

Appendices

Optimization of anesthetic procedure in crustaceans: evidence for sedative and analgesic-like effect of MS-222 using a semi-automated device for exposure to noxious stimulus.

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APPENDIX A: Review of studies on anesthesia with Eugenol and MS-222 in Crustaceans; and illustration of locomotory tracks.

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APPENDIX C: Additional References

APPENDIX A

Table A.1. Review of studies assessing the anesthetic properties of MS-222 and Eugenol (clove Oil) (in bold: studies comparing both)

MS-222	Aim:	concentration	Criteria for anesthesia		Criteria for sedation				Time (min.) (mean or median)	Time (min.) (mean or median)	Dose-dependence of IT and RT	Size- or developmental stage-dependent	Side effects investigated	Reference	Observations
species (weight or size range)	type of stress to be mitigated		sedation	analgesia	immobility	tactile stimuli	other	stages of sedation	"full anesthesia"	full recovery					
<i>Gammarus pulex</i>	handling and transport	200 to 2000 mg/L			x				30 min. at 800 mg/L 14-15°C or 650 mg/L 25°C	16 min at 800 mg/L 14-15°C				Ahmad 1969	criteria for complete anesthesia not detailed - inefficient at 500 mg/L below 25°C- strong temperature-dependent effect
White Indian shrimp - <i>Fenneropenaeus indicus</i> - postlarvae	transport													Akbari et al. 2004 in Akbari et al. 2010	ineffective on juveniles (unless high concentration is used: 3 700 mg/L, compared to 22.5 mg/L for Eugenol)
American horseshoe crab - <i>Limulus polyphemus</i> (0.82–3.18 kg)	invasive procedures (stress and pain)	1 g/L	x	x	x	x	heart rate, gilling rate, dorsal recumbency		mediane 15 min.	25 min.				Archibald et al. 2019	
Crayfish - <i>Oronectes virilis</i>		up to 1000 mg/L				x								Brown et al 1996	ineffective
Giant freshwater prawn - <i>Macrobrachium rosenbergii</i>	transport	25 and 100 mg/L	x											Colye et al 2005	ineffective (induction time > 60 min.)
<i>Artemia franciscana</i>	2 steps euthanasia	4 g/L	x		x	x			35.5 min on average				increase in abnormal behavior	Darbyshire et al. 2019	inconsistent results , but 63% of Artemia became anesthetized at 4 g/L after 60 min.
<i>Corophium volutator</i> <i>Marinogammarus obtusatus</i>		500, 800, 1000 mg/L	x		x		cessation of pleopod beat						no mortality	Gamble, 1969	
Northern kelp crab - <i>Pugettia producta</i>	transportation-manipulation	500 mg/L	x		x		equilibrium loss		25.7 ± 0.9	8.7 ± 0.9			indication of stress (behaviour)	Morgan et al 2001	very low sample size (< 6) - no effect at lower dose, and on two other crab species
Ostracod - <i>Eucypris virens</i>	manipulation	500, 1000, 2000 mg L-1	x		x				2.5 min / 40 s. / 20 s.	5 min. / 10 min. 15 min.				Schmit and Mezquita 2010	(pb full recovery time was underestimated : cf locomotion resumes soon after transfer but increased very progressively - low sample size (4 per group)
<i>Gammarus pulex</i>	anaesthesia	600 mg/L	x	x	x	x			~45	~50	x		survival, respiration, physiological markers	this study	

