consideration of neural organization. Conserved forebrain centers are present across several crustacean lineages, including Ostracoda, indicating a shared neuroanatomical ground plan [24–28]. At the same time, the optic lobe layout varies significantly and does not align neatly across the group [5]. A simplified central complex for ostracods was depicted by [28], and in [5] it was hypothesized that two nested neuropils, representing an ancestral optic lobe, may be found in extant ostracods. However, this hypothesis is based on the histology of *Doloria levis* (Skogsberg, 1920), a myodocopid ostracod likely differing from members of the subclass Podocopa. Despite the available anatomical descriptions (see, e.g., [24, 29–34]), the learning and memory capabilities of this neural architecture in ostracods have never been assessed.

Previous evidence shows that learning is possible even in compact nervous systems [35–37]. Non-associative and associative learning share conserved circuit motifs and neuromodulators [38, 39]. In podocopid ostracods, *Cyclocypris forbesi* Sharpe, 1897 shows light-linked responses in maze experiments and in pseudo-conditioning paradigms (i.e., lacking clear stimulus–reinforcement contingency) [40], whereas *Heterocypris incongruens* displays phototactic behavior coupled with appetitive and aversive associative learning [18]. Notably, memory retention in *H. incongruens* lasts only a few hours, consistent with short- to intermediate-term memory [18]. Although multiple memory forms are well established across invertebrates [36, 37], whether longer retention phases occur in podocopids remains unknown.

Taken together, these findings highlight several unresolved issues: the limited knowledge of opsin repertoires in non-marine podocopids, the uncertain correspondence between spectral tuning and habitat optics, and the lack of data on memory duration and interspecific variation in learning performance. An integrative assessment linking spectral sensitivity, associative memory patterns, and differences among species is still lacking.

In this study, we investigate innate and experience-dependent light-guided behavior in three podocopid ostracods: the cytheroids *Loxoconcha elliptica* Brady, 1868 and *Xestoleberis nitida* (Liljeborg, 1853), belonging to families Loxoconchidae and Xestoleberididae respectively, and the cypridoidean *Vizcainocypria viator* Bisquert-Ribes et al., 2023, belonging to family Cyclocyprididae. The three species differ in habitat preference, salinity tolerance, and eye morphology (Table 1), providing a comparative framework to examine variation in spectral responsiveness and learning performance.

Using controlled miniaturized lab-on-a-chip (LoC) arenas (Fig. 1), which allow precise manipulation of spectral conditions and stable optical backgrounds [18], we combine spectral stimulation with experience-dependent learning assays to assess innate photic

Table 1 Ecological and morphological characteristics of the three podocopid species examined in this study. Further information and supporting references are provided in Additional file 1: Sect. 1.1

Species	Habitat	Salinity range	Adult body length (mm)	Eye structure
Loxoconcha elliptica	Brackish lagoons, estuaries, shallow waters	Oligohaline to euhaline (broad tolerance)	~0.6	Tripartite naupliar eye with separate eyecups and cuticular lenses
Xestoleberis nitida	Sediments within the photic zone and macroalgal mats	Oligohaline to euhaline (preference for mesohaline)	0.5–0.6	Tripartite naupliar eye with separate eyecups and cuticular lenses
Vizcainocypria viator	Wetlands and rice fields	Oligohaline (occasionally low mesohaline)	0.53–0.58	Possibly fused naupliar eye lacking cuticular lenses

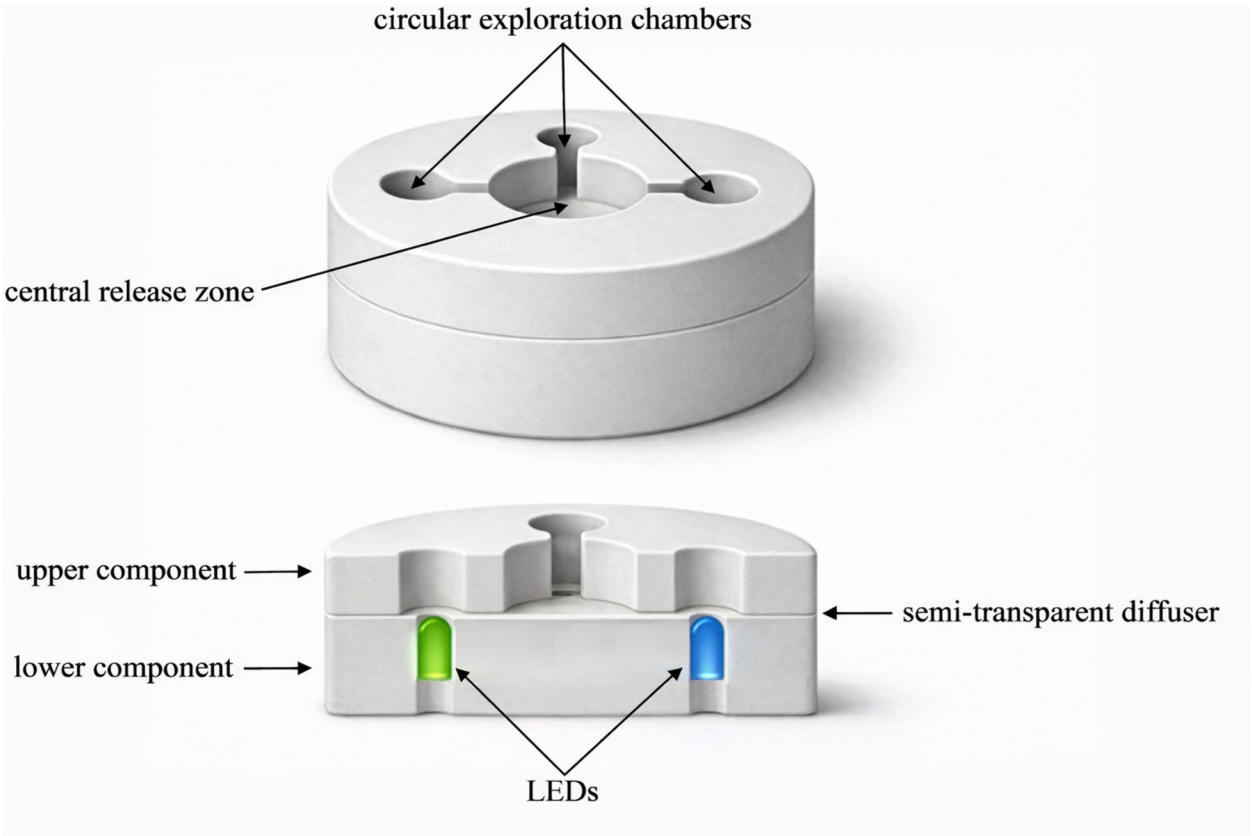


Fig. 1 Schematic representation of the compact three-arm arena (LoC) used in the experiments. Top: external view of the assembled device. Bottom: cross-sectional view illustrating the internal architecture. The arena is composed of upper and lower components that together form a circular central release zone connected by narrow aisles to three circular exploration chambers. The central release zone, the connecting aisles, and the three exploration chambers constitute the core functional architecture of the device, whereas the external peripheral geometry may vary. Visual stimuli are delivered by LEDs housed in the lower component beneath each chamber and covered with a semi-transparent diffuser to ensure uniform light distribution

responses, test sensitivity to different wavelengths, and examine memory retention over time of the three examined species. By adopting a comparative approach, this study evaluates current hypotheses regarding wavelength sensitivity and examines whether experience-dependent plasticity and memory ability shape light-guided locomotory behavior in non-marine podocopid ostracods.

Results

Chamber with maximum total dwell time

A summary of the results concerning the species phototactic behavior for both the naïve and training groups—the latter exposed to either appetitive or aversive stimuli under green LED illumination—is presented in Fig. 2, and in Table 2 whenever the GLM is applicable.

Loxoconcha elliptica

The control group exposed to green light in the single-chamber illumination trials showed a significant preference for the lit chamber (20/40 individual counts, $p=0.0294$). Similarly, the group exposed to blue light in the single-light setting showed an evident attraction to the lit chamber (20/41, $p=0.0457$). The group positively trained with green light showed a significant tendency toward the green chamber in the single-chamber illumination condition (16/32, $p=0.040$). Although not the color of training, the individuals exposed to the blue light also showed a significant preference for the lit chamber in the single-light setting (19/34, $p=0.006$). Conversely, the group trained with an aversive stimulus showed no significant aversive response to the green light (12/33, $p=0.715$), or blue light (18/33, $p=0.9993$). The latter group exposed to blue light in the single-chamber illumination condition in fact seem to have retained a trend of innate preference for the lit chamber. Comparing the experimental groups within each treatment type, no significant pairwise differences were found in their occupation of the illuminated chambers based on color.

In the two-chamber configuration (green vs. blue), the naïve group showed a slight preference for the blue chamber (18/38, $p=0.0839$). Nonetheless, the ostracods overall did not seem influenced by the different stimuli ($\chi^2(2)=3.526$, $p=0.172$). Slight preference for the blue chamber compared to the green chamber (9/38) was observed with less strict significance constraints, $\alpha=0.1$ (OR = 2, 95% CI [0.899, 4.452], $\chi^2(1)=3$, $p=0.083$). No significant difference has been observed for the occupancy of the non-illuminated chamber (11/38), compared

Table 2 Difference in statistical results obtained with the GLM model for the three species: *Loxoconcha elliptica* (LE), *Vizcainocypria viator* (VV), and *Xestoleberis nitida* (XN). Comparisons are reported as follows: one-chamber-lit (1LED) and two-chamber-lit (2LEDs) settings; control (Ctrl), aversive (Trt −), and appetitive (Trt +); blue vs. non-illuminated chamber (B–NL), green vs. non-illuminated chamber (G–NL), and blue vs. green chamber (B–G). *CI*, confidence interval

Species	Light settings	GLM	*p* value
LE	2LEDs, Ctrl B-G	OR = 2, 95% CI [0.899, 4.452]	$p=0.083$
	2LEDs, Trt − B-NL	OR = 0.222, 95% CI [0.075, 0.657]	$p=0.003$
	2LEDs, Trt − G-NL	OR = 0.444, 95% CI [0.193, 1.022]	$p=0.050$
VV	1LED, Trt − B-G	OR = 4.375, 95% CI [1.320, 14.504]	$p=0.011$
	2LEDs, Trt − G-NL	OR = 0.375, 95% CI [0.147, 0.958]	$p=0.033$
XN	2LEDs, Ctrl G-NL	OR = 2.75, 95% CI [1.224, 6.177]	$p=0.011$
	2LEDs, Ctrl B-G	OR = 0.500, 95% CI [0.242, 1.031]	$p=0.056$
	2LEDs, Trt + G-NL	OR = 2.500, 95% CI [0.970, 6.443]	$p=0.050$

