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Ingestion of poisonous eggs from the invasive apple snail *Pomacea canaliculata* adversely affects bullfrog *Lithobates catesbeianus* intestine morphophysiology

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37

38 **Conflicts of interest/Competing interests**

39 We have no competing interests. We have no conflicts of interest.

40

41 **Ethics Statement**

42 Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory
43 Animals (2010) and Frogs and toads as experimental animals (Tyler 2009).

44

45 **Consent for publication**

46 All authors have given their consent for publication.

47

48 **Availability of data and material**

49 Data generated or analysed during this study are included in this published article and its
50 supplementary information files.

51

52 **Authors' contributions**

53 TRB MSD HH conceived and designed the experiments. TBR PEF ELP MSD performed the
54 experiments. TBR PEF ELP MSD HH analysed the data. HH contributed
55 reagents/materials/analysis tools. TBR PEF ELP MSD HH wrote the paper.

56

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62

63 **ABSTRACT**

64 The poisonous eggs of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae) hardly have any
65 predator. This invasive snail, listed among the 100 worst invasive species, is a serious crop pest
66 and a vector of a human parasitosis. Females lay eggs in pink-reddish masses, presumably as a
67 warning coloration of their chemical defenses. Egg ingestion alters rodents' gastrointestinal tract
68 and is lethal if injected, but its effect on other taxa is unknown. Here we explored the toxic effects
69 of *Pomacea canaliculata* eggs on bullfrog *Lithobates catesbeianus* (Anura, Ranidae).

70 Juvenile bullfrogs were gavaged with egg extracts and their digestive tract analyzed after 24 h
71 and 48 h using histological, immunohistochemical and lectin-histochemical techniques. Toxicity
72 was also evaluated by intraperitoneal injection of eggs extracts.

73 Egg extract ingestion adversely affected small intestine of bullfrogs. Short term (24 h) effects
74 included large, reversible changes of the intestinal wall, villi morphology, and changes in the
75 glycosylation patterns of enterocytes. The mucosal area increased and infiltration of inflammatory
76 cells, mainly eosinophils and macrophages, were observed together with a weak hemorrhage.
77 Most of these changes reversed after 48 h. Besides, intraperitoneal injection of egg extract was
78 nontoxic to bullfrog juveniles and no lethality or behavioral changes were observed, a remarkable
79 difference with egg' effect on mammals.

80 As a whole, these results indicate that toxins of apple snail eggs reversibly modify gut
81 morphology, which may alter bullfrog physiology limiting their ability to absorb egg nutrients.
82 This study extends the known targets of the apple snail egg defenses against predation to
83 amphibians.

84

85 INTRODUCTION

86 Eggs of most animals are subject to strong predation (Ricklefs 1969, Kamler 2005). One of the
87 exemptions are the eggs of the freshwater apple snail *Pomacea canaliculata* (Lamarck, 1822)
88 (Caenogastropoda, Ampullariidae). Native from South America, this snail has been introduced
89 worldwide becoming an invasive species and a vector of a human parasitosis (Hayes et al. 2008;
90 Lv et al. 2011). Their invasiveness is partly related to their reproductive strategy (Lowe et al.
91 2000; Martin and Estebenet 2002, Hayes et al. 2012), especially to their egg defenses (Heras et
92 al. 2008, Dreon et al. 2010, 2013, 2014). Females deposit pink-reddish egg masses outside the
93 water on a weekly basis, each containing 30–300 eggs (Albrecht et al. 1996; Cadierno et al. 2018)
94 filled with a perivitelline fluid (PVF) that surrounds the developing embryo. PVF is known to
95 serve the critical functions of embryonic nutrition, protection and defense in *P. canaliculata*
96 (Heras et al. 1998; Heras et al. 2007). Thus, the PVF enable eggs to develop under harsh aerial
97 conditions including desiccation high temperatures, and help coping with terrestrial predators
98 (Dreon et al. 2004; Heras et al. 2007; Dreon et al. 2007). Even more, predators that feed on adults,
99 like the snail kite *Rostrhamus sociabilis* (Vieillot, 1817) or the Norway rat *Rattus norvegicus*
100 (Berkenhout, 1769), systematically discard the albumen gland where most egg PVF is synthesized
101 (Przeslawski 2004, Cadierno et al. 2017b,a, Hayes et al. 2015).

