Symbiosis
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Exploring the mimetic pigmentation of symbiotic shrimps associated with echinoderms

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Abstract

In marine ecosystems, organisms often form enduring associations with other species, creating rich symbiotic communities within benthic hosts. Among these associations, mimetic pigmentation—where symbionts adopt colors resembling their hosts—plays a key role in predator avoidance strategies. Despite its prevalence, this phenomenon remains poorly understood. This research aims to explore pigmentation dynamics in four symbiotic pairs: (i) *Zenopontonia soror* with *Culcita novaeguineae,* (ii) *Synalpheus stimpsonii* with *Phanogenia distincta*, and (iii) *Tulеariocaris holthuisi,* and (iv) *Arete indicus* with *Echinometra mathaei.* The study involves monitoring symbiont survivability, standardized photography to measure color variation, histological analysis to locate pigment-associated cells, and HPLC (High Performance Liquid Chromatography) to characterize pigment chemical composition. Results indicate a strong host dependency for all symbionts, with three species showing reliance on host metabolites. Host-symbiont separation affects symbiont pigmentation, leading to dynamic color changes. Histological analysis reveals chromatophores in all species, with *A. indicus* exhibiting two distinct layers of chromatophores containing different pigments. Chemical analysis shows differences in pigments between partners, with carotenoids detected in both *Z. soror* and *C. novaeguineae*, while *S. stimpsonii* and *P. distincta* show carotenoids predominantly in the symbiont. These findings enhance our understanding of mimetic coloration mechanisms in symbiotic shrimps, advancing knowledge of symbiont ecology and host interactions.

Keywords Crustacean · Ectosymbiont · Coloration · Mimicry · Chromatophore

1 Introduction

The biosphere relies on an intricate interactome, binding biological entities together in dynamic ecosystems (Levin 1998). It includes symbiotic associations, which are defined as intimate and long-lasting interactions between interspecific organisms, called hosts and symbionts (Paracer and Ahmadjian 2000). Symbionts always benefit from an advantage arising from these relationships, often resulting in a particularly strong host dependency (Caulier et al. 2014; Brasseur et al. 2018; Morita and Schmidt 2018; Lourtie et al 2023, 2024). A key factor for increasing the survivability

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of many symbionts is their ability to adopt a mimetic coloration, replicating the texture and color of their host body wall (Maciá and Robinson 2009; Caro 2018). Such mimicry makes them almost invisible and consequently decreases their predation rate for predators relying on visual cues (Maciá and Robinson 2009; Virgili et al. 2020; Bauer 2023). This mimetic coloration has been observed in many symbionts belonging to different taxa, including symbiotic decapods (Gherardi 1991; Horká et al. 2016; Brasseur et al. 2018; Virgili et al. 2020; Bauer 2023). Crustaceans are known to display a large phenotypic plasticity, with two different strategies leading to mimicry. Firstly, a partial or complete translucence of their body allows some decapods to be nearly imperceptible (Britayev et al. 2013; Kumar et al. 2015; Fernández-Lereé et al. 2023). The second strategy involves coloration mimicry, where symbiotic decapods fit to the appearance of their hosts, frequently adopting specific colors that contain shades of multiple colors (de Gier and Becker 2020; Fernández-Lereé et al. 2023). For example, the white coloration may be used by the symbiont to simulate shell fragments of their host, while the vivid colors are more adapted to match some host integument coloration (Gherardi 1991; Caro 2018; Virgili et al. 2020; Caulier et al. 2022; Fernández-Lereé et al. 2023). These adaptive coloration strategies may take the form of uniform shades, textured patterns, or ornamentation, such as distinct colored stripes or spots, which contribute to their effective mimicry (Gherardi 1991; Virgili et al. 2020; de Gier and Becker 2020; Ghory and Kazmi 2021). It has already been observed that crustaceans, whether crabs or shrimps, are capable of adapting to the color of their environment (Stevens et al 2013; Siegenthaler et al 2018) and therefore, for symbiotic organisms, to that of their hosts. This ability has been demonstrated in various species of shrimps such as *Zenopontonia soror* (Fernández-Lereé et al. 2023), *Tulеariocaris holthuisi* and *Arete indicus* (Brasseur et al. 2018), as well as in crabs such as *Lissocarcinus orbicularis*, which can adjust its color according to its environment or host, particularly during molting (Ayotte 2005).

Despite the numerous examples of specific symbiotic coloration and the essential character of this mimetic function on symbiont survival, there is scarce research aimed at understanding its underlying mechanism. Only a few studies have been conducted in applied research to provide a vivid pigmentation to commercialized shrimps in seafood or ornamental shrimp industry to sell the final product at a higher price (Wade et al. 2017; Lu et al. 2022). It has been shown previously that the non-symbiotic crustacean coloration is mainly mediated by carotenoid pigments such as astaxanthin (named after its first characterization in the lobster, *Astacus gammarus* now known as *Homarus gammarus* (Nishida et al. 2023)). Nowadays, it is known that carotenoids can only be synthesized by photosynthetic organisms (Cazzonelli 2011), making de novo synthesis of these chemicals impossible in the vast majority of animals. Therefore, in metazoans, these essential pigments are probably obtained by partial metabolization of their diet or with the help of mutualistic bacteria (Cobbs et al. 2013; Maoka 2020; Lu et al. 2022). In order to achieve an efficient mimicry of their host coloration, some decapod species may acquire pigments by fully or partially feeding on their host's tissues (Zmarzly 1992; Brasseur et al. 2018). Consequently, when kept separated from their hosts, their pigmentation may vary with different time dynamics, from a few hours to several days or months (Ayotte 2005; Brasseur et al. 2018). Moreover, crustaceans coloration can vary depending on abiotic parameters such as the background color or ambient luminosity, and the time period (*i.e.* nychthemeral rhythm) (Joss 1973; Ayotte 2005).

In addition to coloration, carotenoids are also involved in other essential metabolic functions as they are antioxidants, precursors of vitamin A and retinol, photo-protectors, enhancers of immunity, and may also play a key role in mating strategy (Higuera-Ciapara et al. 2006; Maoka 2020; David et al. 2023). Pigments are stored in specialized cells known as chromatophores, present in the integument and containing various pigments such as melanins or pterins for example (Lu et al. 2022; Fernández-Lereé et al. 2023). Some chromatophores, called erythrophores, are characterized by reddish colors and a distinctive dendritic form (Lu et al. 2022; Fernández-Lereé et al. 2023). In general, their pigmentation is due to the presence of carotenoids or their derivatives (Matsumoto 1965; Matsumoto and Obika 1968). Chromatophores may play a role in pigmentation changes in organisms (Fingerman 1985; Fernández-Lereé et al. 2023) through two distinct pathways: (i) a fast pathway involving muscle contractions (*e.g.* in cephalopods; Mäthger et al. 2009), and (ii) a slower pathway involving the endocrine system (*e.g.* in chameleons; Duarte et al. 2017). Considering the endocrine pathway, specific hormones called chromatophorotropins, such as PDH (Pigment Dispersion Hormone) or PCH (Pigment Concentration Hormone), have opposite effects on chromatophore diameter, ultimately impacting the organism coloration (Josefsson 1975; Lu et al. 2022).

Performing host separation experiments on symbiotic shrimps, Brasseur et al. (2018) highlighted the chemical dependency of the shrimp *Tulеariocaris holthuisi* to its sea urchin host, *Echinometra mathaei*. This research suggests the essential role of spinochromes (*i.e.* quinones), a category of pigment specifically produced, in echinoderms, by the echinoid class (Brasseur et al. 2018; Hatate et al. 2002; Hou et al. 2018), in (1) the host recognition and selection and (2) the short-term survival of *T. holthuisi*. Indeed, this study reveals a higher survival rate among isolated *T. holthuisi* individuals when incubated in spinochrome extracts (*i.e.* kairomones) or sea urchin-conditioned water (Brasseur et al. 2018) compared to shrimps isolated in normal seawater. In

this way, kairomones, *i.e.* allomones beneficial to the recipients, can influence not only survival, but also the coloration of the symbiont. This phenomenon was described as a host separation syndrome, defined as a degradation of the health status of a specific symbiotic organism when isolated from its host, potentially leading to the symbiont death. The authors suggested that the pigmentation may be influenced by this dependency, as the isolated shrimps seemed to show less mimetic pigmentation than the healthy ones that remained on their host. In opposition to *T. holthuisi*, the coloration and short-term survival of a second host-specific shrimp, *Arete indicus,* were not affected by the host isolation (Gherardi 1991; Brasseur et al. 2018).

The goal of the present study is to investigate the host separation syndrome, the chemical dependency, and the mimetic coloration of a larger panel of symbiotic shrimps associated with echinoderms. The mimetic coloration of shrimps is investigated using a multi-disciplinary approach, combining (1) the monitoring of color changes over time during the separation from the host with a standardized photographic method, (2) the identification and quantification of pigments in the host and symbiont through chemical extraction and chromatography analyses and (3) the localization of colored cells and their changes in the body wall using histological techniques. Four tropical species of shrimp living on echinoderms are investigated, all harboring typical mimetic coloration (Fig. 1). The first is the shrimp *Zenopontonia soror* associated with the cushion sea star *Culcita novaeguineae* (Fig. 1Aa-d). This crustacean species is known to have three morphotypes: translucent, fully colored and stripe-colored (Antokhina and Sorokin 2010; Antokhina and Britayev 2020; Lourtie et al. 2023; Fernández-Lereé et al. 2023). The second association involves the yellow-orange snapping shrimp *Synalpheus stimpsonii* living on the crinoid *Phanogenia distincta* (Fig. 1Ba-b; VandenSpiegel et al. 1998; Caulier et al. 2022). The third and fourth shrimps are those found on the sea urchin *Echinometra mathaei*: *Tuleariocaris holthuisi* and *Arete indicus* (Fig. 1Ca-c). These symbiotic couples were already investigated by Brasseur et al. (2018) but for a shorter period than the present study. Both symbionts present mimetic pigmentation: *A. indicus* shows a purple coloration with two whiter stripes on the body sides and *T. holthuisi* is characterized by a fully pinkish-purple color (Gherardi 1991; Marin et al. 2005; Brasseur et al. 2018; Dabbagh et al. 2019; Ghory and Kazmi 2021). Both species are known to share common spinochrome pigments with their host, obtained either partially or completely by feeding on their host tegument to gather specific spinochrome pigments (Brasseur et al. 2018).