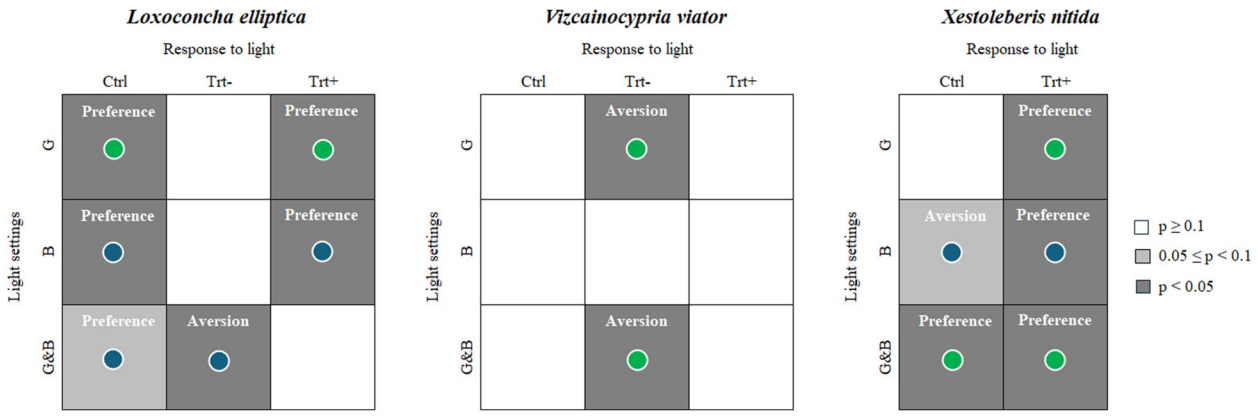


Fig. 2 Significant locomotory behavior, defined as the chamber occupied for the longest time during each trial and tested with an exact binomial test. For each species, columns show control (Ctrl), positive treatment (Trt +), and negative treatment (Trt −). Rows correspond to light settings: single green-lit chamber (G), single blue-lit chamber (B), and two lit chambers (G&B). Dark-gray cells indicate significant responses toward or away from the lit chamber ($p<0.05$); light-gray cells indicate marginal ($0.05\le p<0.1$) responses; white cells indicate non-significant responses ($p\ge 0.1$). Cell labels indicate preference or aversion, and dot color matches the preferred light

to either the green or the blue one. The positive treatment group exposed to the two-light setting was not influenced by the different stimuli ($\chi^2(2)=0.375$, $p=0.829$). Both the green and blue chambers had an occupancy of 15/48 while the non-illuminated chamber had an occupancy of 18/48. Conversely, the negatively trained group in the two-chamber configuration was significantly affected by the stimuli ($\chi^2(2)=10.400$, $p=0.006$). The individuals which occupied the non-illuminated chamber were 18/30, significantly more than those occupying both the blue (4/30; blue vs. non-illuminated chamber: OR = 0.222, 95% CI [0.075, 0.657], $\chi^2(1)=8.909$, $p=0.003$) and green chamber (8/30; green vs. non-illuminated chamber: OR = 0.444, 95% CI [0.193, 1.022], $\chi^2(1)=3.846$, $p=0.050$). In contrast, pairwise comparison between the blue and the green chamber yields no significance. Nonetheless, when considering the result from the binomial test, a significant aversion toward the blue chamber compared to expected occupancy predicted by the null hypothesis ($p=0.012$) was observed.

Vizcainocypria viator

In the single-chamber illumination trials, naïve and positive-trained ostracods showed no significant preference for either green or blue light. For the control groups, occupancy was 14/37 in the green-lit chamber ($p=0.6018$) and 20/46 in the blue-lit chamber ($p=0.1596$). In the positive treatment groups exposed to the single-light condition, 15/33 individuals were observed in the green chamber ($p=0.100$) and 16/50 in the blue one ($p=0.63$). Conversely, the negative treatment group exposed to the green chamber demonstrated a significant aversion to the light (5/30, $p=0.035$). However, no significant response to the light was observed in the negative treatment group exposed to the blue chamber (14/30, $p=0.96$). Between these two negative treatment groups, the occupancy of the green chamber, associated to an aversive stimulus, was significantly lower than the blue chamber (blue vs. green: OR = 4.375, 95% CI [1.320, 14.504], $\chi^2(1)=6.431$, $p=0.011$).

In two-chamber illumination configuration, the control and positive treatment groups were not influenced by the different stimuli (control: $\chi^2(2)=0.280$, $p=0.869$; positive: $\chi^2(2)=1.676$, $p=0.433$). The naïve (control) group showed occupancy rates of 15/50 for blue, 17/50 for green, and 18/50 for the non-illuminated chamber while the positive treatment group showed 11/37 for blue, 10/37 for green, and 16/37 for the non-illuminated chamber. In the negative treatment group exposed to the two-light setting, overall, the individuals were not significantly influenced by the stimuli ($\chi^2(2)=4.545$, $p=0.103$). Nonetheless, the negative treatment group showed a significant aversion to the green chamber (6/33, $p=0.044$), which was occupied significantly less than the non-illuminated chamber (16/33) (OR = 0.375, 95% CI [0.147, 0.958], $\chi^2(1)=4.545$, $p=0.033$). Occupancy of the blue chamber occurred during 11 of the 33 observations. The pairwise comparisons between the blue and green chambers, and between the blue and non-illuminated chambers, were not significant ($p>0.05$).

Xestoleberis nitida

In the single-chamber illumination condition, the control group exposed to green light showed no significant preference or aversion (13/36, $p=0.7258$). The group exposed to blue light showed a slight, marginal aversion (7/36, $p=0.0797$). The groups trained with positive stimuli associated with the green light showed a significant attraction to both the green (25/42, $p<0.001$) and blue (19/30, $p<0.001$) lights when lit separately in the two different single-light settings.

In the two-chamber illumination configuration, the control group was strongly affected by the stimuli ($\chi^2(2)=7.951$, $p=0.019$). The individuals displayed a preference for the green chamber (22/41; from exact binomial test $p=0.0077$), occupied significantly more than the non-illuminated chamber (green vs. non-illuminated: 8/41; OR = 2.75, 95% CI [1.224, 6.177], $\chi^2(1)=6.533$, $p=0.011$), and slightly more than the blue one (blue vs. green: 11/41; OR = 0.500, 95% CI [0.242, 1.031], $\chi^2(1)=3.667$, $p=0.056$). The positively trained individuals were not affected by the stimuli overall ($\chi^2(2)=4.200$, $p=0.123$), but showed a significant preference for the green chamber (15/30, $p=0.043$), which was also occupied, although only marginally significantly, more than the non-illuminated chamber (6/30) (OR = 2.500, 95% CI [0.970, 6.443], $\chi^2(1)=3.857$, $p=0.050$).

Locomotory activity

Chambers explored during the entire experiment

Loxoconcha elliptica The number of chambers individuals visited during the experiments (Fig. 3a) were significantly impacted by the treatment type and light settings (KW: $H=140.907$, df = 8, $p=1.540\times10^{-26}$) with a large rank-based effect ($\varepsilon^2=0.415$). Comparisons are schematically represented by the adjusted p values in Fig. 3a, right panel. Significant differences were localized mainly between negative-trained groups and the control and positive-trained groups. Negative-trained individuals displayed less explorative tendencies, visiting one chamber per experiment on average. The G&B Trt + group was an exception to this trend, showing limited explorative behavior with high counts of single-chamber visits. When compared to other groups, it showed significant differences with all control and positive-trained groups

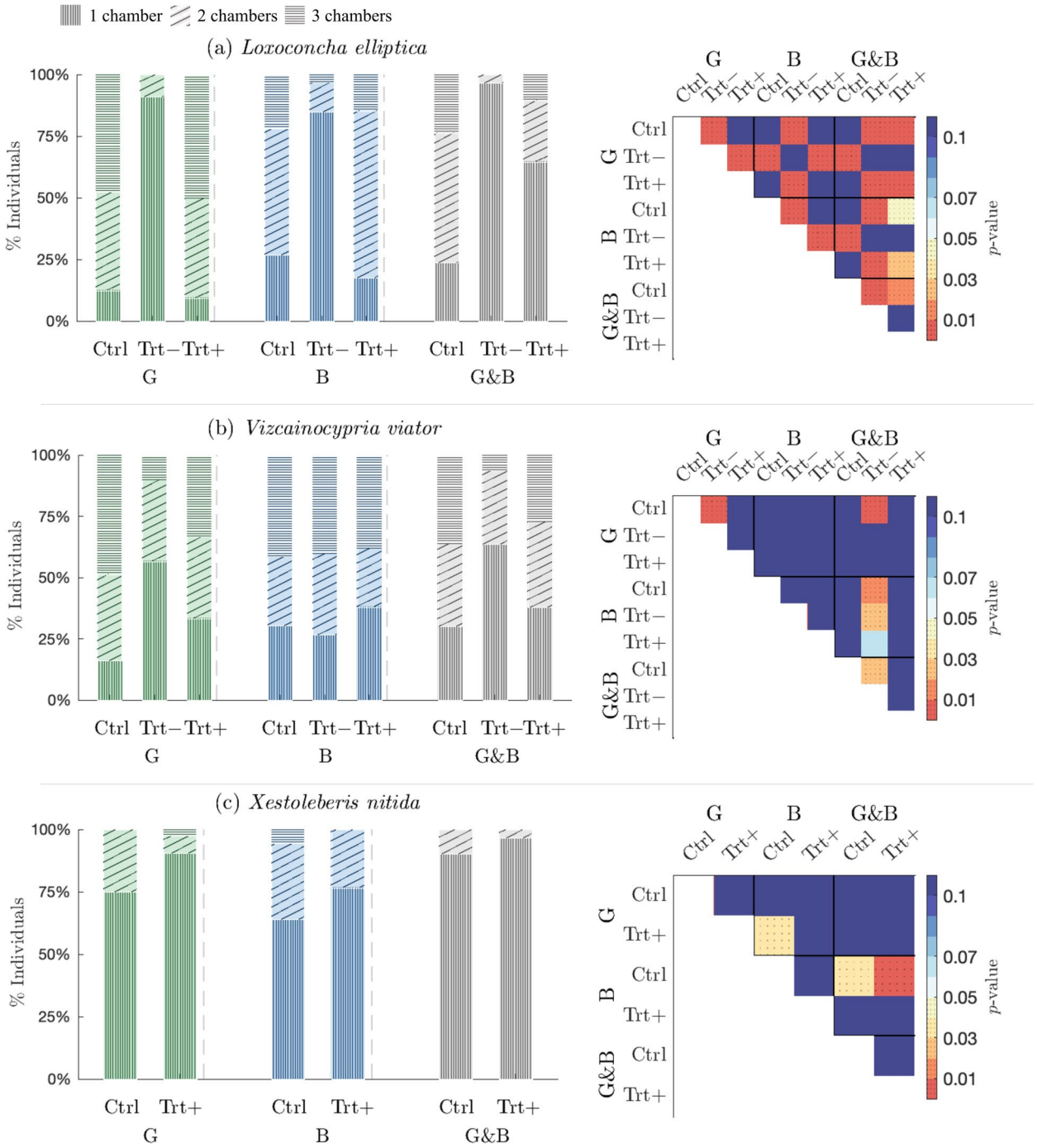


Fig. 3 Exploratory tendency quantified as the number of chambers visited during the experiment. Panels show results for **a** *Loxoconcha elliptica*, **b** *Vizcainocypria viator*, and **c** *Xestoleberis nitida*. Left: for each experimental condition (light setting × treatment), the y-axis reports the percentage of individuals that exhibited each exploratory outcome (from 1 to 3 visited chambers), while the x-axis indicates separately the results for the three light settings: one green-lit chamber (G), one blue-lit chamber (B), or two lit chambers (G&B), and for the three treatments: control (Ctrl), positive (Trt +), and negative (Trt −). Right: heatmaps display *p* values from statistical comparisons among treatments within each light setting