Eugenol	Aim	Concentration	Criteria for anesthesia		Criteria for sedation			Time (min.) (mean or median)	Time (min.) (mean or median)	Dose-dependence	Size- or developmental	Side effects investigated	Reference	Observations	
species (weight or size range)	type of stress to be mitigated		sedation	analgesia	immobility	tactile stimulus	other	stages of sedation	"full anesthesia"	full recovery	of IT and RT	stage-dependent			
white Indian shrimp - <i>Fenneropenaeus indicus</i>	transport	1.3 mg/L to 3.7 mg/L	x		x		stimulation: perturbation - changes in WQ*	yes (4)	8h at 3,7 mg/L		x		respiration, survival	Akbari et al. 2010	100% survival at 2 mg/L and less - 3,7 mg/L necessary to reach full sedation - During 24 hours sedation, significant reductions were found in the oxygen consumption -
American horseshow crab - <i>Limulus polyphemus</i>	invasive procedures (stress and pain)	1 g/L	x	(x)	x	x	heart rate, gilling rate, cardiac contractility, hemolymph pH and pO ₂ WQ*		10 min. max	20.5 min.				Archibald et al 2019	Gilling rate and cardiac contractility decreased during anesthesia, whereas heart rate did not.
<i>Daphnia magna</i>	ecotoxicity of anesthetic drugs used for fish	50, 100, 50, 25, 10 and 1 µL/L.	x		x	x	heart rate and contraction		approx. 55 min. at 50 µL/L (large Daphnia)	approx. 1 min. at 50 µL/L (large Daphnia)	x	x	survival	Bownik et al. 2012	latent toxicity (at 24hs recovery: mortality > 90% above 50 µg/L); decreased heart rate - sedation at concentrations above 50 µg/L - size and dose-dependant
Giant freshwater prawn - <i>Macrobrachium rosenbergii</i> - juveniles (~2 g)	transport	100,200, and 300 mg/L	x			x	equilibrium loss	yes (4)	60 (200 mg/L)-45 (300 mg/L)	85 (200 mg/L)- 70 (300 mg/L)	x		survival	Colye et al 2005	2 stages anesthesia (equilibrium loss-reaction to tactile stimulus) - 100% survival
Norway lobster - <i>Nephrops norvegicus</i>	transport	600 µL/L - 900 µL/L	x		x	x	equilibrium loss		~7.3 - ~4.6	~11.6 - ~12.16	x			Cowing et al 2015	sedation: stationary: pleopod beating, leg movements, no response to stimuli / recovery: passive antennae movement; chelae movements and tail flipping in response to stimuli
<i>Artemia franciscana</i>	2 steps euthanasia	2.5 mg/L or 130 mg/L	x		x	x			inconsistent	inconsistent				Darbyshire et al. 2019	low repeatability and low efficiency: at 130 mg/L, only 20% of Artemia reached an anesthetized state
Australian red claw crayfish - <i>Cherax quadricarinatus</i> (5-37g)	handling	375 and 500 µL/L	x		x	x	equilibrium loss	yes	~13 min. at 500 µL/L	~9 min. at 500 µL/L		x	survival	Ghanawi et al 2019	max. induction time of 10 min. as a criteria for efficiency
Tiger prawn - <i>Penaeus monodon</i> (3.21±0.18 g)	handling and transport	60, 110, 160, 210 µL/L	x			x			30/17/12/12 min.	8/12/18/24 min.	x	x	survival (5 d)	Jiang et al 2020	Temperature-dependent IT and RT
Grass Shrimp - <i>Palaemonetes sinensis</i> (1,5 to 2,5 cm)		100 and 200 µL/L	x			x	complete loss of equilibrium		~20 (stage 2, 16°C, small size, 100 µL/L)	~95 min.	x		survival	Li et al. 2019	body size and temperature: lower survival rate of small shrimp at >= 24°C - no mortality after several days
* ability to right themselves when placed on their back lost: lateral/ dorsal recumbency															