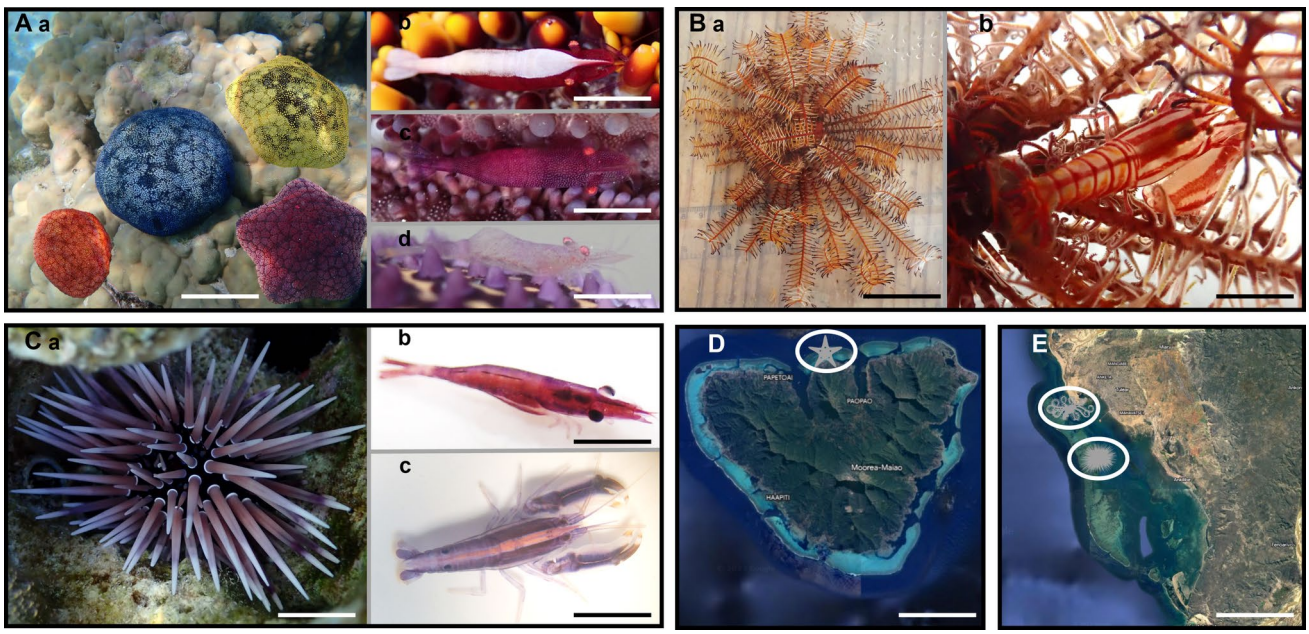


Fig. 1 Symbiotic model organisms investigated for host separation experiments and their respective field locations. (**A**) the association with *Culcita novaeguineae* and *Zenopontonia soror*. (Aa) different color morphotypes of the sea star host, (Ab) stripe-colored shrimp morphotype, (Ac) full-colored shrimp morphotype and (Ad) translucent shrimp morphotype. (**B**) *Phanogenia distincta* associated with *Synalpheus stimpsonii*. (Ba) the crinoid host, (Bb) the symbiotic shrimp. (**C**) the sea urchin host (Ca), *Echinometra mathaei*, that can host two different shrimps named (Cb) *Tuleariocaris holthuisi* and (Cc) *Arete indicus*. (**D**) Moorea island, French Polynesia, where the asteroids were collected (white circle). (**E**) the Great Reef of Toliara, Madagascar, where the crinoids and echinoids were collected (white circles). (**D**, **E**) Modified from ©Google Earth, version 9.181.0.1. Scalebar = 8 cm in Aa; 6 mm in Ab-c and Cc; 4 mm in Ad and Cb; 6.2 cm in Ba; 9 mm in Bb; 2 cm in Ca; 4.8 km in D; 7.2 km in E. The figure was made with Powerpoint

2 Material and methods

Two scientific field trips took place in July—August 2021 and 2022 at the Center for Island Research and Environmental Observatory (CRIOBE) located in Moorea, French Polynesia. In addition, two field missions were conducted in April and May 2022 and 2023, at the Halieutic Institute and Marine Sciences (IHSM, University of Toliara) and the Marine Station of Belaza, Madagascar. Organisms collection, sample preparation and photographic monitoring were performed on site. Carotenoid extractions and subsequent analyzes were conducted at the Marine Station of Concarneau, France. Finally, the assessment of pigmentation variations and the chromatophore analysis in optical microscopy were carried out at the University of Mons, Belgium.

The study of the host separation syndrome, including survival and color change monitoring, and histological analyses was conducted on the four symbiont species: *Z. soror*, *S. stimpsonii*, *A. indicus* and *T. holthuisi*. The HPLC chemical identification of the pigments was only performed on the two symbiotic couples *Z. soror – C. novaeguineae* and *S. stimpsonii – P. distincta* due to limited sample availability.

2.1 Sampling and organisms maintenance

Culcita novaeguineae Müller & Troschel, 1842 hosting *Zenopontonia soror* (Nobili, 1904) were free-diving hand-collected in depths ranging from 1 to 5 m on the northern coast of Moorea near the Opunohu Bay, French Polynesia (Fig. 1D; 17°28′50’’S; 149°50′00’’W). *Phanogenia distincta* (Carpenter, 1888) hosting *Synalpheus stimpsonii* (De Man, 1888) were collected by scuba diving on the north reef pass of the Great Reef of Toliara in Madagascar (Fig. 1E; 23°23′14"S; 43°39′04 "E), at depths ranging from 20 to 28 m. *Echinometra mathaei* (Blainville, 1825) hosting *Tulеariocaris holthuisi* Hipeau-Jacquotte, 1965 and *Arete indicus* Coutière, 1903 were hand-collected at low tide in depths ranging from 0 to 1 m on the flat intertidal zone of the Great Reef of Toliara in Madagascar (Fig. 1E; 23°22′48’’S; 43°38′13’’E). All the collected hosts were stored for a short-term (max. 2 h) in a 3 L hermetic plastic bag filled with seawater to preserve all the symbionts during the collection and transport period. On arrival at the laboratory, an inventory and characterization of the organisms was carried out, including morphotype determination and a count of the number of symbionts per host. When possible, the sex of individuals was identified based on morphological sexual dimorphism or the presence of eggs. To perform that, the host and its associated shrimps were removed from the hermetic plastic bag and transferred to a transparent 4 L tank filled with seawater to perform the inventory. Without being exposed to air, the symbionts were carefully removed by pushing them gently with tweezers and captured within small vials to isolate them from their host. These containers of 30 mL were designed to physically isolate the symbionts from their host, while providing them with sufficient space to move around. The closure lid of the vial is made of mosquito net, which allows a continuous exchange of waters during the following experiment. They are also transparent, which allow the symbionts to benefit from a day/night cycle with natural light. For each shrimp, a picture was taken and considered as the initial standard picture at T_0.

2.2 Host separation experiments

To assess the influence of the host and its olfactory environment on the symbiont fitness and coloration, three to five distinct treatments were established for the four decapod symbiotic species. Those experiments started immediately after the inventories, unless the symbiont died within the first 12 h (if it occurred, it was excluded for further analyses). All experiments were conducted in 6L open water aquarium (25L for *C. novaeguineae* that were the biggest hosts) supplied with 1 µm-filtered seawater, sourced directly from the sea. Aquarium were aerated with an air bubbler and maintained at a constant temperature of 28–29 °C, a salinity of 35 PSU, and a pH of 8. These conditions were designed to minimize stress, other than the experimental stress, on the symbiotic pair during maintenance and throughout the various experiments. Treatments were conducted for a duration ranging from 10 to 60 days depending on symbionts survival:

(1) **Control treatment** where symbionts remained associated with their hosts. We used 22 *Z. soror*, 16 *S. stimpsonii,* 10 *A. indicus* and 10 T. *holthuisi* (Fig. 2A). All organisms were maintained on their host in the 6L aquarium (25L for *C. novaeguineae* that were the biggest hosts), mimicking the natural symbiotic load found in the field. Therefore, we placed 2 hosts per aquarium, and a maximum of two symbionts per hosts for *A. indicus* (Gherardi 1991; Dabbagh et al. 2019), *T. holthuisi* (Marin et al. 2005; Brasseur et al. 2018) and *S. stimpsonii* (VandenSpiegel et al. 1998; Caulier et al. 2022). For *Z. soror*, as this species can be found in large aggregations (Antokhina and Britayev 2020; Fernández-Lereé et al. 2023), we decided to keep them on 2 hosts per aquarium, with a larger number of symbionts composed of 4 adults (colored morphotype) and 4 juveniles (translucent morphotype).

(2) **Isolated treatment** where symbionts were isolated from their hosts in seawater. Twenty-two *Z. soror*, 11 *S. stimpsonii*, 10 *A. indicus* and 10 T. *holthuisi* were used (Fig. 2B). Symbionts were kept isolated individually in

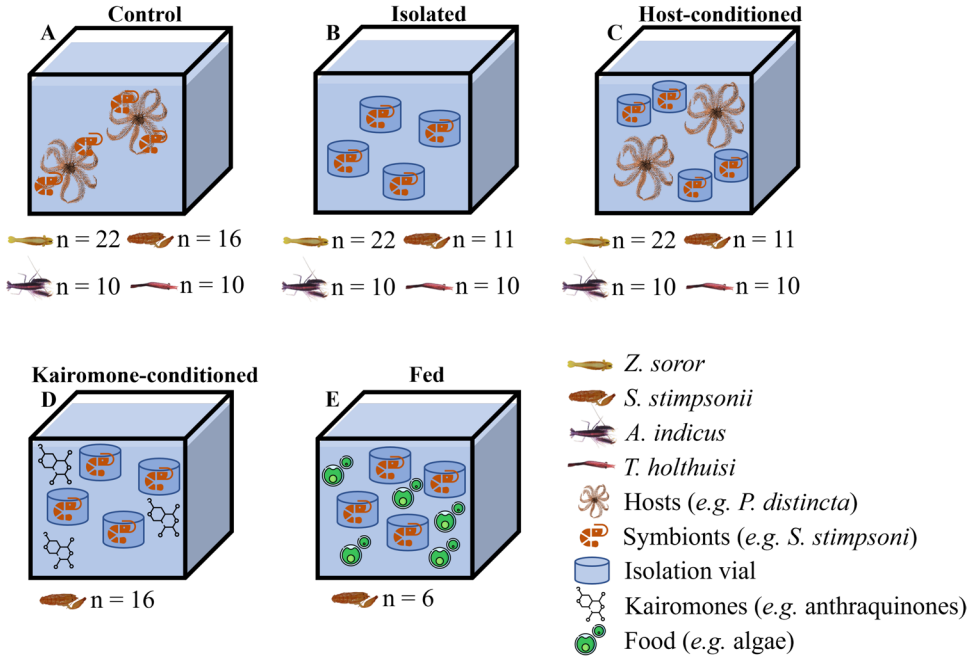


Fig. 2 Scheme displaying the different experimental conditions applied to the 4 symbiotic couples investigated in this study and the number of replicates used for each species and treatment. (**A**) Control treatment with symbionts on their hosts (**B**) Symbiont isolation treatment in pure seawater (**C**) Symbiont isolation treatment in host-conditioned treatment (**D**) Symbiont isolation treatment in kairomone (anthraquinone)-conditioned treatment (**E**) Symbiont fed ad libitum by cultured microalgae. The proportions of the organisms are not to scale. The figure was made with Powerpoint

a vial (see above) and placed in a 6L aquarium containing only filtered seawater (25L for *C. novaeguineae*).

(3) **Host-conditioned treatment** where symbionts were isolated from their hosts in conditioned seawater. Twenty-two *Z. soror*, 11 *S. stimpsonii*, 10 *A. indicus* and 10 T. *holthuisi* were used (Fig. 2C). In this treatment, the symbionts were isolated individually in the same vials used in the previous condition and placed in the aquarium. However, two host individuals that were also placed in this aquarium, continually released metabolites into the seawater, thus conditioning the water. This configuration allowed the exchange of metabolites without direct contact between symbionts and host.

Additionally, two supplementary conditions were introduced for *S. stimpsonii* to further investigate the influence of food and artificial kairomones on symbiont survival:

(4) **Kairomone-conditioned treatment** where symbionts were isolated from their hosts in kairomone-conditioned seawater (Fig. 2D). Sixteen *S. stimpsonii* were isolated in the same vials than (2) and (3), but using seawater conditioned by a 0.01 g/L of commercial anthraquinone (CAS number: 84–65-1; $C_{14}H_8O_2$; Anthraquinone; 98%, Thermo Scientific Chemicals) in seawater. This specific molecule was proven to act as an attractant for the shrimp in a behavioral olfactometer, thus considered to play a kairomonal role allowing host selection (Caulier et al. 2022). After each monitoring, the water contained in these aquarium were completely renewed and 0.06 g (0.01 g/L, for a 6L aquarium) of anthraquinones were added and manually homogenized.