(e.g., vs. G&B Ctrl, $p = 0.020$, $\Delta = -0.413$; vs. G Trt+, $p = 2.476 \times 10^{-6}$, $\Delta = -0.635$) but, notably, not with the negative treatments. Altogether, aversive conditioning reduced exploration by one chamber on average. The full set of effect sizes and directions is provided in Additional file 1: Tables S3–S5 [41–57]. In the G and B

groups, a significant monotonic pattern was found: Trt− < Ctrl < Trt+ (G: Tobs = 2853, $p < 10^{-4}$, maximum resolution of the simulation; B: Tobs = 2723, $p < 10^{-4}$; both with Holm-adjusted $p < 3 \times 10^{-4}$). In the G&B group, a different, but also significant, trend was observed: Trt− < Trt+ < Ctrl (Tobs = 3233, $p < 10^{-4}$, Holm-adjusted $p < 3 \times 10^{-4}$). This result confirms the behavioral patterns observed in Fig. 3a, left panel.

Vizcainocypria viator Significant differences were observed in the exploratory tendency across the experimental groups ($H = 32.897$, df = 8, $p = 6.429 \times 10^{-5}$), with a moderate to low effect size ($\varepsilon^2 = 0.074$). Most groups showed a high rate of exploration (Fig. 3b, left panel), with the exceptions being the G Trt− and G&B Trt− groups (Fig. 3b, right panel). These groups consistently visited fewer chambers than their respective control groups (G Trt− vs. Ctrl G: $p = 0.004$, $\Delta = -0.532$; G&B Trt− vs. G&B Ctrl: $p = 0.022$, $\Delta = -0.425$). The negative treatments did not differ from the positive ones, although within the same light setting the aversive treatments significantly differ from controls, possibly because the positively conditioned groups showed slightly less explorative behavior than the control groups. An exception to this behavior was the B Trt− group, which was not significantly different from either the positive or control groups. The most affected group overall was the G&B Trt−, showing a significant difference in explorative behavior compared to groups in other light settings. The full set of effect sizes and p values is provided in Additional file 1: Tables S17–S19. In the G and G&B groups, we found a significant monotonic pattern: Trt− < Trt+ < Ctrl (G: Tobs = 2241.500, $p < 0.001$, Holm-adjusted $p < 0.001$; G&B: Tobs = 3010, $p = 0.002$, Holm-adjusted $p = 0.003$). No significant trend was observed within the B group.

Xestoleberis nitida The explorative behavior of *Xestoleberis nitida* was significantly affected by the treatments and light settings, leading to heterogeneity across groups (KW: $H = 18.390$, df = 5, $p = 0.003$), with a relatively small effect size ($\varepsilon^2 = 0.064$). As shown in Fig. 3c, left panel, the groups with a single blue-lit chamber and the control group with a single green-lit chamber showed a slightly higher tendency for exploration. However, the Trt+ group under blue light showed no significant difference compared to other groups, and the difference between Ctrl and Trt+ under green light was not statistically significant (Fig. 3c, right panel). Despite the small effect sizes, the most significant differences were found when comparing the B Ctrl group (which had the highest exploration tendencies) to other groups with more limited exploration. The greatest contrast was between the B Ctrl and G&B Trt+ groups ($p = 0.007$, $\Delta = 0.330$), since the positive-trained group subjected to two lit chambers was the one showing the least explorative tendencies. The full set of effect sizes is provided in Additional file 1: Tables S31–S33. No significant difference between treatments was present within light settings.

Fraction of inactive time

Loxoconcha elliptica The time individuals spent resting (not swimming or crawling) was calculated as the ratio of frames showing resting behavior with the total frames available (Fig. 4a, left panel). Light settings and treatments had a significant impact on resting time ($H = 83.745$, df = 8, $p = 8.593 \times 10^{-15}$), with a moderate effect size ($\varepsilon^2 = 0.237$). Visually, we observed a trend in the single-light settings where inactivity was highest in negative treatment groups, followed by control and then positive-trained groups (Fig. 4a, left panel). In contrast, the two-chamber lit setting showed a different trend, where control individuals had the least inactive time, followed by positive and negative-trained groups. The pairwise comparisons displayed a complex pattern that did not clearly link groups within and between treatments and light settings (Fig. 4a, right panel; for full comparison metrics see Additional file 1: Tables S6–S8). The Jonckheere–Terpstra (JT) test supported a Trt+ < Ctrl < Trt− pattern for the G and B groups (Tobs = 2750.500 and Tobs = 2739, both with permutation $p < 10^{-4}$ and Holm-adjusted $p < 3 \times 10^{-4}$). The G&B group showed a different, but also significant, trend of Ctrl < Trt+ < Trt− (Tobs = 2984, permutation $p < 10^{-4}$, Holm-adjusted $p < 3 \times 10^{-4}$).

Vizcainocypria viator Visually, a clear pattern was observed in the single green-lit and two-chamber lit settings, where inactivity was highest in the negative treatment groups, followed by the control and then the positive-trained groups (see Fig. 4b, left panel). In contrast, the single blue-lit chamber setting showed no evident trend. The light settings and treatments had a significant impact on resting time ($H = 20.397$, df = 8, $p = 0.009$), although with a weak effect size ($\varepsilon^2 = 0.037$). After applying the Dunn–Šidák correction, all comparisons were non-significant (see Fig. 4b, right panel). We therefore concluded that the overall difference was small and not driven by any single pairwise effect. Nonetheless, although non-significant, the effect sizes suggested

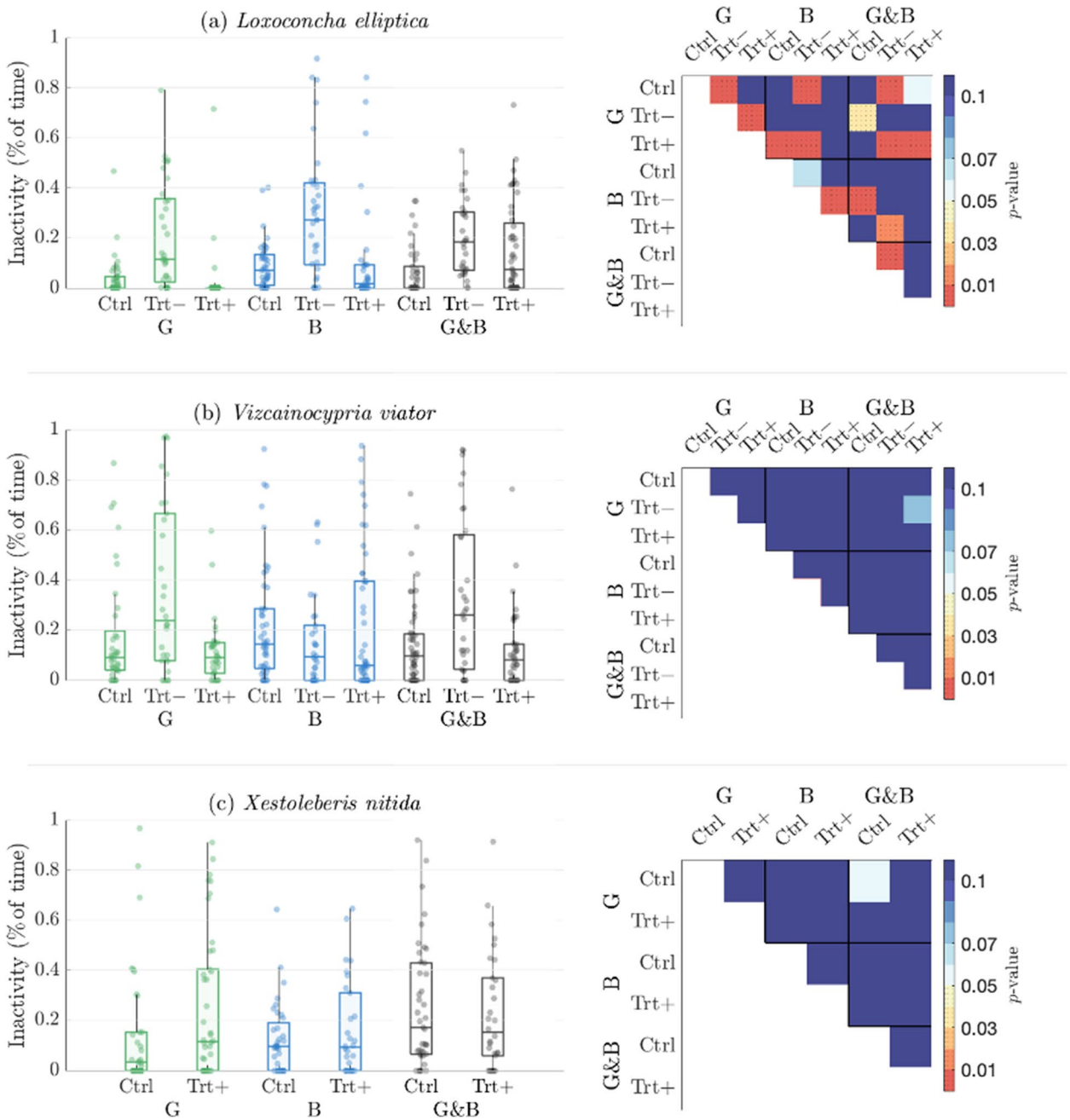


Fig. 4 Fraction of inactive time expressed as the proportion of total time spent inactive. Panels show results for **a** *Loxoconcha elliptica*, **b** *Vizcainocypria viator*, and **c** *Xestoleberis nitida*. Left: boxplots (with individual points shown) showing inactivity across experimental conditions (light settings × treatments as in Fig. 3). Right: heatmaps display *p* values from statistical comparisons among treatments within each light setting

the same directional pattern observed in the green-lit and two-chamber-lit groups (for full effect size and *p* values see Additional file 1: Tables S20–S22). The JT test supported a monotonic increase in inactivity within the single green-light and the two-light setting, where the negative treatment showed the highest resting time. For the G and G&B groups, the hypothesis of Trt+ < Ctrl < Trt− was supported, with Tobs = 2090.500, permutation $p = 0.015$, and Tobs = 2939.500, permutation $p = 0.012$, respectively, and Holm-adjusted $p = 0.037$ for both. In the B group, no ordered trend was observed ($p > > 0.05$ for all combinations). Thus, despite the conservative Dunn–Šidák correction masking individual

contrasts, a coherent treatment-order pattern is present in the G and G&B groups.