Eugenol (cont.)	Aim	concentration	Criteria for anesthesia		Criteria for sedation			Time (min.) (mean or median)	Time (min.) (mean or median)	Dose-dependence of IT and RT	Size- or developmental stage-dependent	Side effects investigated	Reference	Observations	
species (weight or size range)	type of stress to be mitigated		sedation	analgesia	immobility	tactile stimulation	other	stages of sedation	"full anesthesia"	full recovery					
Dungeness crab - <i>Cancer magister</i> - Hairy shore crab - <i>Hemigrapsus oregonensis</i> - Northern kelp crab - <i>Pugettia producta</i>	handling and transport	Cm: 0.5 to 1.5 mL/L; Ho: 1 to 3 mL/L; Pp: 0.015 to 0.25 mL/L	x		x		equilibrium loss		Cm: 68 - 16 min; Ho: 188 - 87 min.; Pp: 54 - 2 min.	Cm: 7 - 11 min.; Ho: 59 - 83 min; Pp: 10 - 20 min	x	x		Morgan et al. 2001	very low sample size (< 6)
White Pacific shrimp - <i>Litopenaeus vannamei</i> - subadult	handling (short-term sedation ST) and transport (long-term LT)	subadults: 50, 100, 200 or 400 µL/L (ST); 5, 10 or 20 µL/L (LT) / post-larvae: 100, 150 or 175 µL/L (ST); 10, 20 or 50 µL/L (LT)	x			x	equilibrium loss		23 min. at 50 µL/L; 4 min. at 400 µL/L (subadults) / 4 min. at 178 µL/L (postlarvae)	4 min. at 400 µL/L	x	x	oxidative-stress, GST (hemolymph)	Parodi et al. 2012	2 stages anesthesia - Eugenol ineffective for long-term anesthesia at low concentrations (> 6 hs)
three spot swimming crab - <i>Portunus sanguinolentus</i> (69.98±3.56 g)	handling and transport	150-200-250 µL/L							20 à 150; 3,5 à 250	26 à 150; 100 à 250				Premarathna et al. 2016	
Giant freshwater prawn - <i>Macrobrachium rosenbergii</i> (32 g±2.1)	metabolic response to handling stress	100, 200, 400 and 800 µL/L	x						65.1 to 21.8	7.6 to 55.5	x		oxygen consumption rate, glucose, glycogen and	Saydmohammed and Pal, 2009	eugenol as a sedative, causing lower levels of glucose, glycogen and stress related enzymes, lower oxygen consumption rate
Green tiger prawn - <i>Penaeus semisulcatus</i> (1.8–2.1 g)	acute toxicity	12.5, 25 (not efficient), 50, 100, 150, 200 mg/L	x		x	x	visual /vibrational	complete loss of equilibrium	20 to 2.2 for "deep anesthesia"	4.5 to 11 min.	x		survival	Soltani et al. 2004	mortality (dose-dependent): LC50 after 1h exposure - effect of salinity and temperature
Giant freshwater prawn - <i>Macrobrachium rosenbergii</i> - post-larvae 13.42±0.84 mm) and juveniles (43.57±0.94 mm)	handling and transport	30 to 75 (post larve) - 125 and 250 mg/l (juveniles)	x		x			complete loss of equilibrium	3.3 (PL. 75 mg/L) - 78.3 (juv. 125 mg/L) - 66.8 (juv. 250 mg/L)	45.2 (PL.75 mg/L) - 4.2 (juv. 125 mg/L) - 16.9 (juv. 250 mg/L)	x	x	survival	Vartak and Singh 2006	mortality a concentrations of 125 mg/L (PL) and 750 mg/l (juv.) and above
<i>Gammarus minus</i> (4.3–14.3 mm)	handling	148 to 590 µL/L	x		x	x			1.7–7 min at 20°C	6.5–11.1 min	x	x	survival at 24hs	Venarsky and Wilhem 2006	size-dependent mortality: 27 to 80% at 590 µL/L for 8 min
American lobster - <i>Homarus americanus</i>		20 to 100 ppm (mg/L)	x		x		loss of aggression (1), and equilibrium (3)	yes	10 min. max	8 (75 and 100 ppm)	x			Waterstrat and Pinkham, 2005	max. induction time of 10 min. as a criteria for efficiency - full sedation reached at 75 and 100 ppm
<i>Gammarus pulex</i>		100 mg/L	x	x	x	x			~45	~70			survival, metabolism	this study	

APPENDIX A (cont.)

Examples of locomotory tracks of gammarids during induction of and recovery from sedation in MS-222 (600 mg.L⁻¹): it illustrates the progressive decline (a) and resumption (b) of locomotory activity under and after exposure to MS-222 respectively, as well as inter-individual variability.

