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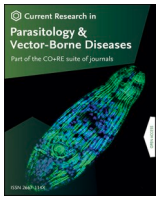

Laser-based killing of a macroparasite inside its live invertebrate host

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ABSTRACT

Clearing infection is an essential step to address many issues in host-parasite interactions but is challenging when dealing with endoparasites of large size relative to that of their host. Here, we took advantage of the lethality, contactless and versatility of high-energy laser beam to achieve it, using thorny-headed worms (Acanthocephala) and their amphipod intermediate host as a model system. We show that laser-based de-parasitization can be achieved using 450 nm Blue Diode Laser targeting carotenoid pigments in the bird acanthocephalan *Polymorphus minutus*. Using proboscis evagination failure and DNA degradation to establish parasite death, we found that 80% *P. minutus* died from within-host exposure to 5 pulses of 50 ms duration, 1.4 W power. Survival of infected gammarids 11 days after laser treatment was 60%. Preliminary tests were also performed with Nanosecond-Green Laser targeting lipids in *Pomphorhynchus tereticollis,* another acanthocephalan parasite. We discuss the efficiency and side-effect of laser treatment in this host-parasite system and highlight the perspectives that this technology more generally offers in parasitology.

1. Introduction

Host-parasite interactions are a driving force in the ecology and evolution of species and, as such, are extensively studied. Curing host from infection is a powerful method of addressing the reversibility of host responses, as well as assessing fitness costs associated with the parasite's strategy of host exploitation. The technology of lasers could offer promising alternatives to pharmacology to clear parasites because it offers a contactless tool to deliver lethal high-energy beam and could be highly specific. For instance, short-wavelength visible light is lethal to many insect pests (Hori et al., 2015), as well as green and infrared laser beam on aphids (Gaetani et al., 2021), mosquitoes (Keller et al., 2016) and salmon lice (ectoparasitic copepods) (Stingray® patent EP2531022, Stingray Marine Solutions AS, Norway; Moura et al., 2018). In these cases, the targets are external and readily exposed to a laser beam. However, targeting endoparasites without killing the host is certainly more challenging.

The aim of the present study was to develop a method to selectively kill a macro-endoparasite while preserving host viability, taking advantage of the great versatility of laser-based technology. Laser-directed optical energy deposition is almost instantly converted into heat. The induced heating is then distributed temporally and spatially in the material according to its thermal properties. Depending on the type of laser used, irradiation can induce thermal effects such as heating, fusion or vaporization, photochemical effect (molecular denaturation) or photo-mechanical effect (fast thermal ablation). The thermal effects correspond to the formation of a thermal gradient of varying duration and possibly to one or more phase changes in the material. Thermal effects can be divided into two main categories according to the relative time of heat generation and propagation: rapid effects correspond to an almost instantaneous supply and transformation of energy into heat and a long-lasting heat propagation; slow effects occur when heat generation time and propagation time are of the same order of magnitude. In addition to absorption, the propagation of light in soft tissues is also guided by diffusion in a tissue-specific manner. The collateral damages consecutive to this diffusion phenomenon can be minimized by using a wavelength matching the optical absorption properties of the target tissues.

Our host-parasite system involves the freshwater amphipod crustacean *Gammarus fossarum* (Gammaridae) as the intermediate host of two species of thorny-headed worms (Acanthocephala): the bird parasite *Polymorphus minutus* (Polymorphidae) and the fish parasite *Pomphorhynchus tereticollis* (Pomphorhynchidae). The infective larval stage of the parasite (cystacanth) is living freely in the gammarid body cavity and appears as an orange or red opaque ball visible though its host translucent cuticle (Kaldonski et al., 2009). The cystacanth is large in