(5) **Food treatment** where symbionts were isolated from their hosts and daily fed (Fig. 2E). Six *S. stimpsonii* were isolated in the same vials used in the previous condition and placed in 6L aquarium filled with seawater. As this species is known to feed during the night on external resources by grazing or filtering (Vanden-Spiegel et al. 1998; Terrana et al. 2024), we decided to feed the shrimps at 10 p.m. every day with a mixture of 10 mL of a *Chaetoceros gracilis* and 15 mL of an *Isochrysis galbana* liquid culture at a concentration of 20,000 cells/mL.

For each species and treatment, survival checks were conducted four times daily at 6 a.m., 12 a.m., 6 p.m., and 12 p.m. Detection of dead individuals was based on three criteria: immobility (*e.g.* shrimp not moving even when stimulated with light and antennae A1 and A2 not moving), unusual body position (*e.g.* shrimp on the back) and, in some cases, an unusual pigmentation (*e.g.*, observed in *T. holthuisi*). Dead organisms were fixed for future analyses such as histological investigations. Hosts and symbionts that were still alive at the end of the experiment were released into their natural environment in order to minimize the impact of this study.

2.3 Monitoring of symbionts pigmentation

2.3.1 Long-term monitoring

All symbiotic shrimps were regularly photographed in standardized conditions to monitor the pigmentation variation throughout the period of separation from their host and to allow comparison between treatments and species. Photos were taken four times a day (6 a.m., 12 a.m., 6 p.m., and 12 p.m.) over a period of 6 to 20 days, depending on the studied species, following the survival monitoring (see above). The experiment was carried out until the death of individuals or, for *T. holthuisi* and *Z. soror* for up to 10 days, *A. indicus* for up to 18 days and *S. stimpsonii* for up to 20 days. Symbionts were isolated or kept in previously described vials containing the water from their respective aquariums (see above). Vials were placed close to a ColorChecker (ColorChecker® mscc model) within a photographic chamber, sealing them from daylight (Fig. 3a). The photography process was conducted under the same LED lighting quality/intensity (1300 lm, 5500 k, ®HAVOX). The distance between the individuals and the camera was kept at 25 cm. Photos were taken with an Olympus TG-6 camera in RAW format, using the BKT (Bracketed Focus Technique), which means that several photos were taken simultaneously at different focus distances. For this experiment, according to the number of individuals available for each symbiont species, a total of 5 individuals of *Z. soror*, 7 individuals of *S. stimpsonii*, 10 individuals of *A. indicus* and 10 individuals of *T. holthuisi* were used. Each photo contained a maximum of six shrimps together with a label inscription and the ColorChecker for color calibration. The ColorChecker is a flat, rectangular chart with a grid of colored patches, each of which representing a specific color with known and standardized values; it was used for color calibration to ensure accurate and consistent color reproduction in images analysis. This allows data to be standardized by creating a photographic profile. The latter enables the creation of a colorimetric profile, as well as brightness adjustment. This methodology makes it possible to standardize photos, eliminate potential biases and ultimately obtain a quantification of the color present in the picture.

In a native file colored digital picture, each pixel can be represented by a vector of three numbers (each ranging from 0 to 255) for the three primary color channels: red, green, and blue (RGB). The color analysis involved the extraction of these RGB values from three to four distinct spots scattered on three different body regions of the symbiotic organisms represented by the blue (anterior part of the body), red (mid part of the body) and green (posterior part of the body) spots on Fig. 3B-E (corresponding respectively to the anterior extremity of the cephalothorax, to the posterior extremity the

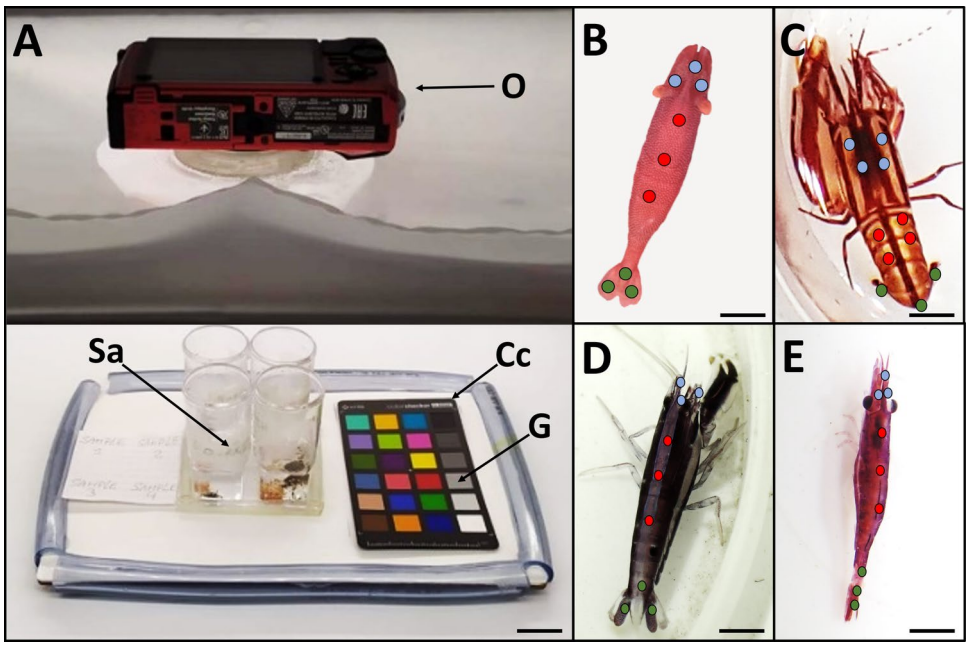


Fig. 3 (**A**) Color monitoring experimental device using a colorchecker in a lightroom. (**B**) Representation of the points sampled on *Zenopontonia soror* (**C**) Representation of the points sampled on *Synalpheus stimpsonii* (**D**) Representation of the points sampled on *Arete indicus* (**E**) Representation of the points sampled on *Tulleariocaris holthuisi.* (**B**, **C**, **D** and **E**) Pictures taken in the represented device in (**A**), with the blue dots indicating points sampled on the cephalon, red dots indicating points sampled on the thorax or abdomen, green dots represent points sampled on the telson/uropods. Cc: ColorChekcker; G: neutral Gray (R: 128, G:128, B: 128); O: camera Olympus TG-6; Sa: Samples. Scalebar = 5 cm in A, 2 mm in B, 3 mm in C, D and E 2 mm. The figure was made with Powerpoint

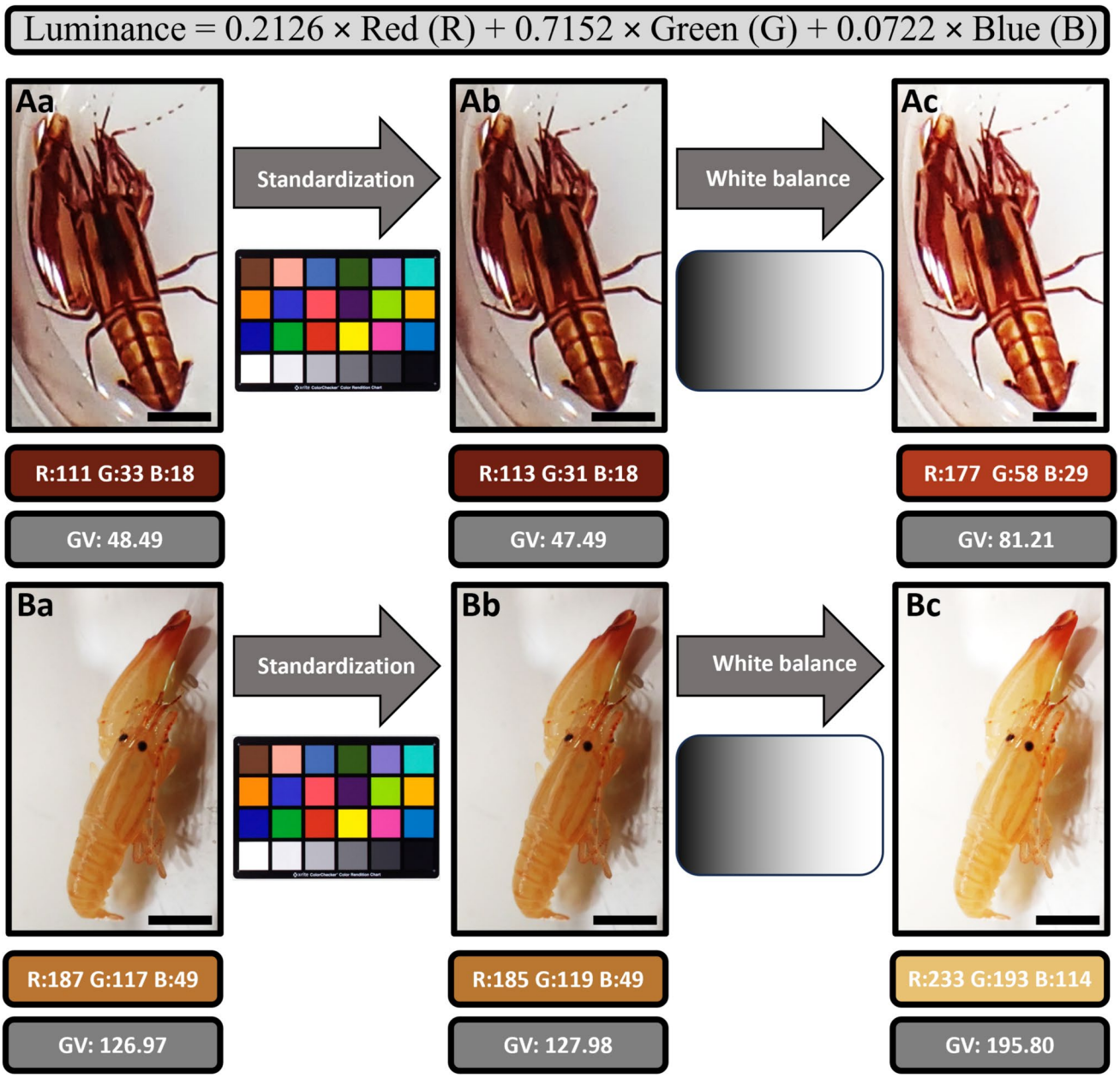


Fig. 4 Illustration of the image processing standardization on the pictures realized during the color monitoring experiment taking a darker (Aa-Ac) and a lighter (Ba-Bc) individuals of *Synalpheus stimpsonii* as examples. RGB represents the Red–Green–Blue values sampled using Adobe Photoshop ®. Gray value (GV) is calculated from the formula: (0.2126 × Red + 0.7152 × Green + 0.0722 × Blue). (**A**) *S. stimpsonii* presenting a vivid pigmentation (**B**) another individual of *S. stimpsonii* presenting a lighter coloration. Aa and Ba: Raw picture of the symbiont taken by the camera without photo standardization; Ab and Bb: picture Aa and Ba standardized using the colorimetric profile using ColorChecker; Ac and Bc: picture Ab and Bb standardized using the white balance. Scalebar = 0.5 cm. The figure was made with Powerpoint

cephalothorax plus the abdomen, and to the telson/uropods, Fig. 3). Subsequently, the lights and colors of the images were standardized using the "white balance" tool (RGB values of the neutral gray aligned to fit the RGB value of 128:128:128) and RGB values of the nine to eleven dots of each shrimp were standardized and measured using Adobe Photoshop software (Adobe Photoshop 2022 V25.5.2). For each triple RGB values obtained for the different regions of the shrimps, the median value was taken and transformed into one average grayscale value (GV) using the formula Luminance = 0.2126 × Red + 0.7152 × Green + 0.0722 × Blue (Gonzalez et al. 2016) (Fig. 4). This formula is used to calculate relative luminance, *i.e.* the variation in gray in relation to the RGB (Red, Green, Blue) values obtained. It is derived

from a two-dimensional Fourier transformation (Shahriar Sazzad et al. 2013).

