Stress response in silver catfish *(Rhamdia quelen)* exposed to the essential oil of *Hesperozygis ringens*

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Abstract

This study investigated the effects of prolonged exposure of silver catfish (*Rhamdia quelen*) to the essential oil (EO) of *Hesperozygis ringens*. Ventilatory rate (VR), stress and metabolic indicators, energy enzyme activities and mRNA expression of adenohypophyseal hormones were examined in specimens that were exposed for 6 h to 0 (control), 30 or 50 µL L^{-1} EO of *H. ringens* in water. Reduction in VR was observed in response to each treatment, but no differences were found between treatments. Plasma glucose, protein and osmolality increased in fish exposed to 50 µL L^{-1}. Moreover, lactate levels increased after exposure to both EO concentrations. Plasma cortisol levels were not changed by EO exposure. Fish exposed to 30 µL L^{-1} EO exhibited higher glycerol-3-phosphate dehydrogenase (G3PDH) activity, while exposure to 50 µL L^{-1} EO elicited an increase in glucose-6-phosphate dehydrogenase (G6PDH), fructose-biphosphatase (FBP) and 3-hydroxyacyl-CoA-dehydrogenase (HOAD) activities compared to the control group. Expression of growth hormone (GH) only decreased in fish exposed to 50 µL L^{-1} EO, while somatolactin (SL) expression decreased in fish exposed to both concentrations of EO. Exposure to EO did not change prolactin expression. The results indicate that GH and SL are associated with energy reorganization in silver catfish. Fish were only slightly affected by 30 µL L^{-1} EO of *H. ringens*, suggesting that it could be used in practices where a reduction in the movement of fish for prolonged periods is beneficial, i.e., such as during fish transportation.

Keywords: Anesthetic; fish; hormones expression; metabolism; ventilatory rate.

1. Introduction

The use of anesthetics for fish is widespread in field studies, laboratory research and commercial aquaculture (Cotter and Rodnick, 2006). In commercial aquaculture, anesthetics are used in diverse husbandry manipulations such as fish selection, measurement, sampling, tagging, surgery procedures and transportation (Weber et al., 2009; Pawar et al., 2011). Live fish transport, as well as other practices in

aquaculture, often induces a physiological stress response that may result in undesirable outcomes such as immune system depression and growth impairment (Rotllant et al., 2001). The addition of sedative agents to the water during transport has the positive effect of improving osmotic and ionic balance, metabolism and oxidative stress parameters (Inoue et al., 2005; Azambuja et al., 2011; Becker et al., 2012, 2013).

Stressors can invoke primary, secondary and tertiary responses in fish (Wendelaar-Bonga, 1997). Fish neuroendocrine stress pathways rely on the adrenergic system and the hypothalamus-pituitary-interrenal (HPI) axis, whose stimulation culminates in the release of catecholamines and corticosteroids into the circulation (Pankurst, 2011; Weber et al., 2011). The primary response can lead to secondary responses such as increased cardiac output, metabolic rate, respiration, plasma free fatty acids and lactic acidosis. Secondary responses can also affect liver glycolysis and electrolyte balance. Tertiary responses also occur in response to stress and include aspects of whole-animal performance such as changes in development and growth, immune capacity, and interference with the storage, biosynthesis and efficient use of energy. Tertiary responses result in alteration of reproductive capacity, overall resistance to disease, and ultimately, survival (Barton, 2002; Schreck, 2010). The activity of enzymes involved in the metabolism of carbohydrates, lipids and proteins can give clues as to how the animal reorganizes its energy status after experiencing a stressful event. However, to date, studies addressing these indicators have been related to the energy demand required for osmoregulatory adjustments that arise due to changes in salinity (Láiz-Carrion et al., 2005; Sangiao-Alvarellos et al., 2005; Polakof et al., 2006). The relationship between exposure to anesthetic and/or sedative agents and activity of metabolic enzymes remains unknown.

The endocrine system plays a key role in the maintenance of homeostasis of a number of functional activities that are related to short or long-term changes in the environment (Laiz-Carrión et al., 2009). The pituitary is considered to be the master gland of the endocrine system and is implicated in the control of a large variety of physiological processes mediated by several hormones and factors released into the bloodstream. Pituitary hormones that are members of the GH/PRL family include prolactin (PRL), growth hormone (GH) and somatolactin (SL). These hormones have

been implicated in the control of pleiotropic biological functions in teleosts (Sudo et al., 2013). In addition to controlling development and somatic growth, GH is involved in adaptation to hyperosmotic environments in fish. In contrast, PRL is an important hormone in freshwater adaptation (Sakamoto and McCormick, 2006). SL has no single regulatory action but appears to be involved in energy mobilization, stress response, calcium metabolism, acidosis and pigmentation in teleosts (Kawauchi and Sower, 2006).