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volume (approx. 0.65–1.00 mm^3 for *P. tereticollis*, two to three times less for *P. minutus*, pers. obs.) relative to its host size (approx. 0.6–1.0 cm in length). Carotenoid pigments and lipids are stored in the radial layer of the cystacanth body wall (Crompton, 1970). We tested two laser sources matching either the chromophore (carotenoids) or the constitutive lipids of cystacanth's tegument, and corresponding to either slow or rapid effects as described above: (i) A continuous laser source at the absorption peak of carotenoids (450 nm) (Gaillard et al., 2004; Kaldonski et al., 2009), with output power around 1 W, possibly chopped, and an exposure time of the order of a second to a few seconds. The cuticle was first drilled by vaporization at low power, and then thermal heating at high power was expected to induce lethal damage to the parasite; (ii) A pulse laser source matching absorption peak of lipids constitutive of acanthocephalan cystacanth at 532 mn, with a very short pulse duration typically in the nanosecond range (< 100 ns), low pulse energy (< 10 mJ) and a low repetition frequency (< 100 Hz). We expected rapid material removal by thermal ablation, i.e. a mechanical wave induced in the tissue. The efficiency of these methods was assessed by observing the mechanical and thermal damage done at the organism level, and the survival of both the targeted cystacanth and its gammarid host.

2. Materials and methods

General background information on laser-material interactions and the physical properties of lasers are necessary to guide specific methodological choices and assess their applicability to de-parasitization, as detailed in Supplementary Text S1. Theoretical models of light-energy transformation and of the spatial profile of laser beam according to its optical shaping (Supplementary Fig. S1) allowed us to predict the performance and features of the laser sources chosen, including laser spot diameter, M^2 beam quality, output energy, fluence and peak illumination.

2.1. Laser sources: Blue Diode Laser and Nanosecond-Green Laser

The Blue Diode Laser (BDL) source was a high-power multimode laser diode emitting 1.6 W @ 450 nm (PL-TB450B OSRAM) with an M^2 beam quality factor of about 4. We added several optical components at the output of BDL to generate a laser beam of diameter as small as less than 100 μm with a working distance to sample around 100 mm and a millimeter Rayleigth distance (see Supplementary Text S2). A low current mode of operation allows the power diode to be used directly as an aiming beam. A simple Arduino Due board provided a user-friendly means of setting a set of parameters: the energy, duration and number of pulses, and the choice of up to five consecutive shots with different specifications.

The laboratory-made Nanosecond Green Laser (NGL) source was converting the natural emission wavelength of 1064 nm - in the near infrared - into green emission at 532 nm with a second harmonic generator, to obtain pulse energy from 1 to 20 mJ and pulse duration around 10 ns with a maximum repetition frequency of 40 Hz. Several laser's parameters such as firing frequency, the number of pulses and energy per pulse were adjustable. The laser beam quality described by the parameter M^2 was less than 2. In this field of intensity, the interactions were highly photomechanical with the possible appearance of a plasma.

2.2. Full setup and exposure of gammarids

The full setup was designed to configure and operate the two laser sources, to pilot sample holder to precisely position the laser beam on the target area, and to allow complete immobility of gammarids during laser treatment, as detailed in the Supplementary Text S2. The setup included the two pulsed and continuous laser sources with their optical shaping and focusing systems, a thermo-regulated sample holder mounted on a motorized XY displacement (precision of 50 μm), and a lighting and aiming system (Fig. 1). We combined cold and anesthesia to immobilize gammarids, by thermo-regulating sample holder at a stabilized temperature of 3 °C, and bathing gammarids in the anesthetic ms222 at 60 mg/l for approx. 30 min prior to laser treatment (Perrot-Minnot et al., 2021).

Uninfected and naturally infected gammarids were collected in River Norges (Burgundy, France: 47°21′40.61″N, 5°9′30.53″E) (see Supplementary Text S4 for maintenance conditions). The optimization of laser treatment was reached by testing different combinations of pulses number and duration per shot. The BDL delivered a laser beam of 1.4 W with a minimum spot size was of around 70 × 30 μm (≈0.002 mm^2) corresponding to an irradiance of 67 kW/cm^2 (Supplementary Text S2; Supplementary Table S1). The exposure of parasite inside host body was facilitated by pre-drilling a hole through the cuticle with a laser beam of 0.5 W for 100 ms, equivalent to an energy of 50 mJ. The setup of the NGL and additional optical components delivered a laser beam at 532 nm with energy per pulse up to 5 mJ, corresponding to a peak irradiance of up to several GW/cm^2 for a 10 ns pulse duration (Supplementary Text S2; Supplementary Table S1).