Xestoleberis nitida The light settings and treatments had a significant impact on resting time, displayed in Fig. 4c ($H = 11.395$, df = 5, $p = 0.044$), though with a very weak effect size ($\varepsilon^2 = 0.030$). All comparisons were non-significant (see Fig. 4c, right panel), suggesting that the overall difference was not driven by any single, clear pairwise effect. No significant difference between treatments within settings were observed (see Additional file 1: Tables S34–S36). Preliminary tests involving negative conditioning revealed that all individuals of this species exhibited a motionless state throughout the entire duration of the experiments. Consequently, individuals exposed to aversive treatments were expected to remain largely inactive, regardless of light conditions.

Median speed

Loxoconcha elliptica The comparison of the median speed sustained by individuals during the experiments (Fig. 5a) resulted in a strong heterogeneity across groups (KW: $H = 216.379$, df = 8, $p = 2.241 \times 10^{-42}$) with a large rank-based effect ($\varepsilon^2 = 0.651$). Comparisons (Fig. 5a, right panel) highlighted a clear pattern where control groups and positive treatments did not generally differ from each other but did differ significantly from the negative treatments, regardless of the light setting. This created a "chessboard effect" in the results displayed in Fig. 5a, left panel. An exception was found in comparisons involving the G Ctrl group, which sustained higher speeds than the B Trt + and G&B Trt + groups (see Additional file 1: Tables S8–S10). Among the positive treatments and controls, the G&B Trt + group had the lowest speeds. The JT test showed a significant monotonic trend consistent with Trt − < Trt + < Ctrl for all light settings (G: Tobs = 3169; B: Tobs = 3129; G&B: Tobs = 3798; for all groups: permutation $p < 10^{-4}$ and Holm-adjusted $p < 3 \times 10^{-4}$). The species' median speed was in the range of 0 to 4 BL s^{-1} (0–0.22 cm s^{-1}; see Additional file 1: Table S12 and Fig. S8a).

Vizcainocypria viator The median speed metric (Fig. 5b) was influenced by treatments and light settings (KW: $H = 30.848$, df = 8, $p = 1.495 \times 10^{-4}$), with a moderately low rank-based effect ($\varepsilon^2 = 0.068$). Overall, the G Ctrl group sustained the highest median speed compared to all other groups (Fig. 5b, left panel). Comparisons (Fig. 5b, right panel) showed that the G Ctrl group was the only one with significant differences, except when compared to the B Trt − group, the G Trt + group, and, marginally, the G Trt − group. The finding of no significant difference with the B Trt − group is consistent with the qualitatively opposite trend observed in the B groups compared to the G groups, and marginally in the G&B groups, where negative-trained individuals showed higher median or spread of speeds than the control and positive groups (see Additional file 1: Tables S23–S25). This led to a lack of a single, general trend across all light settings. To investigate this, we used a Jonckheere–Terpstra (JT) test to analyze trends within each light setting. In the G groups, a clear trend was observed where median speed followed the order Trt − < Trt + < Ctrl (Tobs = 2197, permutation $p = 0.001$, Holm-adjusted $p = 0.004$). In the B groups, a marginal, but not significant, trend was observed where Trt + < Ctrl < Trt − (Tobs = 3065, permutation $p = 0.083$, Holm-adjusted $p = 0.166$). Finally, for the G&B groups, the test identified Trt + < Ctrl < Trt − as the most probable trend, but the result was not significant ($p > > 0.05$). The species' median speed was in the range of 0 to 18 BL s^{-1}, equivalent to approximately 0 to 1 cm s^{-1} (see Additional file 1: Table S26 and Fig. S8b).

Xestoleberis nitida The light settings and treatments had a significant impact on median speed displayed in Fig. 5c ($H = 55.782$, df = 5, $p = 9.012 \times 10^{-11}$) and with a moderate rank-based effect ($\varepsilon^2 = 0.243$). Our comparisons revealed a "chessboard effect" in Fig. 5c, right panel, indicating that control groups generally maintained higher speeds than the positive treatments, regardless of the light setting. The only exception was the G Trt + group which showed no significant difference from the B Ctrl group (see Additional file 1: Tables S37–S39). The Wilcoxon rank-sum pairwise tests confirmed this trend within light settings: the differences between paired control and positive-trained groups were highly significant in all light conditions (G: $Z = 4.395$, $p = 1.107 \times 10^{-5}$, Holm-adjusted $p = 2.214 \times 10^{-5}$; B: $Z = 2.904$, $p = 0.004$, Holm-adjusted $p = 0.004$; G&B: $Z = 4.813$, $p = 1.484 \times 10^{-6}$, Holm-adjusted $p = 4.453 \times 10^{-6}$). The median speed for this species ranged between 0 and 1.5 BL s^{-1}, equivalent to approximately 0 to 0.08 cm s^{-1} (see Additional file 1: Table S40 and Fig. S8c).

Maximum speed

Loxoconcha elliptica The comparison of the maximum speed that individuals reach during the test (Fig. 6a) resulted in a strong heterogeneity across groups (KW: $H = 170.669$, df = 8, $p = 9.340 \times 10^{-33}$) with a large

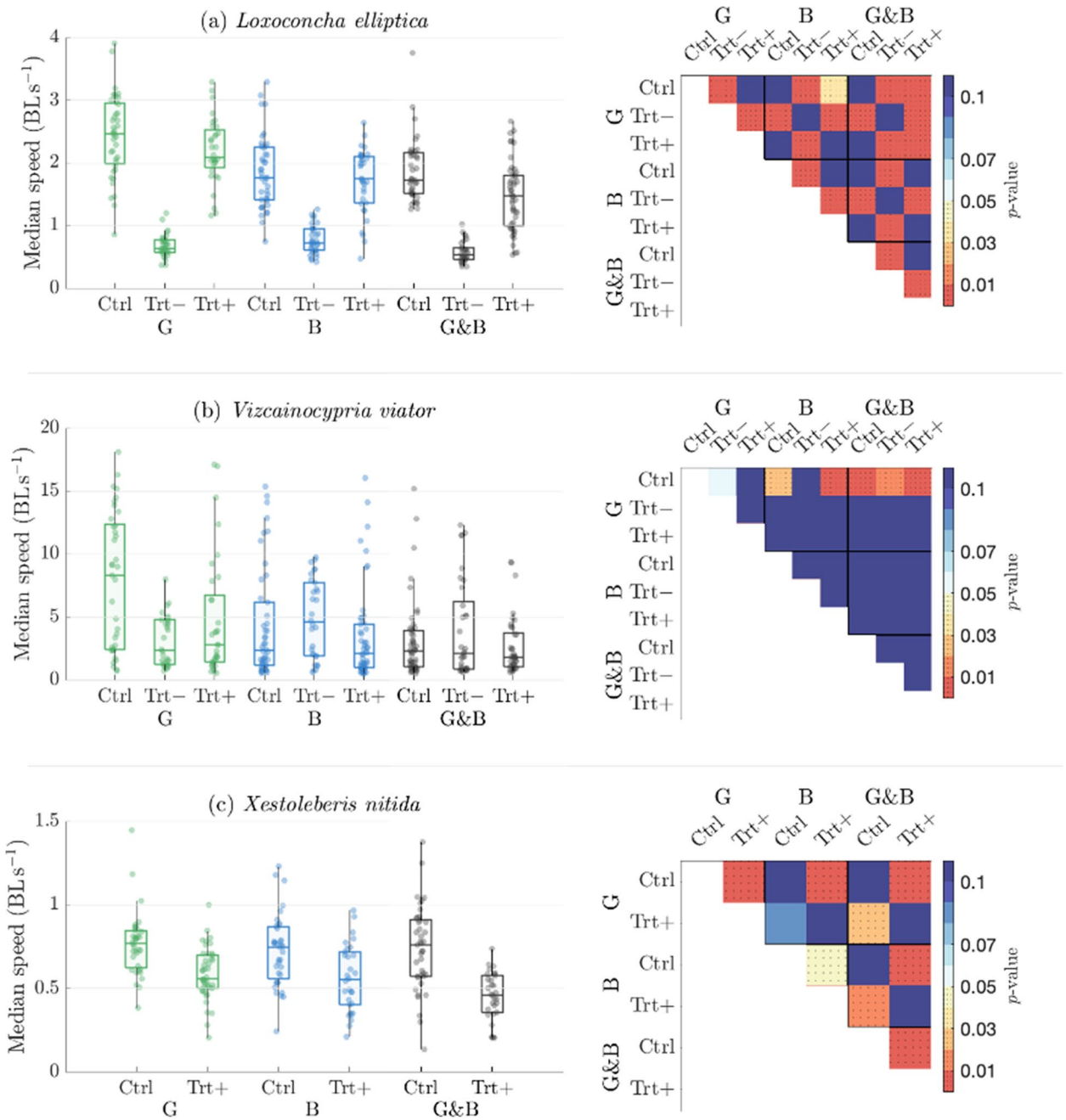


Fig. 5 Median individual speed normalized to species average body length (BL s^{-1}; for all three species, BL ≈ 550 µm). Panels show results for **a** *Loxoconcha elliptica*, **b** *Vizcainocypria viator*, and **c** *Xestoleberis nitida*. Left: boxplots (with individual points shown) summarizing median individual speed across experimental conditions (light settings × treatments as in Fig. 3). Right: heatmaps display *p* values from statistical comparisons among treatments within each light setting

rank-based effect ($\varepsilon^2 = 0.508$). Pairwise comparisons revealed a clear pattern (Fig. 6a, right panel): all control groups and positive treatments did not differ from each other regardless of light setting, but they all differed significantly from the negative treatments. This created a distinct "chessboard effect" with no exceptions. Additionally, controls reached slightly higher median values compared to the positive treatments (see Additional file 1: Tables S13–S15). This trend was also confirmed by a Jonckheere–Terpstra test, which indicated a consistent pattern in the treatments' median within all light settings, following the order of Trt− < Trt+ < Ctrl (G: Tobs = 3052;