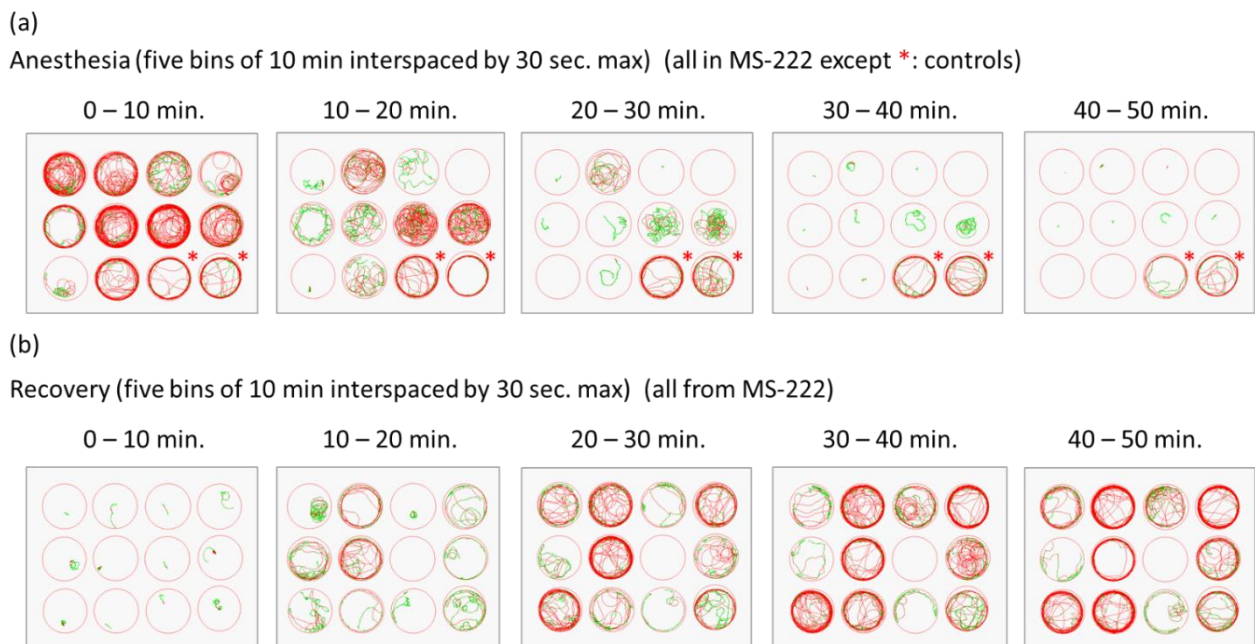


Fig. A1: Examples of locomotory tracks of gammarids recorded over 10 min. time bins (a) during anesthesia in MS-222 at 0.6 g.L⁻¹ and (b) during recovery from anesthesia. Circles represent arena walls (Petri dish, 9cm diameter), red and green tracks correspond to movements at a velocity above 15 mm.s⁻¹ and between 7 and 15 mm.s⁻¹, respectively. The two dishes marked with an asterisk hosted gammarids in water without MS-222 (controls).

APPENDIX B: Physiological markers

Background information: choice of biomarkers

We quantified cellular metabolic capacity by estimating the activity of the mitochondrial enzymatic machinery known as Electron-Transport-system (ETS). Mitochondrial ETS activity is a measure of potential metabolic activity and as such, offers a dynamic measure of host metabolism (Rueda-Jasso et al. 2004), complementary to whole organism actual respiration. For instance, alteration in ETS activity has been reported in aquatic crustaceans in response seasonal changes in temperature (Simčič and Brancelj, 2001) and concentration of contaminants (Olsen et al., 2007; Giusto et al. 2014). We assessed the antioxidant status of individuals by quantifying the total non-enzymatic antioxidant potential (TEAC Trolox-Equivalent-Antioxidant-Capacity assay), We evaluated neurotoxicity by the quantification of Acetylcholine Esterase activity (AChE) in whole gammarids. AChE is key enzyme in the sensory and neuromuscular systems in most animal species and is used in neurotoxicity studies in crustaceans (Xuereb et al., 2009; Gorokhova et al., 2013). Finally, we hypothesized that recovery from anaesthesia could increase cellular metabolism and oxygen consumption as compensatory mechanisms, alter the antioxidant capacity (in response to a putative oxidative stress), and putatively involve neurotoxicity.

Methods

We assessed the effect of exposure to MS-222 and Eugenol on physiological parameters just after 50 min. and 70 min. recovery from anesthesia, respectively. Gammarids were quickly dried on paper, put individually in pre-weighted Eppendorf tube, deep-frozen in N-liquid, and stored at -80°C. At the time of assay, samples (N=156) were analyzed in four batches of 39 samples, balanced with respect to the three treatments. For each batch, gammarids were thoroughly grinded in 450 µL of ice-cold Triton X-100 phosphate buffer (100 mM; pH = 7.8) with three stainless steel grinding balls, using a ball mill (RETSCH MM 400 M Mixer Mill)

during two rounds of 2 min at 30 Hz interspersed with 2 min on ice. The homogenate was then centrifuged at 9000 g at 4 °C for 15 min. Clear supernatants were collected and distributed randomly with respect to treatment and in duplicate (except for TEAC), in a 96-well flat-bottom plate (NUNC). Blanks were done with Triton X-100 phosphate buffer (100 mM; pH = 7.8) instead of supernatant. Biochemical assays were done in two separate experiments: one for AChE and the other for ETS activity and TEAC (supernatants were stored at -80°C meanwhile and defrosted only once); total protein content was therefore assayed twice. All assays were conducted using microplate spectrophotometers (Spectramax Plus384 and Versamax Tunable Microplate Readers; Molecular Devices LLC, Sunnyvale, CA, USA), and analyzed using the software SOFT-Max® Pro. 4.0 (Molecular Devices).