Hesperozygis ringens is a Brazilian native plant whose essential oil (EO) exhibits anesthetic and sedative properties for silver catfish (*Rhamdia quelen*) (Silva et al., 2013), which are an economically important species to southern Brazil (Baldisserotto, 2009). Although the consequences of anesthetic use of EO of *H. ringens* on the physiology and biochemistry of silver catfish has recently been reported (Toni et al., 2013), very little is known about the effects of low concentrations of this EO on fish. Thus, in the present study, we investigated the consequences of long-term exposure to EO of *H. ringens* on expression of adenohypophyseal hormones, plasmatic cortisol, metabolic indicators and osmolality levels in silver catfish. The information obtained in this study could be critical to determining the feasibility and safety of using this EO in aquaculture practices that require a longer handling time of fish, such as during transport.

2. Material and methods

2.1 Plant material and essential oil extraction

Aerial parts of *Hesperozygis ringens* were collected in São Francisco de Assis (Rio Grande do Sul, Brazil). The species was identified by Dr. Solon Jonas Longhi, and a voucher specimen (SMDB nº 13427) was deposited in the herbarium of the Department of Biology, Universidade Federal de Santa Maria (UFSM). Extraction of the EO of the dry leaves of *H. ringens* was performed by hydrodistillation for 3 h using a Clevenger type apparatus (European Pharmacopoeia, 2007). Extract was stored at -4 °C in amber glass bottles. Analysis of the EO extract composition by gas chromatography coupled to mass spectrometry demonstrated that it was similar to

128 the composition of EO used by Toni et al. (2013), with pulegone being the major

129 constituent (81.4%) (Table 1).

130

131 *2.2 Animals and experimental protocol*

132 Silver catfish (93.9 ± 3.9 g, 25.0 ± 0.5 cm) were obtained from Fish Culture

133 Laboratory at the UFSM (RS, Brazil) and transported to the Laboratory of Fish

134 Physiology. Catfish were maintained in continuously aerated 250 L tanks under

135 natural photoperiod and controlled temperature (21.8 ± 0.5 °C) for one week. A semi-

136 static system was used, and 50% of the water volume was changed daily. Fish were

137 fed once a day with commercial feed (28% crude protein). Fish were fasted for 24 h

138 prior to the experiments. Experimental methodologies were approved by the Ethical

139 and Animal Welfare Committee of the UFSM (Process nº 46/2010).

140 Fish (n = 30) were captured from the tanks and allocated to 40 L aquaria, with 5 fish

141 per aquaria. Fish were treated with control (without EO), 30 or 50 µL L^{-1} EO of *H.*

142 *ringens* added to water (2 aquaria per treatment). The EO was previously diluted in

143 ethanol (1:10) for better dissolution in water. Concentrations used in this study do not

144 cause deep anesthesia in silver catfish (Silva et al., 2013). Fish remained in stage 1

145 (sedation) of anesthesia, which is characterized by decreased reactivity to external

146 stimuli according to Small (2003), The fish remained under these conditions for 6 h.

147

148 *2.3 Ventilatory rate (VR)*

149 The VR was quantified by visually counting 20 successive opercular movements and

150 measuring the elapsed time with a chronometer (adapted from Alvarenga and

151 Volpato, 1995). VR was measured 0, 1, 2, 4 and 6 h after treatment exposure.

152

153 *2.4 Sampling*

154 After 6 h of treatment, fish were removed by dip-net and blood was collected into

155 heparinized syringes by caudal puncture. Plasma obtained after blood centrifugation

156 (1000 *g*, 10 min at 4 °C) was immediately stored at -80 °C until assayed. After

157 euthanasia by spinal section, the liver and pituitary were removed quickly from each

158 fish. Liver was stored at -80 °C until the assessment of enzyme activities, while

159 pituitary was immersed in RNAlater (Ambion) solution and stored at -20 °C prior to

160 RNA extraction and hormone expression measurement.

161

162 *2.5 Analytical techniques*

163 Plasma cortisol levels (expressed in ng mL^{-1}) were measured by indirect enzyme

164 immunoassay (ELISA) adapted to microplate as described previously by Rodríguez

165 et al. (2000) for testosterone. Steroids were extracted from 3.5 µL of plasma in 100

166 µL RB (10% v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01% w/v NaN$_3$, 2.34% w/v

167 NaCl, 0.037% w/v EDTA, 0.1% w/v BSA (Bovine Serum Albumin)) and 1.2 mL

168 methanol (Panreac) and evaporated for 48-72 h at 37 ºC. Cortisol EIA standard (Cat.

169 #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific

170 cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol

171 express AChE tracer (Cat. #400370) were obtained from Cayman Chemical

172 Company (Michigan, USA). Standards and extracted plasma samples were run in

173 duplicate. The standard curve range was 2.5 ng/mL to 39.1 pg mL^{-1} (R^2= 0.986). The

174 lower limit of detection (90.4% of binding, ED90.4) was 78.1 pg mL^{-1}. The percentage

175 of recovery was 95%. The intra-assay coefficient of variation (calculated from the

176 sample duplicates) was 4.29 ± 0.48%. The cross-reactivity of specific antibodies with

177 intermediate products involved in steroids synthesis was given by the supplier

178 (cortexolone (1.6%), 11-deoxycorticosterone (0.23%), 17-hydroxyprogesterone

179 (0.23%), cortisol glucurinoide (0.15%), corticosterone (0.14%), cortisone (0.13%),

180 androstenedione (<0.01%), 17-hydroxypregnenolone (<0.01%), testosterone

181 (<0.01%)).