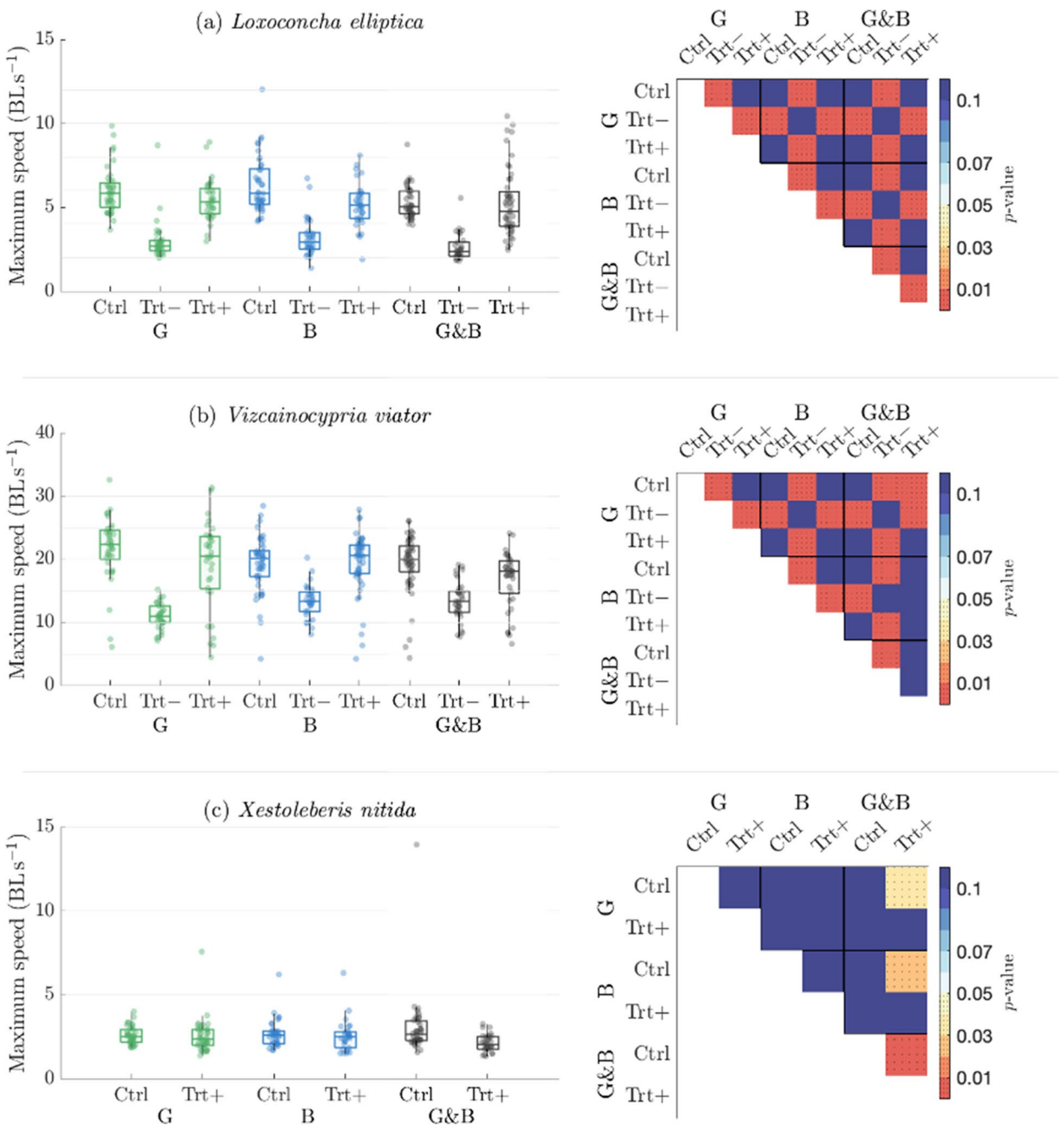


Fig. 6 Maximum individual speed normalized to species average body length (BL s^{-1}; for all three species, BL ≈ 550 µm). Panels show results for **a** *Loxoconcha elliptica*, **b** *Vizcainocypria viator*, and **c** *Xestoleberis nitida*. Left: boxplots (with individual points shown) summarizing maximum individual speed across experimental conditions (light settings × treatments as in Fig. 3). Right: heatmaps display *p* values from statistical comparisons among treatments within each light setting

B: Tobs = 3253; G&B: Tobs = 3539; for all groups: permutation $p < 10^{-4}$ and Holm-adjusted $p < 3 \times 10^{-4}$). The species' maximum speed was in the range of 0 to 12 BL s^{-1} (approximately 0 to 0.7 cm s^{-1}; see Additional file 1: Table S16 and Fig. S9a).

Vizcainocypria viator The maximum speed of individuals appeared to significantly vary across groups (KW: $H = 133.304$, df = 8, $p = 5.84 \times 10^{-25}$), with a large rank-based effect ($\varepsilon^2 = 0.372$) (see Fig. 6b). Pairwise comparisons revealed a clear and consistent pattern, where

individuals from all negative treatments reached a lower maximum speed compared to the control and positive-trained groups, regardless of the light setting. This created a distinct "chessboard effect" in Fig. 6b, right panel, though with one notable exception: the G&B Trt+group reached lower median maximum speeds compared to other controls and positive treatments, with this difference becoming significant with the G Control group, which showed the highest median maximum speed of all groups ($p=0.001$, $\Delta=-0.616$). Moreover, the G&B Trt+group did not differ significantly from the negative treatments of the B and G&B groups (see Additional file 1: Tables S27–S29). A significant monotonic trend was observed for the maximum speed in the order of Trt− <Trt+ <Ctrl for the single-green and green-and-blue light experimental groups (G: Tobs=2611; G&B: Tobs=3647; for both groups: permutation $p<10^{-4}$ and Holm-adjusted $p<3\times10^{-4}$). The B light groups followed a different order, Trt− <Ctrl<Trt+ (Tobs=3785, permutation $p<10^{-4}$, Holm-adjusted $p<3\times10^{-4}$). The species' maximum speed was in the range of 0 to 34 BL s^{-1} (approximately 0 to 1.8 cm s^{-1}, see Additional file 1: Table S30 and Fig. S9b).

Xestoleberis nitida The maximum speed was significantly impacted by treatments and light settings ($H=18.592$, df=5, $p=0.002$), though with a weak effect size ($\varepsilon^2=0.065$) (see Fig. 6c). Comparisons revealed a few significant differences (Fig. 6c, right panel). All significant contrasts involved the G&B Trt+group, which reached substantially lower values than the control groups, regardless of light setting (see Additional file 1: Tables S41–S43). Pairwise comparisons within light setting confirmed this result, showing only a significant difference between the G&B Ctrl and G&B Trt+groups ($Z=3.836$, $p<0.001$, Holm-adjusted $p<0.001$). The maximum speed ranged between 0 and 14 BL s^{-1}, equivalent to, approximately, 0–0.8 cm s^{-1} (see Additional file 1: Table S44 and Fig. S9c).

Speed comparison between species

In order to analyze differences in locomotory activity and treatment responsiveness between species, the median and maximum speed of each experimental group were compared. Overall, the difference in locomotory efficiency between species had a strong impact on both the median ($H=542.624$, df=23, $p=4.609\times10^{-100}$) and maximum speed ($H=770.629$, df=23, $p=5.589\times10^{-148}$), associated with great effect sizes (respectively, $\varepsilon^2=0.600$ and $\varepsilon^2=0.863$). Comparisons revealed significant differences between species in both median speed (Fig. 7a) and maximum speed (Fig. 7b). Full tables of p values and effect sizes for each metric are provided in Additional file 1: Tables S45–S52. From Fig. 7a, it is noticeable that both *V. viator* and *L. elliptica* differed significantly from the median speed observed in *X. nitida*, which is the least locomotory efficient species. Only the negative treatment groups in *L. elliptica*, which had a reduced median speed compared to other treatment groups in that species, aligned with the general median speed of *X. nitida*, resulting in non-significant comparisons. Consistently, these groups showed a significant difference when compared to the more active *V. viator*. Figure 7b highlights

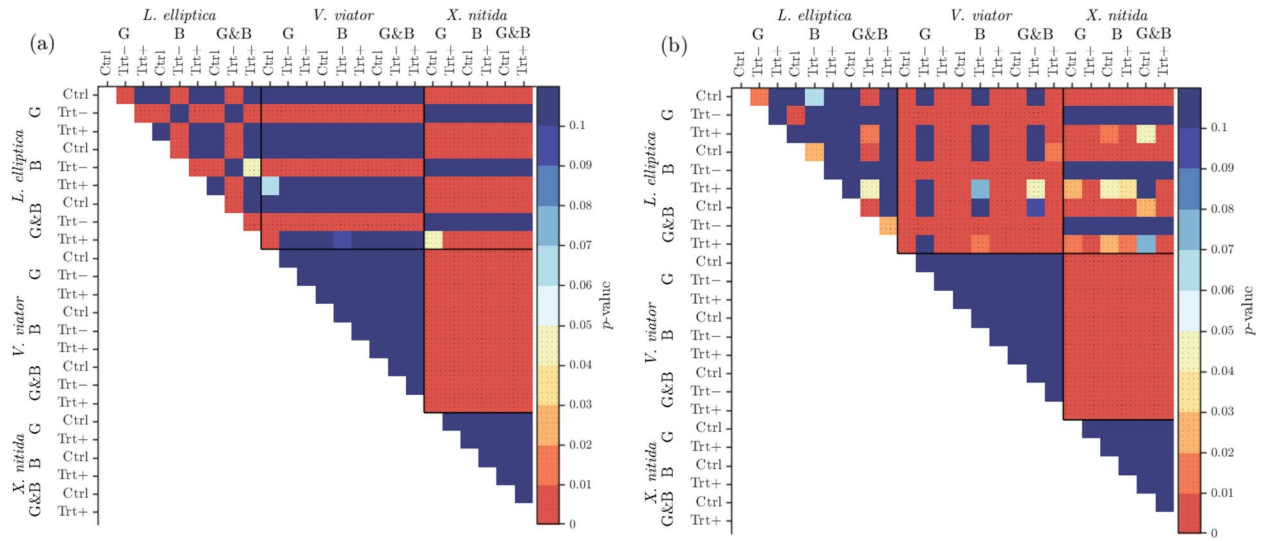


Fig. 7 Heatmaps of p values from comparisons across species, light settings, and treatments (light settings×treatments as in Fig. 3) for **a** median individual speed and **b** maximum individual speed. Comparisons are shown for *Loxoconcha elliptica*, *Vizcainocypria viator*, and *Xestoleberis nitida*

the distinct difference in maximum speed attained between *V. viator* and the other two species. The only non-significant comparisons for *V. viator* were between its negative treatment groups and the positive-trained and control groups of *L. elliptica*. For this metric, *L. elliptica* is also significantly different from *X. nitida*, apart from its negative groups which aligned with the maximum locomotory activity of *X. nitida*. The difference in behavior of median and maximum speed of *V. viator* is consistent with the observation that this species often presented a bimodal speed distribution (see Additional file 1: Figs. S16 and S17), with a higher peak at limited speed values and a smaller one toward the species' maximum attainable speed, considerably greater than that shown by the other two species.