The supernatant was pre-diluted with phosphate buffer (100 mM; pH = 7.8) four times for the dosages of total proteins and TEAC, and twice for the dosage of ETS activity. We quantified total protein concentration in 5 µL of pre-diluted supernatant using the DC™ Protein Assay kit (Biorad) and bovine serum albumin (BSA) as standard (from 0.125 to 2 mg. mL⁻¹), following the manufacturer's instructions. Optical density was read at 750 nm after 15 min. at room temperature.

We measured the activity of Electron Transport System (ETS) following De Coen and Janssen (1997). with minor modifications. We mixed 50 µl of pre-diluted supernatant, 50 µl of buffered substrate solution (BSS; 0.13 M Tris-HCl and 0.3% (w/v) Triton X-100, pH 8.5) and 50 µl NADPH solution (1,7 mM NADH and 250 µM NADPH; Sigma Aldrich: N7505 and N4505, respectively). The reaction was induced by adding 100 µL INT (p-IodoNitro Tetrazolium 8 mM; Sigma Aldrich I8377). The absorbance was read kinetically at 490 nm at 25 °C for 20 min. The amount of formazan produced in µmol min⁻¹ per sample was estimated by dividing the increase in OD₄₉₀ from 0 to 10 min. by the absorptivity of INT-formazan (15.9 abs.units.µmol⁻¹.ml⁻¹.cm⁻¹) to get the molarity in the assay, multiplying by reaction volume

and the proportion of sample used, according to this formula (Maldonado et al 2012) : $\Phi = (\Delta \text{OD}_{490 (10 \text{ min.})} * 0.25 * 0.90) / (15.9 * 0.05 * 1000)$, expressed in $\text{nmol} \cdot \text{min}^{-1}$. We also recorded V_{max} on 10min.

The antioxidant potential was measured using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay, described in Re et al (1999). The antioxidant potential of a sample was estimated from its capacity to quench the free radicals of an oxidized ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] solution. The oxidized ABTS solution (ABTS⁺) was generated by reacting a solution of ABTS (Sigma Aldrich, A1888) at 7 mM in water with 2.45 mM of potassium persulfate, at obscurity and ambient temperature overnight. Just before dosage, the absorbance at 734 nm of ABTS⁺ solution was adjusted to 0.7 by dilution in filtered PBS pH 7.4. The range of standard Trolox (Sigma Aldrich 238813) concentrations was prepared by diluting a 25 mM stock solution freshly made in PBS triton X100 pH 7.8 from 2.5 mM to 0.063 mM, kept on ice and protected from light. The final concentration of Trolox ranged from 1.56 to 25 μM . Ten microliters of pre-diluted supernatant or of Trolox standard solution was then mixed with 240 μL of the ABTS⁺ solution in microplate well, and the absorbance at 734 nm was read every minute for 20 min, at 30 °C. TEAC was estimated by calculating the proportion of change in OD (debleaching) in 10 min. corrected by blanks, both in the samples and in Trolox standard, and by using the Trolox standard curve to derive the Trolox-equivalent antioxidant capacity in the sample in mM. Total antioxidant capacity is expressed in μmol per individual, after correcting by the fraction of supernatant used.

We measured AChE activity using colorimetric assay, following Xuereb et al. (2009). Briefly, 330 μL of PBS (100 mM; pH = 7.8), 20 μL of the chromogenic agent 5,5'-dithiobis-(2-nitrobenzoic acid: DTNB at 0.0076 M (Sigma Aldrich D8130) and 20 μL of supernatant were mixed. The measurement of enzyme activity was initiated by adding 10 μL of acetylthiocholine

iodide (ATCI) solution at 0.076 M (Sigma Aldrich A5751). The absorbance was immediately read kinetically at 405 nm every 60 seconds during 10 min (at 25°C). The increase in nanomoles of substrate hydrolyzed per minute was calculated in the linear part of the reaction (from 0 to 5 min.). AChE activity in nmol min^{-1} in the subsample was estimated by dividing the increase in OD_{405} by the extinction coefficient of DTNB to get the molarity in the assay, and multiplying by reaction volume, according to this formula: $\text{Rate (nmol.min}^{-1}) = (\Delta \text{OD}_{405 (5 \text{ min.})} / 13.6) * 380$. AChE activity in the whole sample was calculated by multiplying by the ratio of total sample volume on subsample volume. We also recorded V_{max} on 10min.