182 Glucose, lactate and triglyceride concentrations were measured in plasma using

183 commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200;

184 Lactate Ref. 1001330; Triglycerides Ref. 1001311) adapted to 96-well microplates.

185 Plasma protein was analyzed by diluting plasma 50 times and measuring protein

186 concentration. Protein concentration was measured using the bicinchonic acid

187 method with a BCA protein kit (Pierce P.O., Rockford, USA), using bovine serum

188 albumin as standard. All of these assays were run on an Automated Microplate

189 Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by

190 KCjunior™ program. Standards and all samples were run in quadruplicate and

191 duplicate, respectively. Plasma osmolality was measured with a vapor pressure

192 osmometer (Fiske One-Ten Osmometer, Fiske-VT, USA) and expressed as mOsm

193 kg^{-1}.

194 Frozen liver was finely minced on an ice-cold Petri dish and homogenized by

195 ultrasonic disruption (Misonix inc., Microson Ultrasonic liquid processor XL-2000) with

196 10 volumes of ice-cold stopping-buffer containing 50 mM imidazole (pH 7.5), 1 mM 2-

197 mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM PMSF, and 250 mM sucrose.

198 The homogenate was centrifuged at 10 000 g, 30 min at 4 °C (Centrifuge 5810R,

199 Eppendorf) and the supernatant was immediately frozen using liquid nitrogen and

200 kept at -80 ºC until enzyme assays were performed. Enzyme activities were analyzed

201 using a Bio-Tek PowerWave 340 Microplate spectrophotometer (Bio-Tek

202 Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft

203 Windows XP. Reaction rates of enzymes were calculated from the increase or

204 decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the

205 addition of homogenates (15 µL) in duplicate, at a pre-established protein

206 concentration, omitting the substrate in control wells (final volume of 275-295 µL,

207 depending on the enzyme tested), and allowing the reactions to proceed at 37 ºC.

208 The specific conditions for the enzymes hexokinase (HK, EC 2.7.1.11), glycerol-3-

209 phosphate dehydrogenase (G3PDH, EC 1.1.1.8), pyruvate kinase (PK, EC 2.7.1.40),

210 phosphorilase (total and active GPase, EC 2.4.1.1), glucose-6-phosphate

211 dehydrogenase (G6PDH, EC 1.1.1.49), fructose-biphosphatase (FBP, EC 3.1.3.11),

212 glutamate dehydrogenase (GDH, EC 1.4.1.2), glutamic-pyruvic transaminase (GPT,

213 EC 2.6.1.2), 3-Hydroxyacyl-CoA-dehydrogenase (HOAD, EC. 1.1.1.35), are

214 previously described (Sangiao-Alvarellos et al., 2005; Polakof et al., 2006).

215 Enzymatic analyses were carried out at conditions meeting requirements for optimal

216 velocities for *R. quelen* (data not shown). Protein levels were assayed in triplicate as

217 performed with plasma samples.

218

219 *2.6 Pituitary expression of GH, PRL and SL mRNA*

220 Total RNA was isolated from pituitary gland using NucleoSpin®RNA XS kit

221 (Macherey-Nagel) and the on-column RNase-free DNase digestion, according to

222 manufacturer's protocol. The amount of RNA was spectrophotometrically measured

at 260 nm with the BioPhotometer Plus (Eppendorf) and its quality was measured in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used for qPCR. Total RNA (250 ng) was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences).

Real-time PCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep *realplex* ^{2} S) as previously described by Baldisserotto et al. (in press). Each reaction mixture (10 µL) contained 0.5 µL of each specific forward and reverse primers at a final concentration of 200 nM, 5 µL of PerfeCTa SYBR$^®$ Green FastMix™ (Quanta Biosciences), and 4 µL of cDNA. The nucleotide sequences of specific primers used for semi-quantitative PCR are in Table 2. The PCR profile was obtained as follows: (95 ºC, 10 min; [95 ºC, 30 sec; 60 ºC, 45 sec] X 40 cycles; *melting curve* [60 ºC to 95 ºC, 20 min], 95 ºC, 15 sec). β-actin was used like a housekeeping gene.

2.7 Statistical analysis

Homogeneity of variance between groups was tested with Levene's test. Data exhibited homogeneous variance, so comparisons between treatments were made using one-way ANOVA followed by the Tukey's test. Analysis was performed using Statistica v. 7.0 software. The minimum significance level was set at $P < 0.05$.

3. Results

No mortality was registered for the duration of the experiments as a consequence of the exposure to EO.