2.3. Monitoring the death of P. minutus and P. tereticollis, and the survival of infected and uninfected gammarids to laser treatment

Two proxies were used to assess the survival of *P. minutus* and *P. tereticollis* cystacanths to laser irradiation: cystacanth's capacity to evaginate the proboscis (Perrot-Minnot et al., 2011), and DNA integrity (Roy et al., 2020) (as detailed in Supplementary Text S3). Evagination assay was performed just after dissection of the infected gammarid at least 24 h after irradiation, by immersion in the dark of *P. minutus* cystacanth in ddH_2O at 42 °C and of *P. tereticollis* cystacanth in bile from barbels *Barbus barbus* at 16 °C. Under these conditions, evagination occurs within 1–1.5 h (Perrot-Minnot et al., 2011). We assessed the integrity of total DNA from deep-frozen cystacanth, at least one day and up to one month after laser exposure. DNA extraction from single cystacanth was restricted to digestion following Park and Patek (1998). Details on DNA digestion, concentration by freeze-dried lyophilization, and visualization, are provided as Supplementary Text S3.

Survival of exposed gammarids was monitored during eleven days in air-conditioned room at 16 °C (Supplementary Text S4).

2.4. Statistical analysis

All analyses were performed with R-Studio, version March 1, 1073 (RStudio Team, 2020). Survival of gammarids to laser treatment was

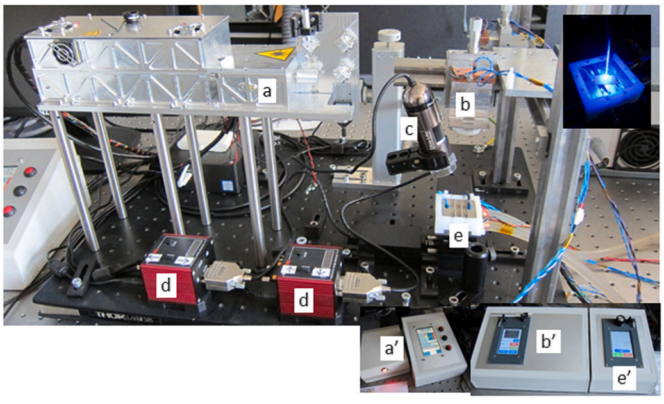

Fig. 1. Complete setup for irradiating acanthocephalan macroparasites inside live crustacean hosts, comprising two laser sources, Nanosecond Green Laser (532 nm) (a) and Blue Diode Laser (450 nm) (b), a video camera (c), the 2D-displacement system of temperature-controlled sample holder (d, e) (connected to computer screen, not on the picture); (a', b', e'): control units of BDL and NGL, and of refrigerated sample holder (thermostat), respectively.

analyzed using the Cox Proportional Hazards regression model with survival time as a dependent variable and laser treatment, infection status (infected or uninfected) and the interaction of both as predictors (*survival* package, v. 2.44-1.1; Therneau, 2021). We computed Type-II likelihood-ratio tests for each covariate in the model using "Anova" function in the *car* package v. 3.0-11 (Fox and Weisberg, 2019). We also checked model assumption of proportional hazards by visual inspection of scaled Schoenfeld residuals against time, for each covariate.

We compared the proportion of evaginated cystacanth in each laser treatment to the unexposed control the using Fisher's exact test on contingency table (*stats* package, v. 4.1.2; R Core Team, 2021). Odds ratio of evagination and associated 95% CI were estimated for each laser treatment. We analyzed the proportion of cystacanth showing DNA integrity similarly.

3. Results and discussion

Cystacanths of *P. minutus* exposed to a laser beam of 5 pulses of 50 ms at 1.4 W exhibited clear wounds in the form of protuberance or lesion (Fig. 2). Failure to evaginate proboscis was dependent on laser treatment, at a rate of 9% ($n = 22$), 72.7% ($n = 22$) and 96% ($n = 25$) for unexposed cystacanths, cystacanths exposed to 3 pulses of 20 ms, and cystacanths exposed to 5 pulses of 50 ms, respectively. The odds ratios (OR) of failure to evaginate after laser irradiation compared to controls were high for both laser treatments and was more than seven times higher at 5 × 50 ms than at 3 × 20 ms (mean OR: 177.1, 95% CI: 16.8–9388.9, $P < 0.0001$, and OR: 24.1, 95% CI: 4.03–273.5, $P < 0.0001$, respectively). DNA integrity assay revealed complete DNA degradation in 50 out of 63 cystacanths exposed to 5 × 50 ms (80%). The large molecular weight genomic DNA was not visible anymore in dead cystacanths, whereas small DNA fragments remained visible (Fig. 3). By contrast, DNA integrity (and RNAs in absence of RNAase treatment) was preserved in cystacanths exposed to 3 × 20 ms ($n = 9$) and in unexposed controls ($n = 38$) (Fig. 3). The odds of DNA degradation following laser irradiation at 5 × 50 ms was 3.5, which is lower than the odds of evagination failure (24.1). Therefore, some cystacanths may not evaginate but still harbor intact DNA. This discrepancy was even more pronounced in the 3 × 20 ms treatment where all cystacanths had intact DNA despite an evagination failure rate of 72.7% ($n = 9$ and $n = 22$, respectively). Laser treatment and its interaction with infection status impacted gammarids' survival, while infection status alone had no significant effect (see Cox Proportional-Hazards regression in Supplementary Text S4). Gammarids survival was lower following irradiation at 5 pulses of 50 ms at 1.4 W compared to unexposed controls but higher than that of uninfected ones (Fig. 4).