102 The PVF of *P. canaliculata* contains more than 30 proteins (perivitellins) (Sun et al. 2012;
103 Sun et al. 2019) including a hyperstable non-digestible carotenoprotein (PcOvo), massively
104 accumulated in the egg. PcOvo has a dual function, on the one hand it can resist proteolysis and
105 withstands passage through the digestive tract of rodents almost unaffected, lowering the
106 nutritional value of the eggs (Pasquevich et al. 2017; Dreon et al. 2010; Dreon et al. 2014). On
107 the other hand, it provides clutches with a vivid coloration (aposematic), probably a warning to
108 visual hunting predators of their toxic defenses. The PVF defensive cocktail also includes a neuro-
109 and enterotoxic lectin (PcPV2), lethal to mice when intraperitoneally injected, that adversely
110 affects their gastrointestinal tract if ingested (Heras et al. 2008, Giglio et al. 2020). Furthermore,
111 oral administration of *P. canaliculata* PVF to rodents alters their gut morphophysiology and

112 decreases rat growth rate (Dreon et al. 2014; Giglio et al. 2018). These unusually efficient
113 biochemical defenses may explain the lack of reported predators for the eggs (Cadierno et al.
114 2017a). However, most of the studies on the defensive system of *P. canaliculata* eggs have been
115 conducted using only mammals as models, and whether the ingestion of these perivitellins affect
116 other taxa is unknown. Amphibians are a likely target of these defenses and their digestive
117 physiology may differ considerably from that of mammals (Sabat and Bozinovic 2000). Besides,
118 many anurans and urodeles overlap in their geographical distribution with those of the apple snail.
119 In particular, the invasive bullfrog *Lithobates catesbeianus* (Shaw, 1802) (Anura, Ranidae)
120 partially overlaps with *P. canaliculata* geographical distribution in both their native and
121 introduced regions (Akmentins and Cardozo 2010).

122 To explore possible targets of *P. canaliculata* egg defense system beyond mammals, and to
123 further understand the biological-ecological meaning of the digestive plasticity in amphibians, we
124 selected *L. catesbeianus* as a model of potential predator of the eggs. This North American frog,
125 introduced to several parts of the world including South America, has voracious habits and a
126 varied diet including eggs of both vertebrates and invertebrates species (Hirai 2004; Snow and
127 Witmer 2010).

128 We report here that oral administration of *P. canaliculata* PVF to juvenile bullfrogs has a
129 strong adverse effect on gut at tissue and organ levels, temporarily affecting their small intestine
130 morphology and inducing an extended cellular reactive reaction. Most of these changes were
131 reversed after 48 h. However, unlike mammals, no lethal or neurotoxic effects were observed
132 when injected with snail egg extracts. As far as we know, the occurrence of toxins that affect
133 intestinal morphology probably affecting absorption, has not been recognized among amphibians
134 before. This is the first report of a poisonous effect by the ingestion of *P. canaliculata* egg extracts
135 on a vertebrate other than mammals.

136

137 **METHODS**

138 **Egg masses and PVF preparation**

139 *P. canaliculata* egg clutches were collected from the field in La Plata city, Buenos Aires,
140 Argentina, between November and March during the reproductive season. Only eggs not beyond
141 morula stage were used. PVF was prepared following Dreon et al. (2014). Briefly, eggs were
142 homogenized in ice cold 20 mM Tris-HCl, pH 7.5 in a buffer: sample ratio of 3:1 v/w. The
143 homogenate was sequentially centrifuged at 10,000 g for 30 min and at 100,000 g for 60 min, and
144 the pellet discarded. The supernatant (egg PVF) was equilibrated in 50 mM phosphate buffer
145 saline (PBS), pH 7.4 using a centrifugal filter device of 50 kDa molecular weight cut off
146 (Millipore Corporation, MA). Total protein concentration was measured by the method of Lowry
147 et al. 1951.

148

149 **Experimental animals**

150 Sexually immature juvenile bullfrogs *Lithobates catesbeianus* were purchased from an aquarium
151 in Buenos Aires, Argentina, and bred in a specially conditioned room at the facilities of the School
152 of Medicine, National University of La Plata. They were housed in plastic 60x40x50 cm
153 containers with natural vegetation and soil. Water provided in plastic containers was changed
154 daily. Frogs were maintained with a 12-h dark/light cycle at 22 ± 3 °C and fed once a day either
155 with *Tenebrio* larvae or adult *Blattella germanica*.

156

157 **Lethality test**

158 To assess the PVF lethality on frogs, a preliminary screening test was performed. Groups of five
159 bullfrog (4.0 ± 1.1 g) were intraperitoneally (i.p.) injected with a single dose of 200 μ l of either
160 PBS (control) or the same volume of a serial dilution of five concentrations of PVF in the range
161 1.5 – 12.0 mg/Kg using a 30G x 13 mm needle. As lethality was not observed, a second test was
162 performed injecting three bullfrogs with 200 μ l of 170 mg/Kg PVF, the highest possible
163 concentration, which was 50 times higher than the LD₅₀ established for mice (Heras et al. 2008).
164 Animals were observed during 96 h after administration for lethality and behavioral changes.