After 6 h, the VR was reduced in all treatments compared to time 0. Although no differences were found between treatments for any time of measurement, it was observed that the breathing amplitude was lower in the fish exposed to 30 or 50 µL L^{-1} EO than the control fish (Fig. 1).

Glucose levels, protein levels and plasma osmolality increased in the specimens exposed to 50 µL L^{-1} EO (Fig. 2A, 2C and 2E, respectively). Similarly, plasma lactate levels were higher in fish exposed to EO compared to control, independent of

255 concentration (Fig. 2B). Triglycerides and cortisol levels were not affected by EO

256 exposure (Fig. 2D and 2F, respectively).

257 The activities of G6PDH, FBP and HOAD all increased in the fish exposed to EO,

258 with a remarkable elevation at the highest EO concentration. In addition, EO

259 increased the hepatic G3PDH activity in both experimental groups, although the

260 highest values were achieved in the 30 µL L^{-1} EO group (Table 3).

261 Expression of GH showed a clear inverse relationship with EO concentration (Fig.

262 3A). PRL expression was not affected by EO exposure (Fig. 3B), while the

263 expression of SL was significantly decreased in fish exposed to 30 or 50 µL L^{-1} EO

264 (Fig. 3C).

265

266 **4. Discussion**

267

268 Stressful conditions in fish culture can result in a poor performance, disease, and

269 even mortality leading to economic losses for the producer. Low concentrations of

270 anesthetics, which cause fish sedation but are not sufficient to cause deep

271 anesthesia, provide a useful means of reducing physical damage and minimizing

272 negative effects of preventing the exacerbation of handling stress (Khalil et al, 2012).

273 It is important to assess the extent of physiological changes related to enhanced

274 energy and metabolite consumption as well as stress processes in fish exposed to an

275 anesthetic.

276 In fish, cortisol is involved not only in stress responses but also in several processes

277 such as the regulation of metabolism, behavior, growth, reproduction and

278 osmoregulation (Mommsen et al., 1999). Given its importance as a modulator of

279 various physiological processes, changes in plasma cortisol are considered to be a

280 primary response to a potential stressor. In the present study, levels of this hormone

281 did not change after 6 h of exposure to EO of *H. ringens.*

282 Plasma metabolites and osmolality were assessed as indicators of a secondary

283 response. Fish exposed to 50 µL L^{-1} EO exhibited elevated plasma levels of glucose,

284 lactate, protein and osmolality. Most studies attribute the physiological changes

285 associated with stress to cortisol, based primarily on the elevated circulating level of

286 this steroid (Mommsen et al., 1999). However, some studies have demonstrated that

287   plasma levels of metabolites might change without changes in cortisol levels

288   (Svobodova et al., 1999; Thomas et al., 1999; Park et al., 2008). As plasma cortisol

289   concentrations reflect the effect of net production and plasma clearance of the

290   hormone (Mommsen et al., 1999), we propose that the primary reaction started in the

291   first minutes of contact with EO. Barcellos et al. (2012) found that *R. quelen* of similar

292   size to catfish used in the present study experienced elevated cortisol levels 5 to 30

293   min after stressor exposure, with a return to baseline concentrations within 60 min. In

294   the present study, even though the fish may still be responding to the stressor after 6

295   h of exposure to EO, plasma cortisol levels were at baseline.

296   The increase in plasma lactate levels verified in fish exposed to both EO

297   concentrations is indicative of tissue hypoxia. This phenomenon occurs when cells

298   receive too little oxygen to function normally. Aquatic animals respond to hypoxia by

299   first attempting to maintain oxygen delivery, (i.e., this response involves increases in

300   respiration rate, number of red blood cells, or oxygen binding capacity of hemoglobin

301   (Wu, 2002). In the absence of oxygen, or even when the aerobic pathways are

302   insufficient to produce the required energy supply, anaerobic glycolysis is the

303   principal energy-generating pathway that results in lactate accumulation (Bickler and

304   Buck, 2007). Therefore, the highest plasma lactate levels observed in fish exposed to

305   EO would result in hypoventilation caused by decreased breathing amplitude and not

306   by reduced ventilation rate.

307   To our knowledge, this is the first report about the effects of exposure to essential

308   oils used as anesthetics and/or sedatives on the activity of enzymes associated with

309   the metabolism of carbohydrates, lipids and protein in fish. Glucolitic pathways were

310   not affected, nor were changes observed in the HK and the PK enzymes (which are

311   the first and the last enzymes in the glucolitic pathway) . There was no mobilization of

312   glycogen stores, as GP activity was undisturbed. Protein metabolism was not

313   affected, as GDH and GPT enzymes were unaltered.

314   The increase in the activity of FBP, G6PDH and HOAD observed in silver catfish

315   demonstrate that intermediary metabolism was affected after 6 h of exposure to 50

316   µL L^{-1} EO of *H. ringens*. Lipids and plasma lactate appeared to be used as energy

317   sources because the activities of enzymes involved in protein metabolism were not

318   altered. These results are indicative of gluconeogenesis Other authors have reported

319 similar gluconeogenic potential in the liver of brook charr *(Salvelinus fontinalis)* and

320 gilthead sea bream *(Sparus aurata)* after the experience of stressful conditions

321 (Vijayan et al., 1991; Polakof et al., 2006). In the present study, the gluconeogenic

322 pathway could be the energetic mechanism that the animal was using in an attempt

323 to restore its homeostasis.