Discussion

Our results revealed species-specific responses to varying light conditions and wavelengths, though the photopigments in the studied species are still unknown. Both innate and learned behaviors were observed, with positive and negative associations more or less evident depending on the species. When considering different metrics, such as total time spent in a chamber and average time per visit, the results remain consistent and do not change significantly (see Additional file 1: Sect. 2), supporting the coherence of observed behavioral responses. Overall, these patterns align with the broader evidence that light-guided behavior in small crustaceans can combine innate response with experience-dependent modulation, with outcomes depending on both sensory constraints and experimental design (e.g., stimulus configuration and retention interval).

In *Loxoconcha elliptica*, naïve individuals showed an innate positive response for illuminated chambers when lit singularly, regardless of whether the light was green or blue. When both chambers were illuminated simultaneously, a slight preference for the blue chamber was observed. Positive training did not significantly alter this behavior. However, aversive (negative) training was more effective. In the single illuminated chamber experiment, the presence of green light (i.e., the color of the association) overrode the trained individuals' innate positive response. However, when only the blue chamber was illuminated, the innate preference persisted. When both chambers were illuminated, the animal exhibited a negative response toward the chamber whose color differed from the aversive training condition, indicating a poor color discrimination. The partial generalization across wavelengths (e.g., avoidance patterns not strictly matching the training color) is compatible with limited spectral discrimination and/or an intensity-driven component to the response.

In *Vizcainocypria viator*, no innate behavior was observed in naïve individuals, and positive training was ineffective under all lighting settings. On the contrary, negative training consistently increased avoidance of green, i.e., the training color. In general, no response to blue light has been observed in *V. viator*. This limited responsiveness to blue light, together with the weak effects of appetitive training, suggests that species-specific sensory tuning and/or ecological relevance of the cue may constrain the effectiveness of conditioning. Such interspecific variability is expected if visual sensitivity and the efficiency of exploring the arena differ across taxa, which can directly influence how readily individuals detect, evaluate, and learn from light cues.

This dominance of aversive conditioning over appetitive training is consistent with the general expectation that avoidance learning can yield clearer behavioral shifts when the reinforcer is salient, and it also parallels the strong impact of stress-related cues reported for several microcrustaceans [58–62], and particularly for non-marine ostracods in response to predator cues [63, 64].

Naïve individuals of *Xestoleberis nitida* showed a slight innate aversion to the blue chamber when singly illuminated, but no innate response to green. Conversely, when both chambers were simultaneously lit, a marked innate preference toward the green chamber was observed, possibly due to a complex behavioral response to the interplay of the two light stimuli. Positive training on *X. nitida* was highly effective, leading to a strong appetitive association and increased occupancy of the colored chambers. When both chambers were illuminated, a green-biased preference was observed. The contrast between weak or absent innate responses under single-light conditions and a strong choice bias under the two-light configuration suggests that the behavioral output may depend on the relative context of stimuli rather than on a simple "attraction/avoidance" rule, a point that is important when comparing assays across studies. Moreover, the strong effect of appetitive training in *Loxoconcha elliptica* and *Xestoleberis nitida* indicates that, at least in some podocopids, light can become a robust predictive cue when paired with a rewarding outcome, consistent with experimental demonstrations of light-cue learning in non-marine ostracods [18]. This difference, together with the lack of an appetitive response in *V. viator*, might be related to their distant phylogenetic origin, as the former belong to the Cytheroidea superfamily and the latter to the Cypridoidea, or perhaps to their distinct habitat conditions and adaptations. These findings suggested a potential simple form of long-term associative memory in the studied ostracods, and a species-specific response to the type of conditioning, wavelengths, and possibly also light intensity. The potential existence of multimodal associative

learning and long-term retention challenges the hypothesis that such cognitive capacities are precluded in animals with a simple central complex brain structure [65]. Nonetheless, our results revealed weaker responses to the stimuli compared to those observed in [18]. This discrepancy may be attributed to the differing experimental protocols and species employed in our study. In particular, differences in reinforcer cues, training schedule, and the duration of the retention interval may modulate the conditioned responses, making direct comparisons across studies challenging even when the cue (light) is conceptually similar. However, this may fundamentally reflect the long retention, which may have imposed higher cognitive abilities than in the shorter memory assays in [18]. Moreover, the differential behavior of *Loxoconcha elliptica* and *Xestoleberis nitida* toward the blue and the green light indicates a slight discrimination ability, perhaps also due to differences in perceived light intensity. Notably, in the single-lit chamber settings, even if based solely on intensity discrimination, the aversely trained individuals of *L. elliptica* exhibited a complex response by avoiding the green light but still displaying a positive, innate response toward blue. Overall, these results are consistent with the idea that conditioning may act by modulating an underlying innate response, rather than replacing it entirely, and that generalization across light conditions may occur when discrimination is limited [66].

The observed behaviors likely reflect species-specific ecological adaptations, and distinct naupliar eye types, which may explain the observed variations in their light-induced behaviors [9, 11, 67]. Compared to the cypridoidean *V. viator*, the two brackish species of the Cytheroidea have a more sophisticated eye type, characterized by a secondary, lateral calcitic lens on the carapace, a feature otherwise restricted to the freshwater family Notodromadidae, whose members display ecological traits markedly distinct from those of other non-marine ostracods [9, 11]. In the two genera of brackish species we considered, variation in light gathering capacity may account for differences in wavelength and brightness perception, with *Xestoleberis nitida* potentially more sensitive to luminescence [10]. This morphological framework provides a parsimonious interpretation for the species-specific patterns observed here: differences in optical structures and light capture likely affect detection thresholds and the perceived contrast between chambers, thereby shaping both innate preferences and the strength of learned associations.

To induce positive learning, we relied on the experimentally demonstrated feeding preference for spinach leaves in *Heterocypris incongruens* and *Eucypris virens* (Jurine, 1820). Nonetheless, other feeding choices may have led to a more efficient positive association. It has been reported that *Xestoleberis nitida* and *L. elliptica* both likely feed on diatoms [68, 69], while the diet of *Vizcainocypria viator* remains undocumented. Podocopid ostracods are known to be generally active foragers, showing selective preference for some food sources over others [63, 70–73]. Because appetitive conditioning depends on motivational state and reinforcer relevance, diet specificity and baseline foraging strategies may contribute to the different success of positive training across species, potentially helping explain why appetitive conditioning was strong in *X. nitida* but weak in *V. viator*.

Overall, our negative training appears to have exerted a marked influence on the animals' choices. Chemical cues released by predators have been shown to function as effective stressors in several microcrustacean taxa, including cladocerans and copepods [61, 74, 75]. Ostracods are known to exhibit diverse antipredator behaviors, including bursts of rapid swimming, concealment, and, in some marine species, bioluminescent flashes used as a deterrent [63, 64, 71, 76, 77]. In this study, a slight salinity change as an additional chemical stressor during the negative training was also introduced. Osmotic pressure is fundamental and critical for ostracod physiology, morphology, and survival [78–80]. For example, *V. viator* has been reported to exhibit a marked decrease in survival probability after several days of exposure to very low salinity water [81]. Nevertheless, our results on motility patterns indicate that the differential behaviors observed cannot be attributed solely to metabolic consequences of osmotic pressure, but rather to a combination of treatment types and light settings. For instance, the total number of chambers visited during the experiment and the amount of active motility time were reduced for all negative treatments in *L. elliptica*, but also for the positive treatment when both chambers were illuminated. In contrast, when exposed to green light, *V. viator* exhibited a monotonic pattern in both the number of chambers visited and active motility, with negatively treated groups displaying reduced exploratory behavior compared to the positive and control groups. However, this pattern was absent when only the blue chamber was lit, regardless of the treatments. Accordingly, we rule out metabolic consequences of osmotic pressure as the primary cause of the differential behavior in the treatment groups. This interpretation is consistent with stressor studies in other microcrustaceans, where behavioral changes are not solely explained by physiology but often reflect context-dependent decision-making under risk [82].

Treatment conditions also had a pronounced effect on the animals' speed. *Loxoconcha elliptica* showed a reduced median and maximum speed in the negative-trained groups, regardless of the light setting. In *X. nitida*, individuals displayed a decrease in median speed

within the positively trained groups. For *V. viator*, as for *L. elliptica*, negative training reduced maximum speed. Yet, median speed decreased in only one of the three light settings, whereas in the others, negatively treated groups displayed greater values. Nonetheless, median speed behavior may be an artifact of the bimodal speed distribution characteristic of *V. viator*, perhaps related to the ability not only to crawl, but also to swim in this species, unlike *X. nitida* and *L. elliptica*, which are unable to swim. Overall, the observed speeds are in the same order of magnitude as those reported for other non-marine podocopid ostracods (e.g., [83]), confirming that these values reflect typical locomotor ranges for these taxa.

While ostracod behavior in the presence of stressors is well documented [63, 64, 71, 77], no studies have quantified speed differences between pre- and post-associative phases. It is therefore possible that the slow aversive directional response observed in the studied species represents an escape reaction following early detection of the light cue.