Statistical analysis

We run linear mixed-effects model to analyze the effect of anesthesia on physiological parameters (package “lme4” v. 1.1.21, Bates et al. 2019). For total protein content, fixed factors were anesthetic treatment, gammarid wet weight (mg) and interaction between treatment and weight, and plate number (N=4) was incorporated as random factor. For ETS activity, TEAC and AChE, fixed effects were anesthetic treatment, total protein content and interaction between treatment and protein content, with plate number as random factor. Acetylcholine Esterase was log₁₀-transformed to meet normality assumption. Backward elimination was performed to reduce the models using the package “lmerTest” v. 3.1.0 (Kuznetsova et al., 2017); and conditional R-squared of the reduced model was estimated using the package “MuMIn” v.1.43.6 (Barton, 2019). A posteriori multiple pair comparison was done using Tukey test with Westfall correction for multiple comparisons (package “multcomp, v. 1.4.10; Hothorn et al. 2008).

Results

The measures of total protein, ETS and AChE were highly to moderately correlated between replicated measures (Pearson correlation: Protein, $r = 0.71$, $N=156$, $P < 0.0001$; ETS, $r = 0.74$, $N=117$, $P < 0.0001$, AChE, $r = 0.34$, $N=117$, $P = 0.0002$). We took the mean of the

two replicated measures for the analysis. The two measures of mean protein content on the same samples were also highly correlated ($N=154$; $r = 0.94$; $P < 0.0001$). Total protein content increased linearly with gammarid's wet weight, with no effect of treatment and the interaction between treatment and weight (generalized linear regression, model reduced to gammarids weight: first dosage, $F = 7.09$, $Df = 1$, $P = 0.009$, $R^2_c = 0.10$; second dosage, AChE experiment: $F = 8.46$, $Df = 1$, $P = 0.004$; $R^2_c = 0.13$).

Variation in ETS activity was positively related to total protein content ($F=42.9$, $Df = 1$, $P < 0.0001$) and differed according to anesthetic treatment ($F = 5.04$, $Df = 2$, $P = 0.007$) with no significant interaction between treatment and protein content ($F = 0.22$, $Df = 2$, $P = 0.81$) (final model: $R^2_c = 0.68$) (Fig. B.1.a). Post-hoc paired comparisons revealed significant difference between MS-222 and Eugenol ($z = -3.17$, $P = 0.004$), but not with controls (Eug. – controls: $z = 1.55$, $P = 0.12$; MS-222 – Controls: $z = -1.76$, $P = 0.08$). The activity of ETS in gammarids recovering from anesthesia was higher after Eugenol bath compared to MS-222 bath.

The variation in total antioxidant capacity (TEAC) was positively related to total protein content ($F = 70.74$, $Df = 1$, $P < 0.0001$; $R^2_c = 0.84$), but independent of anesthetic treatment ($F = 1.11$, $Df = 2$, $P = 0.33$) and its interaction with protein content ($F = 0.25$, $Df=2$, $P = 0.78$) (Fig. Fig. B.1.b).

Finally, AChE activity was positively related to total protein content ($F = 10.23$, $Df = 1$, $P = 0.0017$) and differed according to anesthetic treatment ($F = 3.22$, $Df=2$, $P = 0.04$) and interaction with protein content ($F = 3.28$, $Df = 2$, $P = 0.04$) (Fig. B.1.c) (full model $R^2_c = 0.16$). However, no significant difference between treatments was detected in post-hoc paired comparisons on the ratio of AChE to total protein content (log-transformed). When AChE activity was estimated using V_{max} , only total protein content contributed to its variation ($F =$

12.0, $P = 0.0007$; $R^2_c = 0.12$) with no effect of treatment ($F = 2.70$, $P = 0.07$) nor its interaction with protein content ($F = 2.84$, $P = 0.06$).