324 The main finding in this study is that glycerol-3-phosphate (from lipid reserves) is

325 converted through G3PDH to dihydroxyacetone phosphate (DHAP). DHAP, as part of

326 the gluconeogenic pathway, serves as the initial substrate for the formation of

327 fructose-6-phophate (F6P) by the enzyme FBP. F6P can be derived to the formation

328 of glucose-6-phosphate (G6P), which is the main substrate of the G6PDH. G6PDH,

329 as the first enzyme of the pentose shunt, produces reducing power in the form of

330 NADPH, as well as nucleic acids, nucleotides, gliceraldehyde-3-phosphate and other

331 molecules that could enter the glucolitic pathways. We described an increase in the

332 HOAD activity in liver tissue, further indicating that lipid stores were used. Fatty acids

333 are used as energy stores, producing Acetyl-CoA (Ac-CoA) and reducing power

334 (NADH and $FADH_2$). This Ac-CoA enters in the Krebs cycle, so that more energy can

335 be obtained. In summary, liver, as the main store tissue in fish, does not utilize

336 carbohydrate or amino acid reserves, but lipid ones. These fatty acids are used for

337 the production of reducing power through the pentose shunt and beta-oxidation, and

338 they are also used to produce energy through the Krebs cycle, which could be used

339 for EO detox (for example).

340 Adenohypophyseal hormone expression related to growth, metabolism, and

341 osmoregulation were also investigated in this study. Growth hormone (GH) is an

342 important pituitary hormone known to regulate body growth and metabolism and its

343 release in teleost fish is regulated directly at the pituitary level by a multitude of

344 neuroendocrine factors (Wong et al., 2006). In this study, the lowest levels of GH

345 were found in the pituitary of silver catfish exposed to 50 µL L^{-1} EO of *H. ringens.*

346 Although the consequences of stress responses are dependent on a variety of

347 factors, it is known that tertiary responses to stress (especially chronic stress) include

348 effects on fish growth (Barton, 2002). Reduction in GH expression, as observed in

349 this study, indicates that fish growth may be compromised to address an adverse

350 situation imposed on the body.

351 Since its discovery, the effects of SL on energy metabolism have been widely

352 investigated. These studies demonstrate an active role for this hormone as a marker

353 of energy surplus (availability). Uchida et al. (2009) observed that fasting for 4 weeks

354 produced significant reductions in pituitary SL expression of Mozambique tilapia

355 *(Oreochromis mossambicus)* both in freshwater and in seawater. In another study in

356 gilthead sea bream (*S. aurata*), plasma SL levels decreased in response to reduced

357 nutritive value of diet (Vega-Rubín de Celis et al., 2004). The reduction in SL

358 expression in silver catfish exposed to EO of *H. ringens* is consistent with the

359 increased metabolic activity of enzymes that oxidize organic molecules to the

360 detriment of energy reserves, especially lipids.

361 In summary, these results confirm that GH and SL are associated with the energy

362 reorganization required for homeostatic balance in silver catfish after sedation with

363 EO of *H. ringens.* Furthermore, this study shows greater alterations in metabolic

364 parameters after exposure to 50 µL L^{-1} EO, suggesting that a 30 µL L^{-1} concentration

365 of EO is better and may be advisable for situations that require a long-term exposure,

366 i.e., such as during fish transportation.

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Figure captions

Figure 1 Effects of the essential oil of *Hesperozygis ringens* on ventilatory rate of *Rhamdia quelen* through 6 h of exposure. Data represent the mean ± SEM. An asterisk (*) indicates a significant difference (P < 0.05) from 0 h in the same treatment.

Figure 2 Effects of the essential oil of *Hesperozygis ringens* on plasma levels of glucose (A), lactate (B), protein (C), triglycerides (D), osmolality (E) and cortisol (F) of *Rhamdia quelen* after six hours of exposure. Data represent the mean ± SEM (n = 10). Different letters indicate significant differences (one way-ANOVA, P < 0.05) between treatments.