We failed to adapt the technique to the larger acanthocephalan *P. tereticollis*: although exposure to 5 pulses of 100 ms, 1.4 W resulted in cystacanth's evagination rate below 20% and DNA integrity below 60% (Supplementary Fig. S4), gammarids mortality was very high (close to 90% after 11 days). Interestingly, *P. minutus* has higher concentration of carotenoids compared to *P. tereticollis* (Perrot-Minnot et al., 2011), therefore the higher efficiency of BDL on this species is consistent with the absorption of laser energy by these chromophores and the consecutive lethal thermal effect, as expected from the deleterious effects of laser energy when not absorbed by its target. As an alternative to thermal effect only, we used the NGL to induce photo/thermomechanical damages. However, despite visible wounds, the damages induced to *P. tereticollis* at irradiation levels still compatible with intermediate level of host survival, did not induce instant parasite death (Supplementary Text S5). In fact, many cystacanths were found partly or completely evaginated within the host few hours or days after irradiation, adding another source of mortality to gammarids. It is possible that the high-energy and rapid shock wave produced by NGL resulted in limited superficial thermal ablation of parasite's thick and multilayered body wall, and that average heating was insufficient due to low total energy delivered.

The wound left by BDL irradiation on the amphipod was plugged within 2–3 h and melanized within 24 h (pers. obs.), and the scar was removed together with exuviae at the time of molting (Fig. 2). Gammarids mortality was observed with both laser sources but was much higher with the NGL. This is consistent with the two distinct effects of these laser sources. The BDL possibly produced a mild thermal effect that did not abruptly damage vital organs but rather induced limited heating over a short time. By contrast, the NGL produced a short-in-time mechanical wave at high energy, which could more likely irreversibly damage surrounding host's cells. The observed differences between Blue