2.3.2 Short-term monitoring

To investigate short-term color changes that could occur between the first hour of host isolation (*i.e.* between the two first monitoring times), short monitoring was carried out on *Z. soror*. Symbionts were photographed in the same vials describe above, filled with seawater, renewed every hour to limit variations in temperature or oxygenation. The organisms were kept throughout the short-monitoring within the light-sealed photographic chamber at constant LED lighting intensity (1300 lm) and the distance between the individuals and the camera was kept at 25 cm (Fig. 3A). Photos were taken with an Olympus TG-6 camera in RAW format, using the time lapse program, which means that photos were taken every 5 min throughout the 8 h experimentation. Color variation was analyzed on four individuals of each morphotype of *Z. soror* (*i.e.* 4 translucent, 4 stripe-colored and 4 fully colored) with the addition of the ColorChecker (ColorChecker® mscc model) (Fig. 3). Once the photographs were taken, they were analyzed using the same methodology as explained above in the "long-term monitoring" section.

2.4 Histological analyses

Samples for histology were fixed in formaldehyde 4% (diluted in artificial seawater) in the field. As carotenoids are fat-soluble, it is not possible to use a conventional histology protocol involving solvent baths to characterize them (Krinsky 1994). Therefore, they were frozen-sectioned using a cryostat at the University of Mons. For that purpose, they were placed in plastic boxes specific to the cryostat and then embedded with OCT medium (Optimal Cutting Temperature) (Roth®). The OCT medium becomes solid at -10 °C and is used as a coating medium for the cryostat. The OCT blocks were put in the freezer at -20 °C overnight. The blocks were then sectioned, at a thickness of 8 µm, using a cryostat maintained at -20 °C (Leica ®). The longitudinal sections of the shrimps were collected on SuperFrostPlus® glass slides, mounted with Vectashield® Antifade Mounting Media, and directly observed under a Zeiss Axioscope A1 microscope connected to a Zeiss AxioCam 305 color camera. Pictures were observed with the ZEN Blue Software (ZEN 3.2, ZEN lite). Considering the size of the individuals, this method allows a cross-section of the entire organism.

2.5 Biochemical analyses

Carotenoid pigment extractions and analyzes were only conducted for the two symbiotic couples *Z. soror—C. novaeguineae*, and *S. stimpsonii—P. distincta.* The entire body of the symbiont organisms were preserved for extraction. Concerning the hosts, small parts of their body wall were sampled for extractions without the need to sacrifice the organisms that were later released on their collection spots. A dissection was performed to obtain 40–100 g of the external body wall of *C. novaeguineae* and 5–15 g of the arms of *P. distincta.* In order to preserve the maximum quality/quantity of carotenoids, all samples were directly preserved in a freezer (-20 °C) and lyophilized for 48 h using a Martin Christ alpha 2–4 freeze dryer. Samples were preserved in the dark until extraction to avoid pigment deterioration.

At the Marine Station of Concarneau, 5 to 75 mg of dried samples were placed in 1.5 mL of 95% methanol (buffered with 2% w:v ammonium acetate) along with a stainless-steel bead in a 2 mL plastic tube. The samples were then ground using a MM 400 Retsch bead mill at 30 Hz for 10 min to allow complete and homogenous pigment transfer from the biological tissues to the solvent. The resulting extracts were filtered using 0.2 µm PTFE syringe filters and analyzed using an Agilent 1260 Infinity HPLC system. The HPLC system comprised a quaternary pump (VL 400 bar), a UV–VIS photodiode array detector (DAD 1260 VL, 250–900 nm), and a 100 µl automatic sample injector maintained at 4 °C in the dark. Chromatographic separation was achieved using a reverse-phase C18 column (Supelcosil, 25 cm long, 4.6 mm inner diameter). The mobile phases consisted of A: 0.5 M ammonium acetate in methanol and water (85:15, v:v), B: acetonitrile and water (90:10, v:v), and C: 100% ethyl acetate. The solvent gradient was set according to Brotas and Plante-Cuny (2003), with a 0.5 mL min^{-1} flow rate. Peak identification was performed by comparison with available commercial standards and reference organisms (David et al. 2023) and quantification was performed using 8-apobetacarotene (Roth ®) as internal standard. The number of individuals replicate used for the experimentation were: $n = 3$ for translucent *Z. soror*, $n = 6$ for colored *Z. soror,* $n = 6$ for *C. novaeguineae*, $n = 9$ for *P. distincta* and $n = 7$ for *S. stimpsonii*.

2.6 Statistical analyses

For the survivability experiment, A Mantel-cox statistical test (p-value statistical significance settled to 0.05) was used to compare the potential impact of the different host separation treatments on the survival of symbionts, as performed by Brasseur et al. (2018). Survival statistical analyses and graphs were performed using GraphPad Prism (V10.0.0).

Considering Gray values (GV) variations, linear regressions were performed for each individual for all three body parts (Fig. S1) and for the abdomen part only (Fig. 6). The choice to simplify the analysis to the abdomen was chosen for its higher representativeness of the color changes observed on the organism (Fig. S1). Indeed, the abdomen

was less impacted by carapace ornamentation and color bias caused by an underlying organ. Significant increasing slope of the linear regression reveals a decrease in color intensity (*i.e.* discoloration), while a decreasing slope reveals an increase in color intensity (*i.e.* coloration/ recoloration). An absence of significant slope indicates no change in color intensity (*i.e.* stable coloration). When a line stops, it means the corresponding individual was found dead, stopping the monitoring of this individual (Figure S1, Fig. 6).

Linear regression coefficients and the GV difference between the initial and final GV are then calculated for the abdominal region of each individual (Table 3). A negative coefficient and GV difference indicate recoloration of the individual, while a positive coefficient and GV difference indicate discoloration of the individual (Table 3).

The analysis of the extraction of carotenoids data was made using RStudio software (v2022.12.0).

3 Results

3.1 Symbiotic metrics

A total of 74 sea stars *C. novaeguineae* were collected and revealed hosting 119 shrimps *Z. soror* that were mostly found on the oral face of the host, around the mouth or near one of their ambulacral grooves. The symbiotic prevalence was 60.2% with 1 to 14 shrimps being observed on a single host individual, with a majority being alone (29.4%) or in pairs (25.5%). The three morphotypes (translucent, stripe-colored and fully colored) were part of this symbiotic population (Fig. 1Ab-d). The majority of the shrimps were translucent (72.2%; Fig. 1Ad) while the stripe-colored (Fig. 1Ab) and full-colored morphotypes (Fig. 1Ac) were similarly abundant, representing respectively 14.3% and 13.6% of the population. The stripe-colored morphotype individuals were characterized by a dorsal band that always showed a white coloration while the other part of their anatomy harbored vivid colors, similarly to the fully colored morphotypes (Fig. 1Ab-c).

The three other species investigated in this study presented a unique morphotype with a single, unique pigmentation. A total of 148 crinoids *P. distincta* were collected hosting 134 snapping shrimps *S. stimpsonii*, with a symbiotic prevalence of 50.3%. These shrimps were always found either single (22.2% of single females and 13.8% of single males) or in heterosexual pair (63.8%) on the cirri, calyx or on one of the arms of their host. The body of all individuals presented an orange coloration with dark orange to red horizontal and vertical stripes on their body (Fig. 1Bb). The exact appearance of these stripes could vary among individuals, being wider and more intensely colored when the general coloration of the shrimp was brighter, with sometimes stripes overlapping each other, making their distinction difficult. Usually, on the cephalothorax, four longitudinal stripes were observed, and on the abdomen, there was a unique longitudinal stripe centered on the dorsal side of the organisms (Fig. 1Bb). Transversal stripes, when visible, might not be as distinct as the longitudinal ones, and could vary in their number and appearance, especially in small individuals. They were found only on the abdomen and were positioned in a perpendicular direction to the longitudinal stripes (Fig. 1Bb). These transversal stripes seemed to be located on the boundaries between two abdominal metameres (Fig. 1Bb).

On the 106 sea urchins *E. mathaei*, 44 shrimps *A. indicus* and 18 T. *holthuisi* were found, showing a symbiotic prevalence of 41.5% and 16.98%, respectively. *A. indicus* was always found close to the sea urchin test, at the base of the spine, with its body and chelipeds parallel to a spine and the head toward the exterior of its host. *T. holthuisi* was always located on a spine, with its elongated body parallel to the spine, head toward the test of its host. These two species could be found together on the same host (68.9%), both species being found as a single individual (9%) or in pair on the host (91%). All *A. indicus* presented a similar coloration, characterized by a highly mimetic purple color with a small lighter mid-dorsal band that stretched to the inner edge of the eyestalk and extended from the rostrum to the fifth abdominal segment (Fig. 1Cc). Along each lateral side of the body, there was a minute blue stripe running the entire shrimp length, ultimately converging at the posterior edge of the abdomen where the orange band fades away (Fig. 1Cc). On the fourth abdominal segment, two prominent dark blue spots could be observed symmetrically placed at each side of the mid-dorsal band (Fig. 1Cc). For *T. holthuisi*, all individuals exhibited a specific elongated body shape, highly similar to a host spine, making them difficult to find on the host. Their coloration consisted of a purple-pink uniform color, with a minute mid-dorsal stripe that had a darker coloration and two-minute lateral white stripes running from the cephalothorax to the end of the abdomen (Fig. 1Cb).

3.2 Survivability and chemical dependency

Figure 5, Tables 1 and 2 show the different survival curves and statistical parameters resulting from the host separation experiment for the four symbionts investigated. For *Z. soror*, individuals separated from their host exhibited a rapid mortality for both isolated and host-conditioned treatments (Fig. 5A; Tables 1 and 2). Indeed, after 10 days, 90% of the individuals in the control were still alive (20/22 symbionts)