Figure 3 Effects of the essential oil of *Hesperozygis ringens* on expression of growth hormone (GH) (A), prolactin (PRL) (B) and somatolactin (SL) (C) in the pituitary of *Rhamdia quelen* after six hours of exposure. Data represent the mean ± SEM (n = 10). Different letters indicate significant differences (one way-ANOVA, P < 0.05) between treatments.
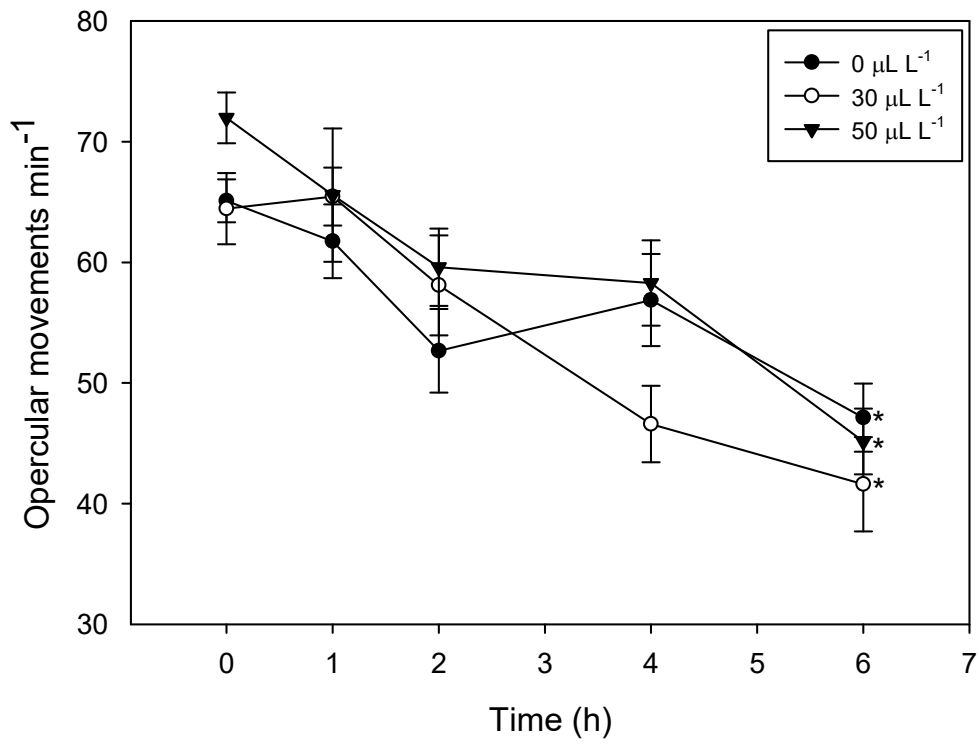
533   **Figure 1.** *Toni et al.*