In other microcrustaceans, changes in swimming speed have been reported in response to predator-related chemical stimuli (e.g., [60, 84]), although these responses were measured during direct exposure to the stimulus and did not involve associative processes. In our case, the speed measurements refer to phases associated with learned light cues, thus potentially reflecting behavioral modulation linked to associative learning rather than an immediate reaction to a concurrent stressor.

Differences in locomotor efficiency among species likely reflect morphological and functional traits (e.g., presence or absence of swimming setae), but the key point is that speed modulation appears to be context-dependent and associated with the learned light stimulus. Importantly, because baseline locomotor mode (crawling vs. swimming) can constrain how individuals actively explore the arena, interspecific differences in speed and exploration are not merely "performance" differences but also influence the opportunity to express choice and learning under the same assay conditions.

In summary, the studied species showed distinct behavioral patterns across light responses, conditioning types, and locomotor activity. These differences likely arise from their distinct phylogenetic placement within families and superfamilies, which in turn affects their morphological and ecological traits (Table 1). Accordingly, interspecific divergence in both visual structures (e.g., naupliar eye design) and locomotor traits provides a coherent biological basis for interpreting the combined patterns of chamber occupancy, exploration, inactivity, and speed observed across light settings and treatments. In this framework, placing these differences within a broader phylogenetic context is essential to understand how lineage-specific evolutionary histories have shaped the architecture of sensory systems and ecological strategies, thereby contributing to the interspecific behavioral divergence documented in this study. The higher-order phylogenetic relationships between the two living ostracod subclasses, Myodocopa and Podocopa, remain a subject of debate [3, 4, 85–88]. Even within the Podocopa, phylogenetic reconstructions based on morphology (such as soft part morphology, shell characteristics, and pore systems) and molecular sequences differ significantly, possibly uncovering high complexity [4, 89–96]. For example, from molecular studies, the family Notodromadidae, which is the only one possessing a sophisticated naupliar eye compared to the other taxa in the Cypridoidea, seems phylogenetically nested within this superfamily [97]; additionally, the Loxoconchidae, to which *L. elliptica* belongs, seems to be a more basal family than that of the Xestoleberidae [98]. Moreover, opsins expression may also differ among close relatives, with decapods being a prime example within Crustacea [8, 16, 99–101].

To date, no phylogenetic analyses of opsins within Oligostraca have incorporated different podocopid ostracod species. The recently available genomes of *Darwinula stevensoni* (Brady and Robertson, 1870), *Notodromas monacha* (O.F. Müller, 1776), and *Cyprideis torosa* (Jones, 1850) are anticipated to substantially enhance future investigations in this area and to help clarify the relationship between opsin evolution and ostracod systematic position [102]. It is plausible that different ostracod species possess distinct opsin types or exhibit shifts in absorbance spectra resulting from substantial sequence modifications. Crustaceans generally possess several mechanisms, in addition to opsin expression, that enable modulation of their absorbance spectrum; therefore, the presence of similar mechanisms in ostracods cannot be ruled out [103–108]. Other mechanisms, such as modifications in opsin–chromophore interactions, are also known to cause sensitivity shifts [109, 110]. These mechanisms provide testable hypotheses for the contrasting wavelength-dependent behaviors observed here (e.g., weak or absent responses to blue in *V. viator* versus clear wavelength biases in the other two species). Future integration of behavioral assays with comparative morphology and genomic data will be essential to clarify how photoreception and habitat specialization have evolved within Podocopida.

Ultimately, from an ethological perspective, the experimental results highlight the adaptive significance of the observed learning patterns. At the same time, their relevance extends to ecological contexts, as visual perception plays a central role in shaping ecological interactions and behavioral decisions across taxa [111]. In this context, wavelength discrimination and visual learning likely

facilitate microhabitat selection, predator avoidance, and reproductive behavior. The ability to distinguish among different spectral cues could allow ostracods to optimize their positioning within heterogeneous light environments, thereby enhancing camouflage or reducing exposure to visually hunting predators. Furthermore, visual learning may facilitate the recognition of conspecifics or mating-related signals, contributing to reproductive success. Overall, these findings underscore the ecological and evolutionary relevance of visual plasticity in ostracods, suggesting that even relatively simple visual systems can sustain flexible, experience-dependent behaviors with clear fitness advantages in natural environments.

Conclusions

This study reinforces the importance of photoreception in the survival and ecological success of non-marine ostracods. By investigating wavelength perception, discrimination, and learning and memory capabilities in three podocopid species, we extend previous work to include broader tests of memory retention and spectral sensitivity. The evidence presented here highlights that a simple form of long-term associative learning, when coupled with species-specific photoreceptive abilities, represents a crucial yet still underexplored dimension of ostracod biology. Although research on these topics remains in its infancy, the evolutionary and ecological implications are substantial. The light-mediated behaviors observed likely support niche differentiation and adaptive success across diverse aquatic environments.

A further strength of this study lies in the methodological approach. The integration of a LoC technology with a custom body tracking system for recording and analyzing trajectories proved to be a powerful and versatile approach for investigating behavioral and sensory processes in small aquatic invertebrates. Its precision, scalability, and adaptability facilitated experimental designs that are often challenging with traditional methods, providing a versatile tool. We anticipate that LoC platforms will play an increasingly important role in advancing the study of ostracod functional aspects, particularly in areas such as fine scale behavioral assays, multisensory integration, and the ecological relevance of learning and memory.

Taken together, our results also underscore the need for a more integrative perspective on ostracod biology, one that combines molecular, morphological, and behavioral approaches to better understand the interplay between sensory systems and ecological adaptation. Future research on non-marine ostracods should aim to expand taxonomic coverage, incorporate genomic and transcriptomic data, and explore the evolutionary trajectories of opsin diversity and photoreceptive mechanisms. Such efforts will not only clarify the role of photoreception in this crustacean group, but also contribute to broader discussions on the evolution of sensory systems in aquatic invertebrates.

Methods

Ostracod sampling

Specimens of *Xestoleberis nitida* and *Loxoconcha elliptica* were collected using hand nets in June–July 2024 from two sites in the Marjal de Pego–Oliva wetland (Valencian Community, Spain), along an oligohaline to mesohaline gradient: (i) Riu Bullent (locally "Riu del Vedat," 38.889476° N, 0.078496° W), where in situ measurements gave electrical conductivity of 3.65 mS cm^{-1}, temperature 23 °C, water transparency (measured with a Snell tube) 46 cm, and water depth 0.80 m; and (ii) Font Salada (Ullal del Burro, 38.889476° N, 0.078496° W), a coastal thermal spring that discharges to the marsh, which drains seaward via the Riu Bullent–Vedat. For Font Salada, published measurements report 26.4 °C and 18.27 mS cm^{-1} [79].

The species *Vizcainocypria viator* was sampled by hand nets in June–July 2024 from Tancat de la Ratlla (39.329251° N, 0.392207° W), a shallow marginal pond at the edge of the Albufera de València lake; in situ values for electrical conductivity, temperature, turbidity, and depth were, respectively, 2.15 mS cm^{-1}, 22.6 °C, water transparency (measured with a Snell tube) 25 cm, and depth 4 cm.

More information on the biology and ecology of all three species is provided in Table 1 and in Additional file 1: Sect. 1.1. Adult individuals of all species were identified and isolated using a stereoscope, ensuring a balanced representation of both sexes in the experimental groups considered.

Miniaturized testing arena and illumination

LoC arenas, typically small glass or polymer devices, are increasingly used in behavioral assays of micro-invertebrates because they allow low-volume experiments under controlled environmental and optical conditions [18, 112]. These systems are particularly useful for parallel behavioral experiments and have been applied across multiple research domains [113–115]. In this study, a compact three-arm arena was used, consisting of a circular central release zone (diameter 20 mm; height 5 mm) connected by aisles (5×5 mm; height 5 mm) to three circular exploration chambers (diameter 10 mm; height 5 mm) (Fig. 1). This arena is a modified version of that described by [18], with a 5-mm reduction in height. During the experiments, three arenas were placed in close proximity, enabling parallel experiments. The experiments were recorded using a fixed camera (Xiaomi

Redmi A2 rear camera 8MP, 1080p) at 30 frames per second (fps) that were consequently undersampled at 15 fps for the trajectory analysis.

Each exploration chamber had a 5-mm LED that could be lit independently and driven by an Arduino Uno microcontroller board. Choice of LED wavelengths was motivated by previous results on vision in Podocopida [8, 16]. The reference wavelength was chosen in the green range, represented by the narrow-band green diode of the LED (515–520 nm). As a comparison, a wavelength in the blue range was chosen (LED 460–465 nm). The LEDs in the arena were coupled with a thin foil of semi-transparent white diffuser to minimize point-source cues. Unlike standard approaches that randomize training colors, in this study only green light was used for the training, avoiding potential phototoxicity from prolonged blue-light exposure [116, 117], while also using a wavelength relevant to all studied habitats.

Illuminance (lux) at the animal plane was measured with a handheld luxmeter (BTMETER, BT-881D). Regarding the LEDs used during the testing phase, we explicitly interpret behavior under photometric (lux) matching rather than radiometric equivalence, adjusting to a photometric near match at the animal plane to matched within $\Delta\text{lux} \leq 10\%$, equivalent to ~ 1.5 klux total per LED. During the training phase, the green channel of the RGB LED matrix panel (Adafruit 64 × 64) was used, fitted with a white opal diffuser and placed beneath a 5-cm riser supporting the arenas. The LEDs matrix panel was set at a lower illuminance level than the testing LEDs (~ 0.4 klux). This approach allows us to reduce the importance of brightness as a cue during the testing phase. Even if the training and testing phases were photometrically matched, blue light consistently delivers a higher photon flux than green, which could lead to a significant brightness bias. Conversely, a radiometric match would not solve the issue, as a long-wavelength-sensitive bias is hypothesized for podocopid ostracods, meaning they would still perceive the green light as brighter, resulting again in unequal photoreceptor excitation [8, 16]. Thus, matching the lux between the test LEDs while allowing a different absolute level of brightness at the training phase should, in practice, highlight the animal's response to wavelength. Nonetheless, slight difference in light intensity may still contribute to the animal behavior.