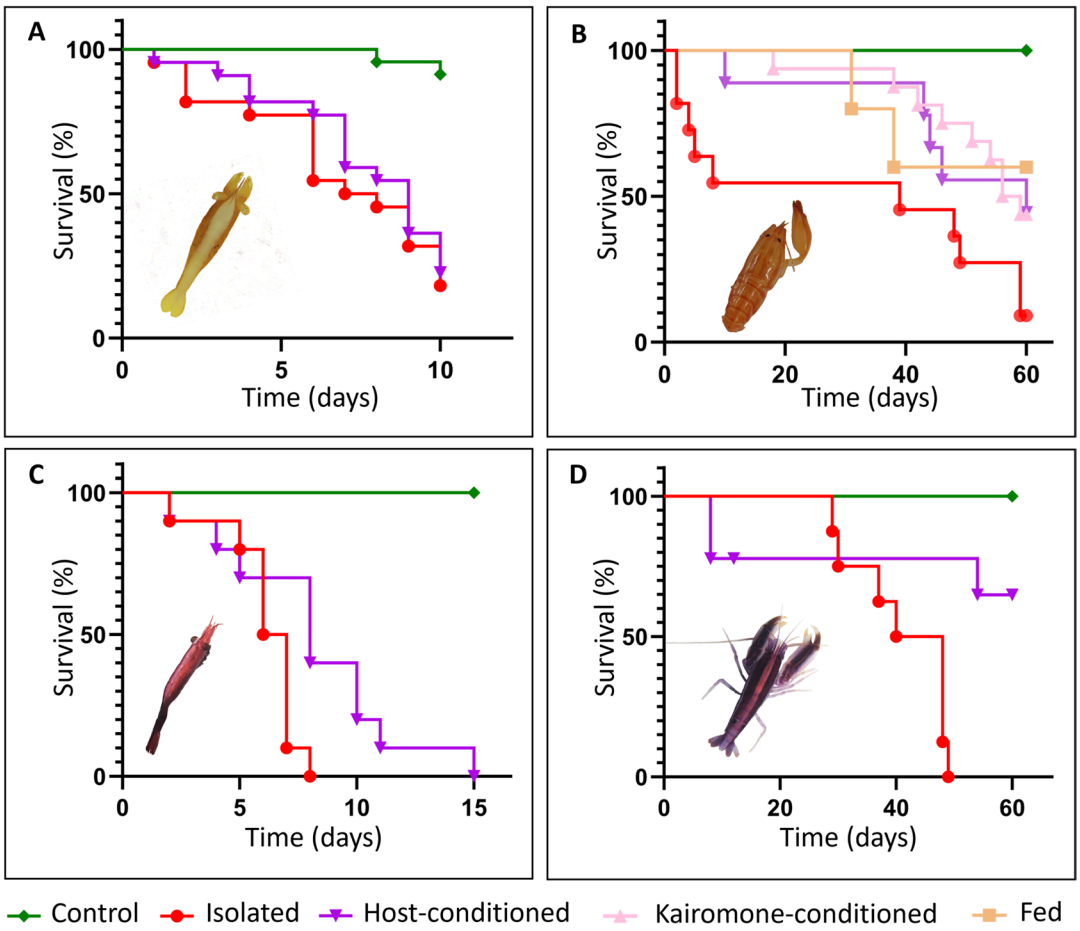


Fig. 5 Survival curves of the four species of shrimps associated with echinoderms. (**A**) *Zenopontonia soror*, (**B**) *Synalpheus stimpsonii*, (**C**) *Tuleariocaris holthuisi* and (**D**) *Arete indicus*. For all species, three conditions are represented with (i) the control shrimps in green, (ii) the isolated treatment in red and (iii) the host-conditioned in purple. For *S. stimpsonii*, two supplementary treatments were applied: (iv) kairomone-conditioned and (v) Fed with algae. The x-axis represents the number of days of the experimentation and the y-axis shows the percentage of survivability of the species. Number of replicas for each treatment can be seen in Fig. 2 or in the Material and methods section. The figure was made with Prism

while only 22.7% in the host-conditioned treatment (5/22 symbionts) and 18.1% in the isolated treatment (4/22 symbionts). The Mantel-Cox test indicated that the survival curve distribution of the controls was significantly different from the distributions of the two other treatments (both p-value $< 10^{-4}$) but there was no significant difference between the survival curve distributions of symbionts maintained isolated or in water conditioned by the host (p-value = 0.5).

Table 1 Median number of days of survival after a host separation experiment using four symbiotic shrimp species associated with echinoderms

	Z. soror	*S. stimpsonii*	*T. holthuisi*	*A. indicus*
Control	Undefined (90% of survival at the end of the test, 10 days)	Undefined (100% of survival at the end of the test, 60 days)	Undefined (100% of survival at the end of the test, 10 days)	Undefined (100% of survival at the end of the test, 60 days)
Isolated	7.5	39	6.5 days	44
Host-conditioned	9	60	8 days	Undefined (64.8% of survival after 60 days)
Kairomone- conditioned	N/A	57.5	N/A	N/A
Food treatment	N/A	Undefined (60% of survival after 60 days)	N/A	N/A

Replicates associated to the number of species tested per condition can be obtained in Fig. 2. N/A = Not Applicable (as not tested)

Table 2 Statistical analyses comparing the different host separation treatments displaying *P*-values obtained using Mentel-Cox statistic tests (see material and methods for more information)

	Z. soror	*S. stimpsonii*	*T. holthuisi*	*A. indicus*
Control VS Isolated	**$< 10^{-4}$**	**$< 10^{-4}$**	**$< 10^{-4}$**	**$< 10^{-4}$**
Control VS Host-conditioned	**$< 10^{-4}$**	**$6\ 10^{-4}$**	**$< 10^{-4}$**	**$4.6\ 10^{-2}$**
Isolated VS Host-conditioned	0.5	**$4.1\ 10^{-2}$**	**$2.8\ 10^{-2}$**	**$1.2\ 10^{-2}$**
Control VS Fed	NA	**$7.5\ 10^{-3}$**	NA	NA
Control VS Kairomones-conditioned	NA	**$5\ 10^{-4}$**	NA	NA
Host-conditioned vs Fed	NA	0.8	NA	NA
Host-conditioned VS kairomones-conditioned	NA	0.9	NA	NA
Fed VS Kairomone-conditioned	NA	0.7	NA	NA

P-value < 0.05 are highlighted in bold. N/A = Not Applicable (as not tested)

Considering the three other host species, the survival rates are positively impacted by the chemical environment produced by the hosts (Table 2). Indeed, after 60 days of experiment using *S. stimpsonii*, we observed a survival rate of 100% in the control (16/16 symbionts), 45.4% in conditioned sea water (5/11 symbionts) and only 9.1% in the isolated treatment (1/11 symbionts) (Fig. 5B). The Mantel-Cox test revealed a significant difference between the three different treatments, each corresponding *p*-value being under 0.05. In pairwise comparisons, the control condition is significatively different from the isolated treatment (p-value $< 10^{-4}$) and from host-conditioned treatment (p-value $= 6\ 10^{-4}$). The isolated and host-conditioned symbionts survival curves were also significatively different (p-value $= 4.1\ 10^{-2}$). In addition, shrimps in the anthraquinone-conditioned sea water presented a survival of 56.25% (9/16 symbionts) while daily fed symbionts survival was of 60.0% (3/5 symbionts) (Fig. 5B). These two additional conditions were both significatively different in comparison to the control (p-value $= 5\ 10^{-4}$ and $7.5\ 10^{-3}$ respectively) but not significatively different of each other (p-value = 0.7) or from the host-conditioned treatments (p-value = 0.9 and 0.7, respectively).

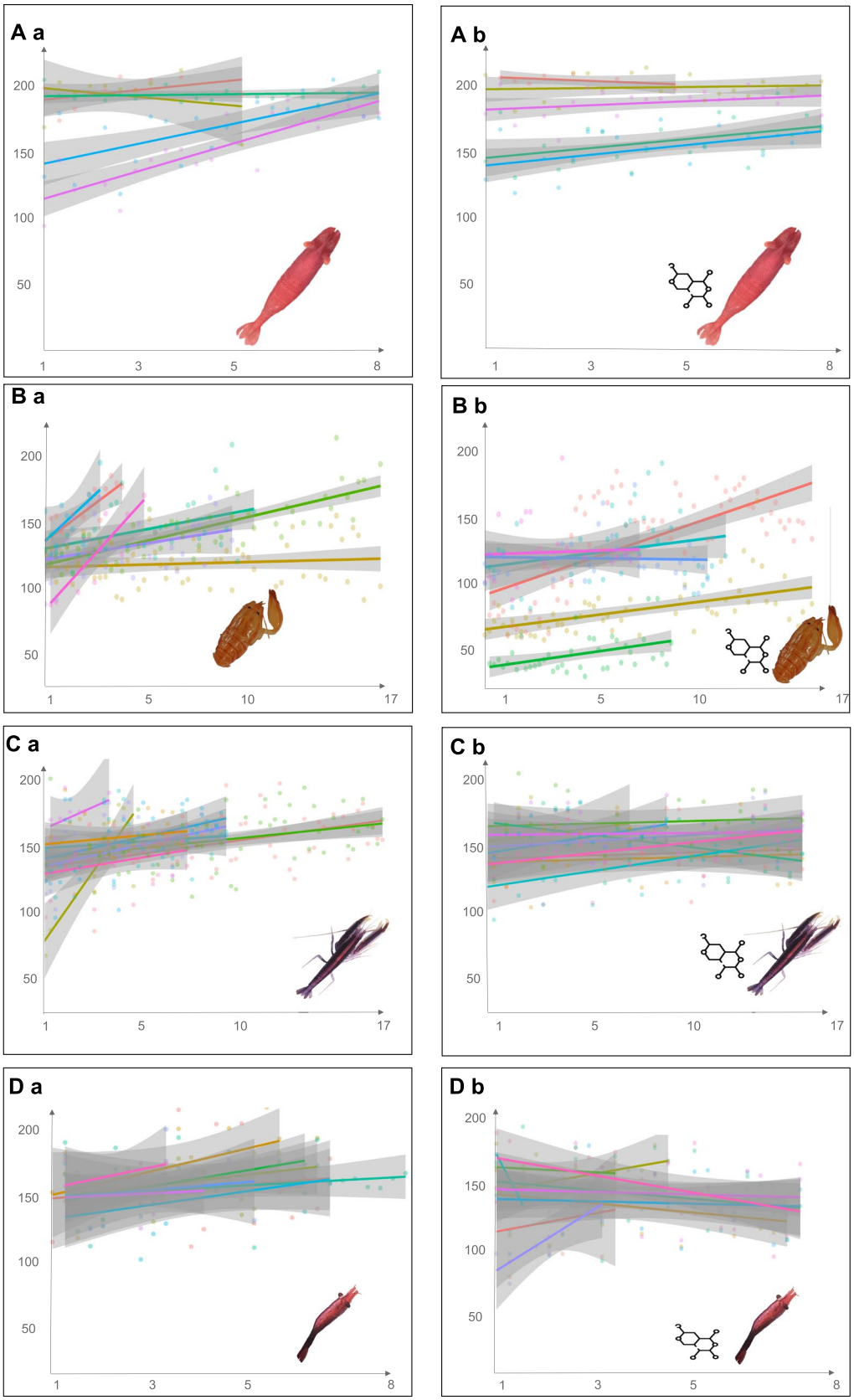
Concerning *T. holthuisi,* the isolated symbionts survived up to a maximum of 8 days (0/10 shrimps) (Fig. 5C). After 10 days, only 20% of host-conditioned individuals remained alive (2/10 symbionts) while 100% of the control symbionts remained (10/10 symbionts) (Fig. 5C). The Mantel-Cox test showed significant differences between the control and the isolated symbionts (p-value $< 10^{-4}$), between the control and the host-conditioned treatment (p-value $< 10^{-4}$) and between the isolated and host-conditioned symbionts (p-value $= 2.8\ 10^{-2}$). Concerning *A. indicus,* after 48 days of experimentation, all isolated symbionts did not survive (0/10 symbionts), while 64.8% of conditioned shrimps (6/9 symbionts) and 100% of the control (10/10 symbionts) remained after 60 days (Fig. 5D). Statistically, the Mantel-cox test also shows a significant difference with a p-value of $< 10^{-4}$ in control versus isolated treatments, a p-value of $4.6\ 10^{-2}$ in the comparison between control and host-conditioned symbionts and a p-value of $1.2\ 10^{-2}$ in the remaining comparison.

3.3 Symbiont pigmentation variation associated with host separation

3.3.1 Long-term monitoring

During long-term isolation, observations revealed diverse discoloration and coloration events, depending on the treatment (isolated or host-conditioned) or the species investigated. When individuals were maintained in complete isolation, mainly discoloration was monitored for all four species studied, with some exceptions being observed at the individual level. For *S. stimpsonii*, *A. indicus* and *T. holthuisi*, discoloration was less pronounced when individuals were exposed to kairomones produced by their respective hosts but there was no difference in color variation between these treatments for the asteroid shrimp *Z. soror*. For *S. stimpsonii*, a nychthemeral cycle was highlighted, resulting in a more pronounced discoloration usually observed during the night, and recoloration starting from the sunrise until the sunset. In addition, when using artificial lights (*i.e.* during the midnight monitoring), some individuals could recolor rapidly, varying from transparent to redder individuals in a few seconds to a few minutes (pers. obs.). It must be pointed out that some individuals of *T. holthuisi* and *Z. soror* especially, seemed to show a specific recoloration pattern before dying prematurely (pers. obs.).