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Figure 2. *Toni et al.*

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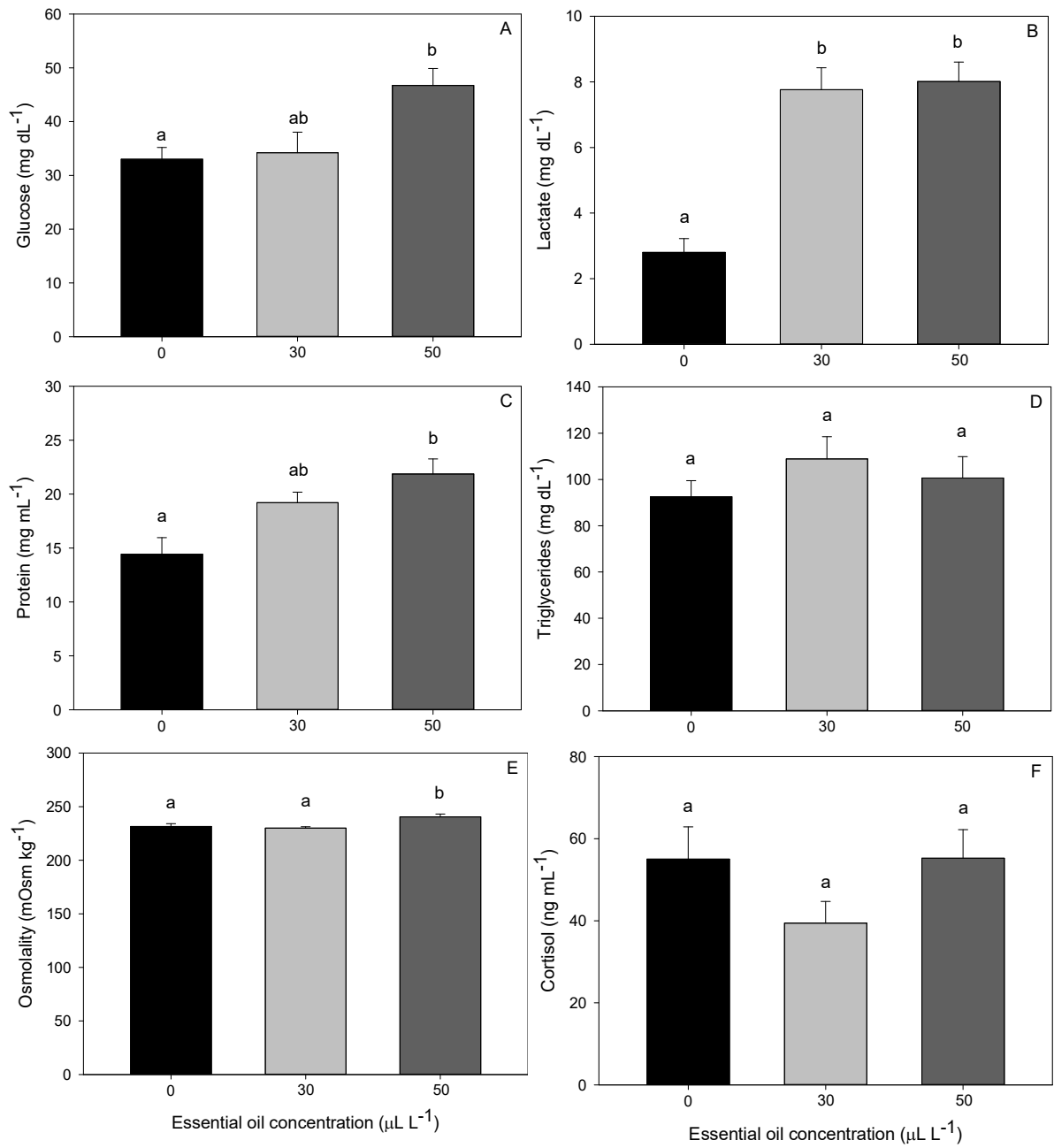561
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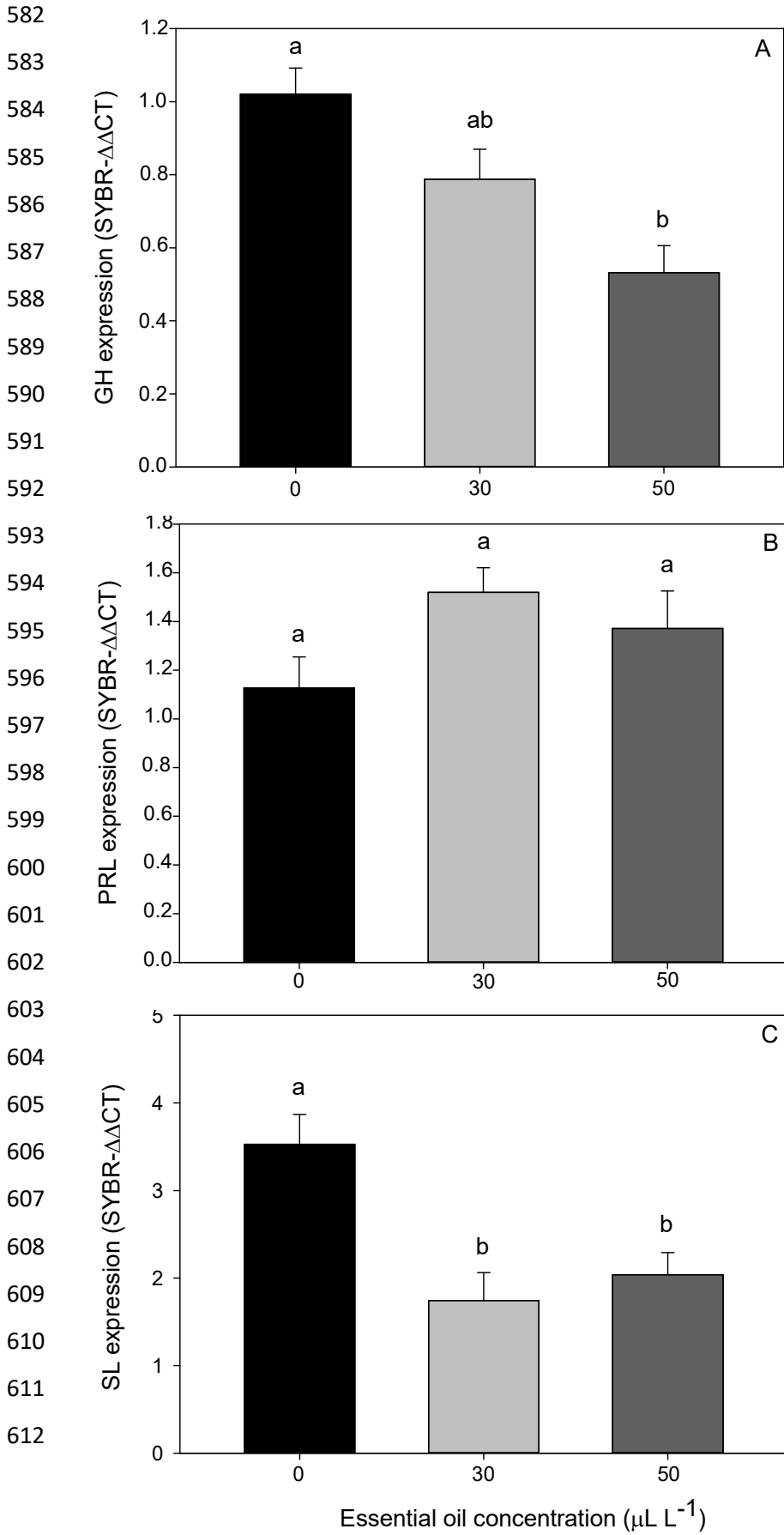563
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Figure 3. *Toni et al.*