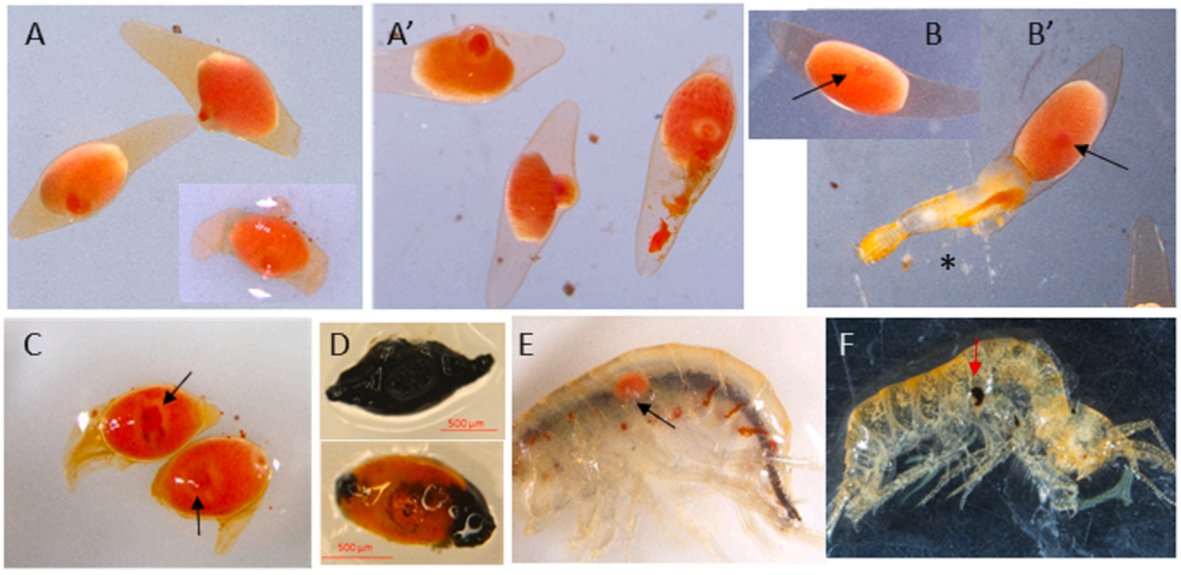

Fig. 2. Appearance of *P. minutus* cystacanths following exposure within live *G. fossarum* host, to a Blue Diode Laser (BDL) beam of 1.4 W at 450 nm. Cystacanths were dissected out of gammarids after exposure to BDL for 3 pulses of 20 ms (**A**, **A′**, **B**, **B′**) or 5 pulses of 50 ms (**C**, **D**). After exposure at 3 pulses of 20 ms, the same cystacanths were observed before (**A**, **B**) and after (**A′**, **B′**) evagination assay at 42 °C for 1 h: **A′** failure to evaginate; **B′** evagination of trunk and proboscis (*). After exposure at 5 × 50 ms, cystacanths were observed either few hours after irradiation (**C**) or a month later (**D**) showing complete (top) or partial (bottom) melanization of the cystacanth on exceptional occasion. **E, F** Live *G. fossarum* infected with *P. minutus* several days after laser treatment (**E**) and its exuviae after molting (**F**): the melanized scar is no more visible (**E**, *arrow*), as it has gone with the exuviae upon molting (**F**, *arrow*). Gammarid length is approximately 6–10 mm.

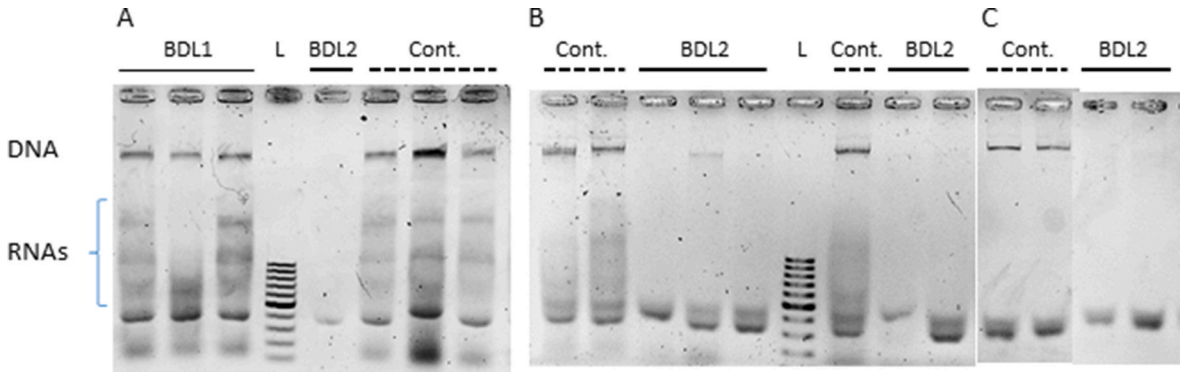

Fig. 3. DNA and RNAs integrity in *P. minutus* cystacanths following exposure through the cuticle of infected *G. fossarum,* to a Blue-Diode Laser beam of 1.4 W at 450 nm for 3 pulses of 20 ms (BDL1) or 5 pulses of 50 ms (BDL2), or not exposed (Cont.). Cystacanths were deep frozen in liquid-N just after dissection (less than 3 h after laser exposure) (**A**, **C**) or after evagination test (1.5 h at 42°C) (**B**). **A**, **B** no RNAse treatment, **C** RNAse treatment after DNA purification and before lyophilisation. L: 500 bp ladder.