Gray values (GV) variations of the abdomen are illustrated in Fig. 6 (only the abdomen) and Figure S1 (all body parts considered) for isolated and host conditioned symbionts. Considering the five isolated *Z. soror* individuals (Fig. 6Aa), three showed an increase in GV of their abdomen, suggesting a tendency towards discoloration; one individual showed a relatively stable GV, indicating little color change; and one individual showed a decrease in GV over its abdomen, suggesting potential recoloration (Fig. 6Aa). It is important to note that this last individual died prematurely compared to the other replicates. Similarly, *Z. soror* individuals conditioned by the host environment (Fig. 6Ab) showed comparable patterns of GV variation. Four of these individuals showed a tendency towards discoloration, while


A a
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A b
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B a
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B b
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C b
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◂**Fig. 6** Long term analysis of the abdomen color variations in four species of ectosymbiotic decapods associated with echinoderms in isolated (Aa, Ba, Ca, Da) and host-conditioned (Ab, Bb, Cb, Db) treatments. Each graph represents the gray-scale values of the transformed RGB values obtained from photos taken at the time of the experiments on (**A**) *Zenopontonia soror*, (**B**) *Synalpheus stimpsonii*, (**C**) *Arete indicus*, and **D**) *Tulearіocaris holthuisi*. Each colored line represents the variation in gray values of an individual. Lines that extend to the end of the graph represent individuals that survived throughout the experiment, while lines that do not reach the end of the graph indicate that these individuals did not survive to the end of the experiment. These trajectories were generated from the linear regression of grey variation values, represented by the points on the graph. The x-axis represents time with day, the y-axis represents grey values. Each line represents an individual. The figure was made with RStudio

another appeared to undergo partial recoloration before prematurely dying. In summary, a trend towards increased mean GV, indicative of discoloration, was observed in the abdomen, whether the individual was isolated or conditioned by the host's olfactory environment (Fig. 6Aa-b).

For the isolated *S. stimpsonii* (Fig. 6Ba), a trend toward discoloration of the abdomen was observed. Indeed, on seven individuals examined, six showed a marked increase in gray values (GV), while one individual exhibited stagnation in GV. For shrimp conditioned in host-specific seawater (Fig. 6Bb), GV revealed greater diversity in coloration patterns among specimens**.** Indeed, two individuals showed a tendency to discoloration, three appeared to have brighter coloration, and one individual remained relatively stable. In conclusion, in isolated *S. stimpsonii*, a general discoloration can be suggested. In contrast, when conditioned by the host's olfactory environment, isolated individuals appear to show variable responses in their coloration.

Considering *A. indicus*, important variations are observed depending on the treatment, whether the shrimps are isolated in seawater or in conditioned seawater (Fig. 6C). The individual number 4 died prematurely (yellow slope) and showed a substantial increase in GV, indicating significant discoloration, while the other eight individuals also showed an increase in GV associated with discoloration (Fig. 6Ca). In the host-conditioned shrimps (Fig. 6Cb), the linear regression of GV appeared to be more stable, with a smaller linear coefficient. Nine individuals remained more stable in their GV values, reflecting more consistent coloration, while one individual showed a decrease in GV, suggesting potential discoloration**.** In summary, despite a general increase in GV in both conditions, this trend appears to be more pronounced in totally isolated individuals, indicating more marked discoloration in this condition (Fig. 6Cb).

In isolated *T. holthuisi* (Fig. 6Da), isolated individual 3 is not shown as it died rapidly. The other individuals show a general increase in GV. For host-conditioned shrimps (Fig. 6Db), the graph reveals nuanced results as seven individuals show an intensification of coloration, while three others show a discoloration**.** Therefore, there is a notable difference in the trend of GV variations between the two conditions.

Table 3 shows the differences observed in grey values (GV) between the initial and final points, together with the linear regression coefficients obtained from the graphs in Fig. 6. For *Z. soror,* an increase in GV is observed both in total isolation and in host-conditioned environments, with no difference visualized between the 2 conditions. For *S. stimpsonii*, this numerical increase corresponds to the graphical observations. Similar trends are observed for *A. indicus* and *T. holthuisi*.

3.3.2 Short-term monitoring

As a reminder, short-term color monitoring was only realized on the three morphotypes of *Z. soror* for 8 h. For the three morphotypes and all body part investigated, including the abdomen, color intensity seems to decrease when individuals were isolated from their hosts, as all the GV linear regression tend to increase (Fig. 7A-C, Fig. S2, Table 4). Therefore, in this short-term monitoring, results indicate that all *Z. soror*, independently of their morphotypes or the body part investigated, have a discoloration when separated from the hosts.

3.4 Chromatophore analysis with histological sections

Under light microscopy, histological sections revealed the presence of chromatophores located within the dermal layer of the shrimp, under their transparent cuticle. Chromatophores were identified in all species with significant variation in size and pigment covers. They ranged from single chromatophore measuring 20 ± 8.5 µm (in *Z. soror*; Fig. 8Aa) to a maximum of 36 ± 13 µm (in *S. stimpsonii*; Fig. 8Bd). Multiple chromatophores generally packed together making cell boundaries difficult to identify because of their proximity (in colored *S. stimpsonii* for example: Fig. 8 Bb). Their shape can vary from amoeboid, characterized by radial arrangement with elongated and pigmented arms extending outward from a central cell body (*e.g.* Fig. 8Aa or Cc) to spherical presenting a condensed uniform round shape that have less cell protuberances (*e.g.* Fig. 8Bd or Cd). The chromatophore displayed varying degrees of pigmentation, with some appearing densely colored (*e.g.* Fig. 8Bb), while others were relatively pale (*e.g.* Fig. 8Cb). Usually, these chromatophores arbored different tints of orange/red. This coloration was observed in all four species. However, one species (*A. indicus*) also presented a second type of chromatophores containing pigments with purple coloration (Fig. 8D).

Table 3 Table showing the differences in gray variations (GV) of the long-term analysis of the abdomen color variations in four ectosymbiotic decapod species associated with echinoderms in isolated and host-conditioned treatments

	Z. soror									
ID	**1**		**2**		**3**		**4**		**5**	
δ GV (I)	-4.6		-13.6		6.3		45.6		88	
δ GV (HC)	-8.5		-5		25		41.6		-1.2	
LR coefficient (I)	-25.2		35		-12.3		49.6		51.2	
LR coefficient (HC)	-28.2		38.5		39		22.9		-2.6	
	S. stimpsonii									
ID	**1**	**2**		**3**		**4**	**5**		**6**	**7**
δ GV (I)	24.2	-25.2		67.4		30.4	37.7		49.4	84.8
δ GV (HC)	71.3	-0.3		-0.5		22.2	-2.3		19.6	NA
LR coefficient (I)	1.6	4.5		-9.6		12.2	-33.5		16,5	2.1
LR coefficient (HC)	9.2	2.8		-0.8		11.9	7.3		4	NA
	A. indicus									
ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
δ GV (I)	63.4	59.5	-5.1	NA	-26.9	-33	36.2	19.8	13	23.7
δ GV (HC)	4.05	27.7	8.7	40.8	18.7	52.2	29.7	32.8	29.8	46.3
LR coefficient (I)	5.9	8.6	-20.6	NA	-41.3	-66.7	-2.1	-13.7	-12.6	23.6
LR coefficient (HC)	-19.4	21.7	-30.7	15.1	4	2.8	-1.4	9	0.3	33.5
	T. holthuisi									
ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
δ GV (I)	61.2	39.4	NA	25.6	7.7	-22.6	-14.8	-47	0.3	-2.5
δ GV (HC)	26.5	-28.9	11.8	-4.5	-32	-37.2	4.1	25.3	16.8	-23
LR coefficient (I)	30	29	NA	-26.4	-28.4	-65	-46.9	-53.5	13.8	-37.7
LR coefficient (HC)	-65.9	-67.4	-67.8	-4.45	-50.3	-37.2	-43.2	-22.3	11.3	-6

The initial and final GV and the linear regression coefficients obtained of each individual for the 4 species studied. Differences of GV are in RGB units, linear regression coefficients have not units

δ, differences; *GV*, Gray variation; *ID*, individual; *LR*, Linear regression; *I*, Isolated and *HC*, Host-conditioned

In the case of *Z. soror*, two morphotypes were investigated: colored (*e.g.* Fig. 8Ae) and translucent (*e.g.* Fig. 8Af). In colored morphotypes, numerous spherical and amoeboid chromatophores were visible with a pigmentation covering 50.7% (±37.9%) of the total dermis surface (Fig. 8Aa-b). When translucent, no chromatophore was observed in the entire histological section (pigmentation covering = 0%) (Fig. 8Ac-d).

For *S. stimpsonii,* individuals with a vivid coloration (*e.g.* Figure 8Be) or discolored because of the nychthemeral cycle of this species (*e.g.* Figure 8Bf) were investigated. The dermal surface covered by chromatophores was 75.2% (±26.6%) in the colored individuals and 35.4% (±19.2%) for the discolored ones (Fig. 8Bc-d). For this species also, spherical and amoeboid chromatophores of various sizes were apparent throughout the dermal and muscular layers

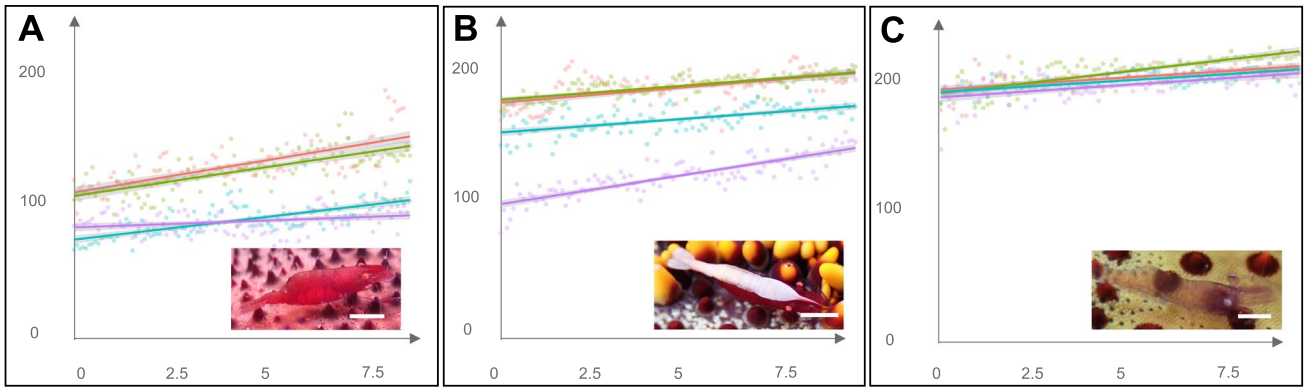

Fig. 7 Short term analysis of the abdomen color variations in three morphotypes of the decapod shrimp *Zenopontonia soror* in isolated conditions. Color variation during a short time under constant light (1300 Lumen) (**A**) Graph showing the variation in RGB values of 4 *Z. soror* individuals of full-colored morphotype separated from their hosts. The x-axis represents time in hours, the y-axis represents RGB values transformed into gray. (**B**) Graph showing the variation in RGB values of 4 *Z. soror* individuals of stripe-colored separated from their hosts. The x-axis represents time in hours, the y-axis represents RGB values transformed into gray. (**C**) Graph showing the variation in RGB values of 4 *Z. soror* individuals of translucent morphotype separated from their hosts. The x-axis represents time in hours, the y-axis represents RGB values transformed into gray. Scalebar = 0.3 cm. The figure was made with RStudio

Table 4 Table showing the differences in gray variations (GV) of the short-term analysis of the abdomen color variations in the three morphotypes of *Zenopontonia soror* associated to *Culcita novaeguineae* in isolated conditions

	Full colored morphotype			
ID	**1**	**2**	**3**	**4**
δ GV	26.4	33.8	52.4	19.3
LR coefficient	4.7	4.2	3.3	1
	Strip-colored morphotype			
ID	**1**	**2**	**3**	**4**
δ GV	29.1	32.3	28.3	66.2
LR coefficient	2.4	2.2	2.1	4.5
	Translucent morphotype			
ID	**1**	**2**	**3**	**4**
δ GV	28.6	37.8	26.5	50.2
LR coefficient	2.5	4.5	2.4	5.6

Table represents the initial and final GV and the linear regression coefficients obtained of each individual. Differences of GV are in RGB units, linear regression coefficients have not units

δ, differences; *GV*, Gray variation; *ID*, individual; *LR*, Linear regression; *I*, Isolated and *HC*, Host-conditioned

(Fig. 8Ba-b). During discoloration, a noticeable aggregation of chromatophores could be noticed (Fig. 8Bc-d).