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613 **Table 1.** Chemical composition of *Hesperozygis ringens* essential oil.
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Peak	Rt (min)	Compounds	%	RI calc	RI ref
1	9.718	α-Pinene	0.61	931	930[2]
2	11.355	Sabinene	0.37	970	971[2]
3	11.419	β-Pinene	0.51	972	971[2]
4	12.176	β-Myrcene	0.36	990	991[2]
5	13.613	Limonene	1.92	1025	1026[2]
6	13.705	Eucalyptol	0.18	1027	1027[2]
7	14.454	NI	0.19	1046	
8	16.621	Linalool	1.33	1099	1099[2]
9	17.177	Octen-3-yl acetate <1->	0.09	1113	1113[1]
10	18.690	Menthone	0.04	1152	1154[2]
11	18.749	(-)- Isopulegol	0.06	1153	1156[2]
12	19.061	Menthofurane	0.16	1161	1163[2]
13	19.547	Isopulegone	0.93	1174	1177[2]
14	20.154	α-Terpineol	0.39	1189	1189[2]
15	20.889	Verbenone	0.51	1209	1207[2]
16	21.140	NI*	0.29	1215	
17	21.666	NI	0.17	1230	
18	22.188	Pulegone	**81.37**	1244	1244[2]
19	22.383	NI	0.39	1250	
20	22.876	NI*	0.13	1263	
21	23.471	NI	0.28	1279	
22	23.613	NI	0.39	1283	
23	23.817	NI	3.32	1288	
24	25.620	Eucarvone	0.19	1340	1343[2]
25	25.913	NI	0.24	1348	
26	27.197	NI	0.27	1385	
27	27.519	NI	0.18	1394	
28	27.825	NI	2.32	1403	
29	28.291	Caryophyllene	0.39	1418	1418[2]
30	28.633	NI	0.14	1428	
31	28.806	NI	0.31	1433	
32	29.136	NI	0.23	1443	
33	32.678	NI	0.11	1555	
34	33.370	(-)-Spathulenol	0.65	1577	1578[2]
35	33.538	Caryophyllene oxide	0.98	1583	1581[2]
		Total	99.99		
		Identified	91.03		

615 Rt: retention time; %: percentage; RI calc.: calculated Kovats retention index; RI ref.: reference Kovats
616 retention index; NI: not identified. [1]Adams (2001), [2]NIST (2002).
617 *Compounds with fragmentation model indicative of structure derived from oxidation of pulegone.
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619 **Table 2.** Sequences for the primers of *Rhamdia quelen* used in real-time PCR.
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Gene	Primer	Nucleotide sequence
GH	Fw	5'-GGACAAACCACCCTAGACGAG-3'
	Rv	5'-TTCTTGAAGCAGGACAGCAGA-3'
PRL	Fw	5'-CCTGTCTCTGGTTCGCTCTCT-3'
	Rv	5'-GTCCTGCAGCTCTCTGGTCTT-3'
SL	Fw	5'-TCCAGCACGCTGAGCTGATCT-3'
	Rv	5'-AAGAGTTTCCCCCATGACCTT-3'
β-ACTIN	Fw	5'-GAAGTGTGACGTCGATATCCG-3'
	Rv	5'-CCTGAACCTCTCATTGCCAAT-3'

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624 **Table 3.** Enzymatic activity in the liver of *Rhamdia quelen* after 6 h of exposure to
625 essential oil of *Hesperozygis ringens*.
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Enzyme	Metabolism involved	Treatments		
		Control	30 µL L^{-1}	50 µL L^{-1}
HK	Carbohydrate	0.13 ± 0.02	0.18 ± 0.03	0.14 ± 0.02
G3PDH	Carbohydrate/Lipid	8.00 ± 0.49[a]	11.23 ± 0.43[b]	10.65 ± 1.11[ab]
PK	Carbohydrate	9.90 ± 0.86	10.59 ± 0.80	10.90 ± 0.77
GPtotal	Carbohydrate	3.60 ± 0.39	3.32 ± 0.31	3.28 ± 0.31
GPactive	Carbohydrate	2.61 ± 0.24	1.98 ± 0.38	3.01 ± 0.26
G6PDH	Carbohydrate	5.66 ± 0.58[a]	6.59 ± 0.78[ab]	8.18 ± 0.83[b]
FBP	Carbohydrate	1.56 ± 0.16[a]	2.01 ± 0.21[ab]	2.26 ± 0.29[b]
GDH	Amino acid	7.25 ± 1.07	9.43 ± 1.80	10.51 ± 2.37
GPT	Amino acid	19.50 ± 1.72	17.71 ± 2.12	17.32 ± 2.53
HOAD	Lipid	0.31 ± 0.07[a]	0.68 ± 0.12[ab]	0.89 ± 0.22[b]

627
628 Data represent the mean ± SEM (n = 10).
629 Different letters indicate significant differences (P < 0.05) between treatments.
630 Enzymatic activity expressed by U mg^{-1} protein.
631