A daylight LED ring was positioned above the experimental setup in the camera plane to provide uniform natural illumination (~ 200 lx, 6500 K) to the animal plane. Infrared illumination was not employed; while infrared illumination would ideally eliminate any potential interaction between the recording light and the animals' photic responses, the use of spectrally neutral white light better approximates the natural photic background experienced by the animals during observed daytime activity. Thus, the white ring light, which was applied uniformly across all chambers from above, was symmetric and created no directional luminance gradient between chambers, thus not accounting for any lateralized chamber preference. The spectrum approximates natural daylight, maintaining ecologically relevant ambient conditions during testing while providing substantially dimmer ambient light than the test LEDs, meaning the test LEDs dominated the photic environment experienced by the animals. It is thus possible that ambient light reduced or diluted the animal responses to the LED cue, but it seems unlikely that it severely masked or overrode the wavelength-specific stimuli being tested; rather, it provided an ecologically meaningful background noise. Experiments were conducted during daytime hours, consistent with observed activity patterns of the species under natural conditions. Both the training sessions and the test phase were performed in lab temperature conditions (25 ± 3 °C).

Training phase

The training phase included both appetitive (positive) stimuli, such as food availability, hereafter referred to as the positively conditioned group (Trt +), and aversive (negative) stimuli, including chemical threat cues like low salinity and fish kairomones, hereafter referred to as the negatively conditioned group (Trt −).

LoC-based behavioral experiments 1 (appetitive association)

For every species considered, adult ostracods were kept individually in one well of a 24-well multi-dish plate. Each well contained 1 mL of the sampling site water, filtered using Branchia™ (47 mm, medium flow rate, pore size 1.2 µm) microfiber glass filters (Labbox Labware, Premià de Dalt, Barcelona, Spain) to remove particulate matter while retaining the natural ionic composition of the habitat water. To pair light with food, a small spinach fragment was added to each well; the feeding choice is motivated by the finding that two podocopid ostracod species exhibit a strong preference for spinach during multi-choice feeding assays [72, 73]. Plates were positioned above the green LED matrix panel, a diffuser, and a 5-cm riser, providing a broad, uniform field at the animal plane. During training, the platform was enclosed within an opaque box to exclude ambient light, so that the only visual cue was the stimulus emitted by the matrix panel, minimizing potential confounds during the associative phase.

Training spanned two consecutive days, solely during daylight hours (no earlier than 09:00 and no later than 19:00). On each day, the green panel was illuminated for 6 h starting at food delivery, after which the panel was

switched off and any leftover food removed. Between daily sessions, individuals were transferred to containers with clean filtered water from the sampling site.

LoC-based behavioral experiments 2 (aversive association)

Following the same procedure as the appetitive association training, a separate group of adult individuals were kept in wells of 24-well multi-dish plates and positioned above the green LED matrix panel. To assay avoidance learning with a general stressor, each well contained 1 mL of predator-conditioned freshwater. Conditioned water was produced by collecting water (~0.85–0.95 mS cm^{-1}, tap water) from an aquarium occupied by *Gambusia holbrooki* Girard, 1859, and then mechanically filtering it with a 1.2-µm filter. The conditioned water was used immediately after filtration to prevent the odor from decaying over time. Reported persistence of such chemical cues varies widely, from 3–6 h to several days [118–120]. This choice allowed us to use the same general stressor for all three ostracod species, which differ in their ecology and salinity tolerance. For the freshwater species, the stressor acted as a predator-odor (kairomone) cue. Fish kairomones reliably drive avoidance/defense and learning across invertebrates [121–124]; *G. holbrooki* is notably an efficient predator feeding on small invertebrates, such as ostracods (e.g., [125, 126]). For the brackish species studied here, besides the signal of fish presence, the same stimulus plausibly added a hypo-osmotic deviation, as it is known that salinity changes have a central role as a stressor in crustaceans, with a few examples explicitly considering podocopid ostracod species [18, 81, 127]. However, as all three species display broadly overlapping salinity tolerance ranges spanning the oligohaline zone, the osmotic component of the conditioned water was unlikely to represent a markedly differential stressor across species.

Training spanned two consecutive days, solely during daylight hours. On each day, the session lasted for 6 h, after which individuals were transferred to containers with clean sampling site-filtered water as a recovery phase.

Given its low motility and benthic lifestyle, *X. nitida* was not subjected to negative conditioning, as individuals typically exhibit thanatosis (motionless state) under stress, making experimental assessment impractical.

Testing phase: LoC-based behavioral experiments 1 and 2 (appetitive and aversive)

After being trained to associate a wavelength with either a positive (food) or a negative (stressor) stimulus, adult individuals of each species were transferred by micropipette to the releasing chamber of the miniaturized arena and individually tested once. Memory retention was assessed at 16–24 h post-training, classifying it as medium- to long-term memory. Testing took place solely during daylight hours, ensuring temporal consistency between training and testing phases. Each experiment started once the individual began to move (accounting for latency due to stress-induced immobility) and lasted for 4 min. At test initiation, light cues were activated and one of these three configurations was assigned per individual: (i) one exploration chamber lit with the green (training, or "correct" light) color, shown in the results as "G," and the other two chambers not lit; (ii) one exploration chamber lit with the blue (non-training, or "different" light) color, shown as "B," and the other two chambers not lit; or (iii) two exploration chambers, each lit with one of the two colors ("G&B") and one not lit chamber.

The spatial assignment of colors to exploration chambers was rotated across replicates to control for directional bias. The test was considered successful if the animal entered at least one of the exploration chambers. For all the testing configurations, animals could freely explore the three chambers of the arena; thus, all light configurations consist of three-choice tests. For each paradigm (appetitive or aversive), at least 30 trained and naïve individuals were tested per configuration; exact sample sizes per species and treatment are provided in Additional file 1: Table S1. Naïve individuals, hereafter referred to as controls (Ctrl), were not subjected to the same pre-testing LED exposure protocol as conditioned animals. They were isolated and kept under natural light, compared to conditioned animals, which were subjected to the matrix LED light. Thus, they were included solely to characterize baseline unconditioned behavior and innate color preferences, rather than serving as fully matched procedural controls for associative learning. Establishing such a control would require exposing naïve individuals to identical pre-testing protocols without the associative learning stimuli, which represents a limitation of the current study. For this reason, throughout this work we refer cautiously to associative learning and, where appropriate, prefer the broader term "experience-based behavioral modulation".

Spatial choice was measured as the exploration chamber with the highest total dwell time across the trial. The number of distinct chambers visited was also recorded to quantify the animals' exploration tendency. The locomotor behaviors were characterized by analyzing individual trajectories extracted using a custom dark-body tracker in MATLAB. More detailed information on the extrapolation of trajectories, with examples, is provided in Additional file 1: Sect. 1.2. The trajectories were then segmented into inactive and active intervals. The proportion of time spent inactive was calculated separately from the motility metrics, which included the maximum and

median speed for each individual. Speed metrics were normalized to body length and expressed in body lengths per second (BL s^{-1}) to reflect locomotor efficiency. An average carapace size of 550 μm was used for all three species, as they are very similar in shell length [67, 128]. Other metrics are presented in Additional file 1: Sect. 2.

Statistical analysis

For control experiments, a two-sided exact binomial test was applied to assess whether the number of individuals choosing a specific chamber deviated from chance expectation of 1/3, with no directional hypothesis. For treatment experiments, one-sided exact binomial tests were used: upper-tail for positive conditioning where attraction was expected, and lower-tail for negative where avoidance was expected. Significance was set at $p < 0.05$; results with $0.05 \leq p < 0.10$ were considered marginal trends and are discussed where relevant. Differences in occupancy between chambers within the two-light configurations were quantified using a Generalized Linear Model (GLM) with a binomial error structure [18, 129]. The GLM was also used to assess differences between treatments. Effect sizes for chamber occupancy are reported as odds ratios (OR).

Kinematic outcomes within species, namely median and maximum speed, number of exploration chambers visited, and fraction of inactive time, were compared between treatments and light configurations using Kruskal–Wallis tests with Dunn–Šidák post hoc contrasts. The same nonparametric framework was applied to cross-species comparisons of maximum and median speed. For each test, we report the Kruskal–Wallis χ^2 statistic, epsilon-squared (ε^2) effect size, and Cliff's delta for pairwise contrasts. Within each light setting, treatment trends were assessed using the Jonckheere–Terpstra test. Specific pairwise differences within light settings were analyzed by Wilcoxon rank-sum tests.

Abbreviations

LWS	Long-wavelength-sensitive
MWS	Medium-wavelength-sensitive
SWS	Short-wavelength-sensitive
LoC	Lab-on-a-chip
LED	Light-emitting diode
fps	Frames per second
RGB	Red–green–blue
Trt +	Positively conditioned group/appetitive treatment
Trt −	Negatively conditioned group/aversive treatment
G	Green
B	Blue
G&B	Green and blue
Ctrl	Control
BL	Body length
GLM	Generalized Linear Model
OR	Odds ratio
CI	Confidence interval
KW	Kruskal–Wallis
JT	Jonckheere–Terpstra
LE	*Loxoconcha elliptica*
VV	*Vizcainocypria viator*
XN	*Xestoleberis nitida*
B–NL	Blue versus non-illuminated chamber
G–NL	Green versus non-illuminated chamber
B–G	Blue versus green chamber
NL	Non-illuminated chamber

Supplementary Information

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Additional file 1. Characteristics of the species used in the experiments: Sect. 1.1. Trajectory extraction, zone assignment, and locomotor descriptors: Sect. 1.2., Figures S1–S7. Sample size per treatment: Sect. 1.3., Table S1. Results for the between-treatment comparisons of chamber occupancy: Table S2. Additional metrics for chamber with maximum dwell time in total, chamber with maximum dwell time per visit, locomotory activity, median speed, maximum speed, turning angles, velocity autocorrelation function, mean squared displacement, collective distribution of individual speeds: Sect. 2.1. *Loxoconcha elliptica*, Tables S3–S16, Figures S8a, S9a, S10; Sect. 2.2. *Vizcainocypria viator*, Tables S17–S30, Figures S8b, S9b, S11–S13; Sect. 2.3. *Xestoleberis nitida*, Tables S31–S44, Figures S8c, S9c, S14. Speed comparisons between species: Sect. 2.4., Tables S45–S52.

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Authors' contributions

E.B. conceptualized the study, curated the data, performed the formal analysis and investigation, developed the methodology, contributed to the original draft, and led the review and editing of the manuscript. F.M.J. conceptualized the study, contributed to the formal analysis, investigation and methodology, and contributed to writing, reviewing and editing the manuscript. D.R. conceptualized the study, contributed to data curation, formal analysis and methodology, supervised the work, and contributed to writing, reviewing and editing the manuscript. G.R. conceptualized the study, contributed to the formal analysis and methodology, supervised the work, and led the review and editing of the manuscript. All authors read and approved the final manuscript.

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Data availability

Raw and processed data are accessible from https://zenodo.org/records/18145262 (https://doi.org/10.5281/zenodo.18145262) under the license Creative Commons Attribution Non Commercial No Derivatives 4.0 International.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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