For the third species *T. holthuisi,* histological sections were performed on colored individuals (*e.g.* Figure 8Ce), as well as host-isolated individuals that have discolored (*e.g.* Figure 8Cf). The pigmentation covering in colored individuals represent 54.4% (± 13.4%) of the total dermal surface. In comparison, in discolored organisms, they only had 16.5% (± 10.5%) of their dermal surface covered by pigmentation (Fig. 8Cc-d). In colored *T. holthuisi*, chromatophores do not exhibit a unique and distinct shape, harboring different degrees of amoeboid and spherical chromatophores, usually densely concentrated together in unstructured aggregates (Fig. 8Ca-b). Conversely, in discolored individuals,

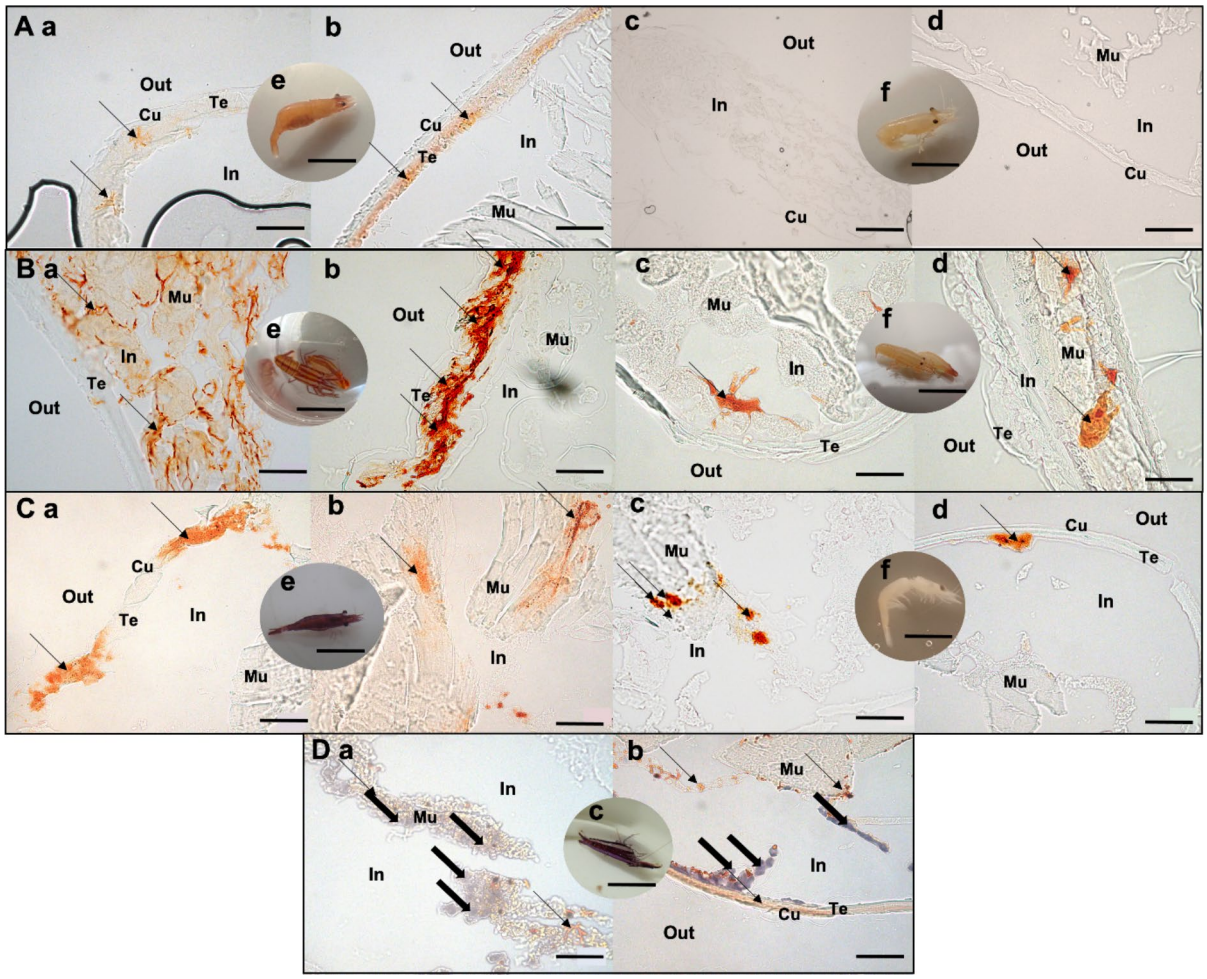

Fig. 8 Histological sections reveal chromatophores in four symbiotic shrimp species: (**A**) *Zenopontonia soror.* (Aa-b) histological sections of a fully colored *Z. soror* individual. (Ac-d) histological sections of a translucent *Z. soror* individual. (Ae) represents a fully colored and (Af) represents a translucent adult *Z. soror.* (**B**) *Synalpheus stimpsonii.* (Ba-b) histological sections of a colored *S. stimpsonii* individual. (Bc-d) histological sections of a discolored *S. stimpsonii* individual. (Be) represents a colored adult *S. stimpsonii.* (Bf) represents a discolored adult *S. stimpsonii.* (**C**) *Tuleariocaris holthuisi*. (Ca-b) histological sections of a colored *T. holthuisi* individual. (Cc-d) histological sections of a discolored *T. holthuisi* individual. (Ce) colored adult *T. holthuisi*. (Cf) discolored adult *T. holthuisi*. (**D**) *Arete indicus*. (Da-b) histological sections of a colored *A. indicus* individual. (Dc) colored adult *A. indicus*. Thin arrows indicate erythrophores, while the thick arrow indicates cells containing spinochromes. Out: Outside the individual; In: Inside the individual; Te: tegument; Mu: muscles; Cu: Cuticle. Scale bar = 20 µm in Aa-d; 200 µm in Ac; 1 cm in Af; 20 µm in Ba-d; 1 cm in Be-f; 20 µm in Ca-d; 1 cm in Ce-f; 20 µm in Da-d; 1 cm in Dc. The figure was made with Powerpoint

chromatophores coalesce into well-defined, intensely orange spherical formations (Fig. 8Cc-d).

On the latest species, *A. indicus*, no observation was made on the "discolored" morphotype as this species did not show any transparent morphotype and only colored individuals were investigated (*e.g.* Figure 8Dc). Histological sections highlight two distinct pigment layers in the dermis: an inner purple layer and an outer red layer (Fig. 8Da-b). The purple layer represented 65.8% (± 19.6%) of the total surface of the dermis and the red layer covered a surface of 59.7% (± 37%).

3.5 Pigments characterization by HPLC

As pigments of red/orange colors were observed in histological section, we suspected they may be composed of carotenoids, a major group of pigments found in shrimps and usually obtained through their diets. To investigate this, HPLC characterization was performed on two of the four symbiotic couples: *Z. soror—C. novaeguineae* and *S. stimpsonii—P. distincta.* Depending on the species, several carotenoids were separated and highlighted using the HPLC system (Fig. 9; table S1 and S2). The sea star *C. novaeguineae* presents the most complex carotenoid assemblage, with 11 to 15 different peaks corresponding

to two isomers of astaxanthin, lutein, zeaxanthin, canthaxanthin and their derivatives (Fig. 9A). The associated shrimps *Z. soror* present fewer pigments, with a carotenoid assemblage of both isomers of astaxanthin and a few traces of zeaxanthin and beta-carotene in the colored morphotype (Fig. 9B). In addition, no pigments were detected in the translucent morphotype of this species. The second host, *P. distincta* has the least complex assemblage, with only few traces of canthaxanthin detected (Fig. 9C). However, its symbiotic shrimps, *S. stimpsonii*, contain several pigments dominated by zeaxanthin with astaxanthin (both isomers), beta-carotene (both isomers), and traces of canthaxanthin, lutein and other pigment derivatives (Fig. 9D). As most derivatives could not be precisely identified and many compounds co-eluted, we decided to report the sum of all carotenoids (Table S2). *S. stimpsonii* are organisms presenting the biggest proportions of pigments per sample mass (mean: 97.7 ± SD: 23.4 mg per sample g), followed by *C. novaeguineae* (mean: 64.6 ± SD: 9.7 mg per sample g). *Z. soror* presents significatively fewer pigments, with a mean of 7.1 (± SD: 2.2) mg per sample gram and *P. distincta* is the species that contained the smallest proportion of carotenoids (mean: 1.5 ± SD: 0.8 mg per sample g).

4 Discussion

The shrimps *Z. soror*, *S. stimpsonii*, *T. holthuisi* and *A. indicus* are four obligatory ectosymbionts associated with 3 different classes of echinoderms. These symbionts share a similar way of life, and all present a mimetic pigmentation as well as a specific morphology resulting from co-adaptation with their host. As an example, more than harboring similar colors than their host, *T. holthuisi* also shows an elongated morphology that allows it to be confused with an urchin spine (Marin et al. 2005; Horká et al. 2016). The level of morphological adaptation is usually related to the level of specificity/dependency of the association. *Z. soror* is the most generalist symbiont being found on at least 23 asteroid species (Antokhina and Britayev 2020; De Grave et al. 2021; Lourtie et al. 2023; Fernández-Lereé et al. 2023). *S. stimpsonii* is also a generalist that can be found on at least 10 crinoid species (VandenSpiegel et al. 1998; Virgili et al. 2020; Caulier et al. 2022). However, these two species remain specifically associated to the class level, being only associated with asteroid and crinoid hosts, respectively. In contrary, both *A. indicus* and *T. holthuisi* are highly specific symbionts, primarily or exclusively found on *E. mathaei* (Gherardi 1991; Brasseur et al. 2018; Dabbagh et al. 2019;