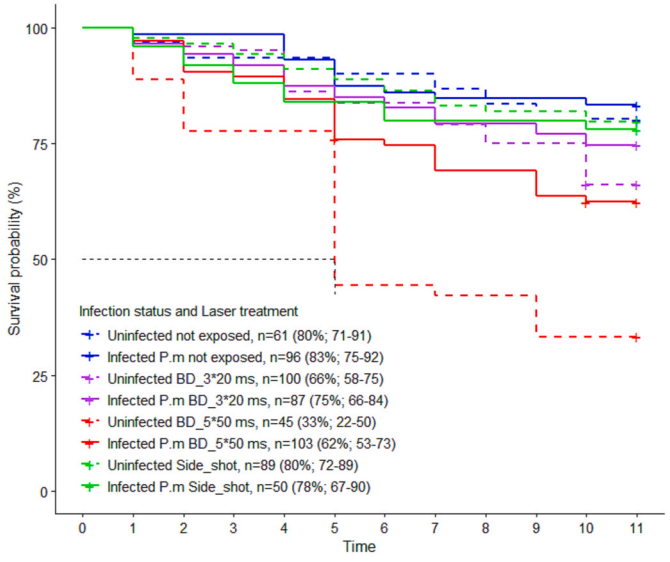

Fig. 4. Diagnostic plot for Cox Proportional Hazards Model representing the survival rate of uninfected gammarids (*dashed lines*) and gammarids infected with *P. minutus* (*plain lines*) up to 11 days post-irradiation to laser beam at 450 nm. Gammarids were exposed to one of two treatments: 3 pulses of 20 ms at 1.4 W interspaced by 100 ms (*purple lines*), or 5 pulses of 50 ms at 1.4 W interspaced by 100 ms (*red lines*). In both treatments, gammarid's cuticle was pre-drilled by irradiation at 0.5 W, 100 ms. Two controls were processed in the same way (anesthesia and handling) but either not exposed (*blue lines*) or exposed to a single pulse at 0.5 W, 100 ms (next to *P. minutus* for infected ones) (Side_shot). *Abbreviations*: *n*, sample size per treatment; P.m., *Polymorphus minutus*; BD, Blue Diode Laser.

Laser Diode and Nanosecond Green Laser in their relative efficiency and their deleterious side effect to the non-target organism, confirm the importance of optimizing wavelength, energy, and pulse duration to the absorption properties of targeted tissues, as emphasized in previous studies (Hori et al., 2015; Gaetani et al., 2021).

Laser-based technologies are increasingly used in biology, and offer prospects for applications to pest control, as exemplified by the automation of laser treatment on aphids (Lacotte et al., 2022) and salmon lice (Moura et al., 2018). To go further, the development and application of laser-based de-parasitization could also be expended to other host-macroparasite systems with various physical characteristics, such as parasitoids inside their insect hosts or insect larvae inside plant seeds. Indeed, the various thermomechanical and thermal effects of high-energy, short-wavelength visible light can be exploited and adapted to a range of host-parasite systems, providing that an appropriate imaging and aiming system allows targeting the parasite inside its living host. Such sighting system includes direct visualization, as used in the present study, but also X-ray scanner or micro-computed tomography. The temporal and spectral characteristics of the lasers as well as the irradiance and the tempo of the beam should then be optimized to choose the laser-tissue interaction modalities best suited to the types of pigments or physical structures that must absorb the energy delivered, while minimizing damage to the host's surrounding tissues.

4. Conclusions

We provide evidence for the lethal effects of high-irradiation energy from BDL on the carotenoid-rich *P. minutus* acanthocephalan with low associated mortality of its amphipod host. Laser-based de-parasitization could be achieved in other host-parasite systems provided that the target's absorption properties are taken into account, as illustrated here by the differences observed between BDL and NGL in their relative efficiency and their deleterious side effect. Killing the cystacanth inside its live intermediate host will allow us to evaluate the reversibility of phenotypic manipulation by *P. minutus*. The same goal applies to *P. tereticollis* providing that laser treatment can be optimized to minimize the death of *G. fossarum* associated with higher energy irradiation.

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Ethical approval

The study complies with the rules of ethics on crustaceans, as prescribed by the French legislation and the Université de Bourgogne Franche-Comté.

CRediT authorship contribution statement

Olivier Musset: Conceptualization, Methodology, Resources, Software, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Aude Balourdet:** Validation, Investigation. **Marie-Jeanne Perrot-Minnot:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary file. Raw data are available in Mendeley Data, at https://doi.org/10.17632/8j78n2znjb.2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2023.100135.

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