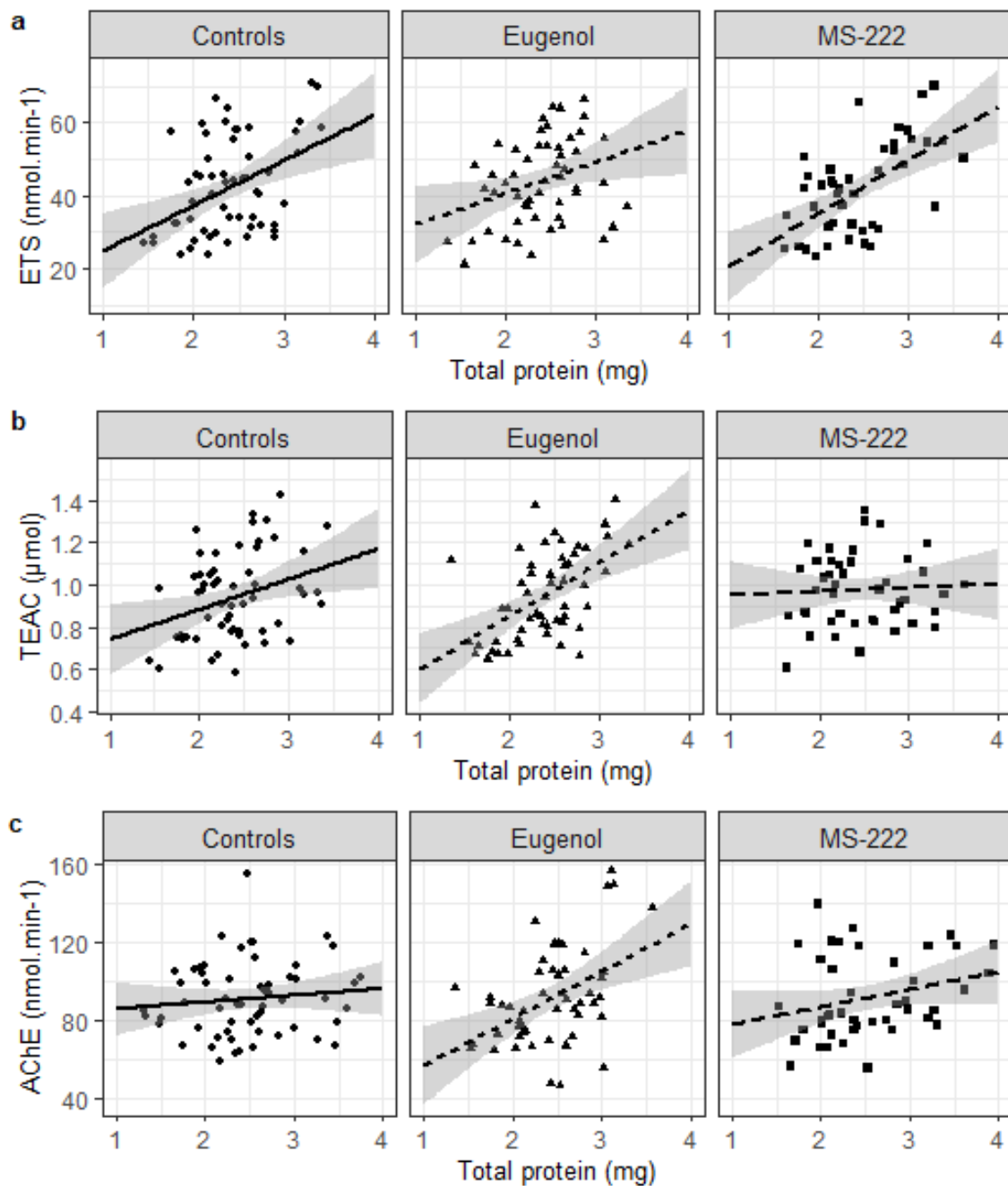


Fig. B.1. Physiological markers of cellular aerobic metabolism (Electron Transport System) (A), total antioxidant capacity TEAC (B), and activity of AChE (C), according to anesthetic treatment and total protein content in *G. pulex* (whole body). Dosages were done after anesthesia in Eugenol bath ($100 \mu\text{L} \cdot \text{L}^{-1}$) or MS-222 bath ($600 \text{ mg} \cdot \text{L}^{-1}$) for 45 min., and a recovery time of 70 min. or 50 min. respectively.

APPENDIX C: Assessing the efficiency of cold/freeze bath for anesthesia.