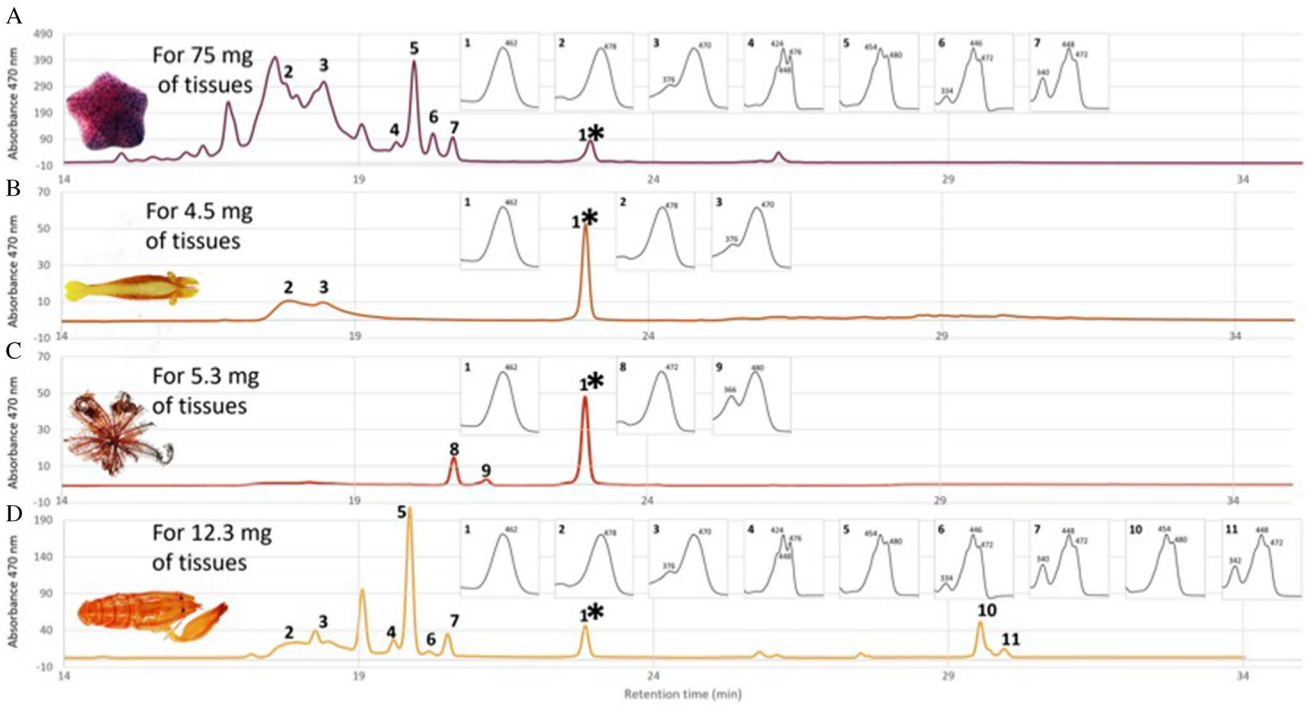

Fig. 9 High-performance liquid chromatography (HPLC) carotenoid profile with y-axis as absorbance at 470 nm and x-axis as retention time. (**A**) *Culcita novaeguineae,* (**B**) *Zenopontonia soror,* (**C**) *Phanogenia distincta* and (**D**) *Synalpheus stimpsonii*. Only peaks identified with experimental standards were highlighted. Peak 1* highlight the peak obtain with 20 µg of internal standard (8-apobeta-carotene (Roth ®)). Peak 2 + 3 = Astaxanthin; 4 = Lutein; 5 + 6 + 7 = Zeaxanthin; 8 + 9 = canthaxanthin; 10 + 11 = Beta-carotene. The figure was made with Powerpoint

Al-Kandari et al. 2020; Ghory and Kazmi 2021; Hayes et al. 2022).

The results obtained in this study support the concept of host separation syndrome introduced by Brasseur et al. in 2018. Indeed, the obligate ectosymbionts investigated here reveal a decline in their health condition when isolated from their hosts, leading ultimately to the organisms death, particularly in the absence of the chemical environment produced by the host. However, our multi-species data offer a more nuanced understanding of the dynamics of host separation and olfactory dependence, with a particular emphasis on coloration variation. All four decapod species are impacted by host separation, but notable differences exist. Firstly, *Z. soror* does not appear to be strongly dependent on the chemical signals produced by its host seastar, unlike the other symbiont species studied (*i.e.* no significant differences were measured between isolated and conditioned treatments). Also, *T. holthuisi* and *Z. soror* react more quickly to separation from their host than *A. indicus* and *S. stimpsonii,* probably because of their diets that depend on their hosts and because of their smaller body size than the latter species (Fautin et al 1995). This divergence also raises questions about the link existing between survivability and loss of coloration. *T. holthuisi*, *A. indicus* and *S. stimpsonii* show distinct coloration variation between the two isolated treatments. When isolated in seawater, discoloration is observed, but when isolated in the presence of the host-produced chemical environment, their coloration remains more stable. This suggests that host-derived chemicals, including sea urchin spinochromes (Brasseur et al. 2018) or crinoid anthraquinones (Caulier et al. 2022), may contribute to maintaining coloration stability. Both of these molecules were proven to be specific quinones used notably as colored molecules in these respective classes of echinoderms (Hatate et al. 2002; Zhu et al. 2008; Brasseur et al. 2018; Hou et al. 2018; Mishchenko et al. 2020). Interestingly, for *Z. soror*, survival data show host dependence, but this does not appear to rely on host semiochemicals. However, its separation from the host leads to discoloration, even when the shrimps are in the presence of semiochemicals produced by the sea star. Those semiochemicals are asterosaponins, which are defensive allomones against predators but also attractive kairomones for *Z. soror* (Lourtie et al. 2023). Contrarily to the attractive kairomones of crinoids, the anthraquinones (Lourtie et al. 2023), and the attractive kairomones of sea urchins, the spinochromes (Brasseur et al. 2018), asterosaponins are no pigments, they do not absorb UV and/or visible light and have never been associated with any other function than chemical defenses (Rideout et al. 1979; Netala et al. 2015; Brasseur et al. 2018; Caulier et al. 2022).

Considering that essential pigments for symbionts are carotenoids and quinones, future research should focus on the potential trophic link between symbionts and their hosts (Latscha 1989; Maoka 2020; Wade et al. 2017). Whereas *T. holthuisi* feeds exclusively on host tissues, *A. indicus* feeds only partially on them and may rely on other food sources to obtain its spinochromes (Brasseur et al. 2018). But we suspect that at least a part of their pigmentation may be attributed to additional carotenoids. A similar hypothesis may be true for *S. stimpsonii*, which feeds on particles available in the water column or by grazing close to the cirri of their host, rather than on host tissue (VandenSpiegel et al. 1998; Terrana et al. 2024). In particular, our HPLC results suggest that this shrimp contains a high proportion of carotenoids, with diverse pigments such as zeaxanthin, astaxanthin or beta-carotene, whereas its crinoid host has only a very low proportion of carotenoids. Previous studies suggest that the symbiont mimetic coloration, and the persistence of this coloration in the presence of host kairomones, may be facilitated by the transfer of anthraquinone pigments produced by the host (Caulier et al. 2022). Concerning *Z. soror*, some results suggest that it may be a kleptoparasite, feeding both on the same food source as its host and even potentially on the host itself (Mussoi et al. in prep.). Notably, they are usually found close to the ambulacral system and the mouth of the host (Lourtie et al. 2023). Since it shares common pigments with its host, such as astaxanthin or beta-carotene, it is possible that it obtains these carotenoids either by feeding at the same source as its host, or by directly consuming its host.

Also, the possibility of coloration being influenced by the color of the environment cannot be ignored. Previous research shows that the holothuroid associated crab *Lissocarcinus orbicularis* was able to adapt its pigmentation and shell ornamentation to match the colors of its host or the color of the bottom of the aquarium (Ayotte 2005. When isolated in uniform environments, such as aquariums without a host, symbionts may undergo discoloration to reduce visibility to potential predators (Gherardi 1991; Ayotte 2005). In contrast, in conditioned treatments, the shrimps are isolated in the same aquarium as their host where they can see them or be close to them. Therefore, they may remain more colored in response to the presence of the host.

Intraspecific characteristics may also play a role, particularly in *A. indicus* and *S. stimpsonii*. *A. indicus* exhibits a unique two-layered colored tissue, potentially containing carotenoids (red layer) in the first layer and spinochromes (purple layer) in the second layer. A previous study highlights the presence of spinochromes in this species by chemical extraction (Caulier et al. 2022). During isolation, carotenoids may be metabolized to ensure survival while maintaining the purple layer's coloration remained, inducing only a slight coloration change. For *S. stimpsonii*, which exhibits a nychthemeral coloration/discoloration cycle, the color variations can be significantly influenced by the light during monitoring and may be more related to the quantity of light present during the experiment (Joss 1973).

According to the literature, carotenoids are usually suggested as the dominant pigments in shrimp species (Latscha 1989; Higuera-Ciapara et al. 2006; Maoka 2020; Wade et al. 2017). Our results indicates that the pigments are found in mainly one or two layers of pigmented tissue located just beneath the transparent cuticle. Histological sections reveal erythrophores containing red–orange pigment dendritic cells, probably containing carotenoids, in the first layer found in all species, and a second layer with a purple coloration that we attributed to spinochromes only in *A. indicus*. The erythrophores are present in large quantities in colored individuals of all species but appear less abundant in *Z. soror* translucent morphotype and in a condensed form in discolored *S. stimpsonii.* For *Z. soror* translucent morphotype, the HPLC analyses highlighted that the lack of coloration is linked to the absence of carotenoids. However, for *S. stimpsonii*, carotenoids are still present in the organisms, but their chromatophores seem to have the ability to change their size/diameter by condensing or decondensing, influencing their coloration. This dynamic system may be regulated by different mechanisms (i) hormonal regulation, (ii) neuronal regulation or (iii) direct impact on the metabolism of carotenoids. In shrimps, mechanisms involving PCH (Pigment Concentrate Hormone) or PDH (Pigment Dispersion Hormone) were already observed (Fingerman 1969). These mechanisms may be involved in reversal color variation as a fast coloration/discoloration event or nychthemeral cycle. However, the discoloration observed in response to host separation may be primarily due to a lack of pigments obtained through feeding or through the host's chemical environment. As carotenoids are involved in various functions of metabolism (Matsuno 2001; Vershinin 1999; Wade et al. 2017), symbionts may reuse them to more fundamental functions leading to an overall depigmentation of the organisms. But as often in complex and dynamic environmental systems, it is evident that the observed color variations in these symbionts are influenced by a combination of factors, including perhaps hormonal regulation, neuronal regulation, and direct impacts on pigment metabolism. Further research is needed to unravel the precise mechanisms underlying these coloration variations and their ecological significance.

5 Conclusion

Our study reveals a host dependence for all four species and a dependence on host semiochemicals in *A. indicus*, *T. holthuisi* and *S. stimpsonii*. We observed discoloration in all four species studied upon separation from the host. Complete isolation of these symbionts revealed a noticeable discoloration, which seems to be mitigated in the presence of the chemical environment produced by their host. This discoloration was attributed to the presence of carotenoids, or other pigments such as spinochromes, stored in chromatophores. These dendritic pigment cells were found just beneath the transparent cuticle and were red–orange pigmented. This study highlights several potential drivers of coloration variation. The presence of host-derived chemicals (carotenoids, spinochromes and/or anthraquinones), dietary choices, environmental coloration adaptation, and intraspecific characteristics all seem to play a role in the mimetic color variation.

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Data and material availability Data and material can be requested by e-mail by contacting lisa.mussoi@umons.ac.be. Unused samples of the various species are available in the Marine Organisms and Biomimetics unit (UMONS, Belgium).

Code availability Not applicable.

Declarations

Ethics approval Animals used for the experiments were maintained and treated in compliance with the guidelines specified by the French Polynesia's Environment Department (DIREN) and the Belgian Ministry of Trade and Agriculture.

Consent to participate Not applicable.

Consent for publication All authors provide their consent for the publication of this scientific article.

Conflicts of interest The authors declare no potential conflicts of interest.

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