Methods

We used a fully crossed design to assess the efficiency of melted-ice bath as an anesthetic. For sedation in melted-ice, individual gammarids were immersed in a mixture of conditioned-water and ice flakes until immobility and lack of reaction to a tactile stimulus were observed. Anesthetic controls were handled in the same way but immersed in CW only. Gammarids were anesthetized in melted-ice and exposed to electric-shocks in the same 9-cm petri-dish equipped with two electric wires, as described in Perrot-Minnot et al. (2017). Upon reaching full sedation, three short impulses of 2 s. at 50Hz (9V) were delivered at 5 min intervals for 10 min. Control gammarids (either anesthetized or not) were placed in the same conditions but did not receive the electric stimulation. Refuge use was scored upon full recovery, assessed by the resumption of locomotion and reaction to tactile stimulus.

Results

Hypothermia did not mitigate the effect of ES: gammarids exposed to ES during ice-bathing exhibited an increased level of refuge use compared to ice-bathed gammarids unexposed to ES (Dunn test: $Z = 3.04$, $P = 0.0044$). This behavioural response to ES was not significantly different from unanesthetized gammarids exposed to ES (Dunn test: $Z = 0.26$, $P = 0.98$; Fig. C.1a). The effect size of electric shocks was comparable whether gammarids were exposed to ES during immersion in melted-ice or not (Fig. C.1b).

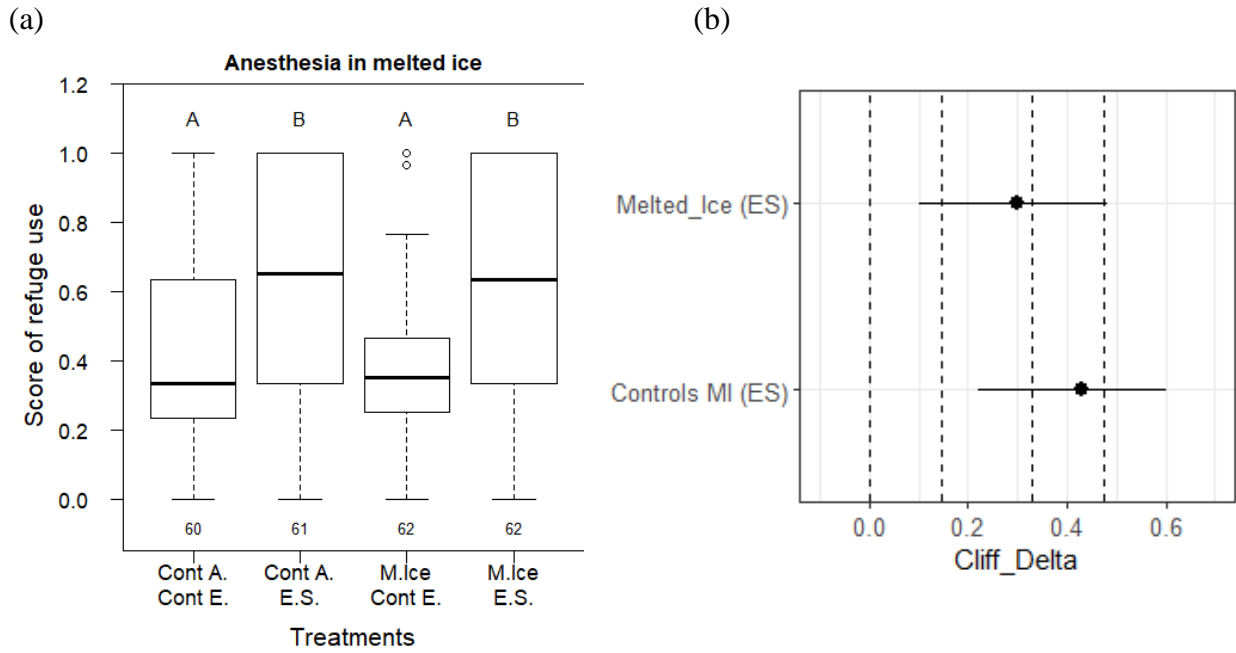


Fig. C.1. Assessing the anesthetic effect of hypothermia: (a) Refuge use of gammarids following exposure to a nociceptive stimulus (electric shock: E.S.) or not (Cont. E) under anesthesia by immersion in melted-ice (M. ice) or not (Cont. A). (b) Effect size of electric shock in gammarids exposed during sedation in melted-ice, and in controls ones (no sedation). Sample size is given below bars (a).

APPENDIX C: Reference list (additional references for appendices A and B)

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