165

166 **Histology analysis**

167 Bullfrogs (n=6) were gavaged with a single dose of 200 µl of PVF (9 mg/ml) using a flexible
168 plastic cannula. Control animals (n=3) were administrated with the same volume of PBS. Groups
169 of three animals were euthanized either after 24 h or 48 h post-administration by immersion in a
170 water bath containing Tricaine methane-sulfonate (MS-222) 0.2 mg/ml, following the procedure
171 described by Tyler (2009). Intestines were removed and samples of the first part of the small
172 intestine were cut, rinsed several times with PBS and then fixed in 10% neutral buffered formalin
173 for 48 h for histological examination. After 48 h fixation, samples were transferred into 70°
174 ethanol and dehydrated before being embedded in paraffin wax at 60°C for 1 h. Five to seven µm
175 thick sections were obtained from the paraffin blocks using a manual microtome and routinely
176 stained with haematoxylin and eosin (HE) according to Bancroft and Gamble (2008). Analysis
177 was performed using an Olympus BX51 microscope. Digital images were obtained using an
178 Olympus DP-70 video camera and then captured with an image analyzer (ImagePro Plus v.6.3,
179 Media Cybernetics, USA).

180

181 **Morphometric analysis**

182 Changes in small intestine morphometry caused by PVF administration were measured from
183 images (4x magnification) obtained using the image analyzer (ImagePro Plus) from 9 properly
184 oriented HE stained sections per animal, a total of 27 images for each experimental group.
185 “Section area” (SA) was measured considering both mucosa (epithelium, and lamina propria) and
186 the lumen area, but excluding muscular layer. “Tissue area” (TA) was measured considering the
187 relative area of the mucosa to SA and excluding lumen area. The relation between lumen area and
188 SA was also measured. To estimate the absorptive surface, perimeter of the epithelium facing
189 lumen was measured in relation to SA.

190

191 **Presence of eosinophils and macrophages cells**

192 A total of 27 small intestine sections for each group were analyzed to determine the presence of
193 eosinophils and macrophages, which were counted in 5 randomly chosen fields of 0.05 mm² from
194 each section (60x magnification, 135 fields per experimental group). The presence of eosinophils
195 was determined in HE stained sections. Macrophages were determined by immunohistochemistry
196 (IHC). Briefly, sections were deparaffinized, hydrated through graded ethanol and incubated
197 overnight at 4°C with a mouse anti-human CD68 clone KP1 monoclonal antibody (1:100, Dako,
198 Denmark). Then, sections were revealed with a commercial kit (LSAB System-HRP, Dako,
199 Denmark) that includes a streptavidin-biotin labeled secondary antibody. The colored reaction
200 was developed using 3,3'- diaminobenzidine tetrahydrochloride (DAB, Dako, Denmark)
201 (chromogen) and H₂O₂ as reaction substrates. All sections were then counterstained with Mayer's
202 hematoxylin before analysis. Dark brown stained cells were considered positive.

203

204 **Binding assay of PcPV2 toxic lectin to gut**

205 The binding and location of PcPV2 to epithelial cells of the small intestine was performed on
206 control, 24 h and 48 h post-gavage with PVF as described in Histology analysis section. Nine
207 sections for each treatment were mounted on positively charged slides, deparaffinized,
208 dehydrated, and incubated in 3% H₂O₂ in methanol for 30 min at room temperature. Slides were
209 rinsed with PBS (pH = 7.4) and then subjected to antigen retrieval procedure using proteinase K
210 1:30 (Dako, Denmark). Nonspecific binding sites were blocked with 2% BSA for 30 min in a
211 humid chamber at 4°C, followed by overnight primary anti-PcPV2 polyclonal antibody (1:200)
212 incubation. Slides were then revealed using the Envision plus kit (Dako, Carpinteria, CA,
213 USA). Polyclonal antibodies against PcPV2 were prepared in rabbits as previously described
214 (Dreon et al. 2002). Positively immunostained regions showed a golden dark brown color using
215 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen and H₂O₂ reaction substrates
216 (DakoCytomation, Glostrup, Denmark). All sections were counterstained with Maeyer's
217 hematoxylin.

218

219 **Collagen determination**

220 Five 5 μm thick sections of the intestine of bullfrogs were stained using the Picosirius red
221 technique (Direct Red 80, Aldrich, Milwaukee, WI 53233, USA) for collagen evaluation, as
222 described elsewhere (Portiansky et al. 2002). Briefly, sections were deparaffinized, hydrated
223 through graded ethanol and stained for 1 h in a 0.1% solution of Sirius Red dissolved in aqueous
224 saturated picric acid. Sections were then rapidly washed in running tap water and counterstained
225 with Harris hematoxylin.

226 Samples observed under polarized light, using an analyzer (U-ANT, Olympus) and a polarizer
227 (UPOT, Olympus) were used to study the birefringence of the stained collagen. Type I collagen
228 reflected red to yellow light, while type III collagen reflected green light. Histological (20 \times
229 magnification) images were digitized using a digital video camera (Olympus DP73, Japan)
230 mounted on a widefield microscope (Olympus BX53, Japan). Captured images were saved in TIF
231 format using an image analysis software (Olympus cellSens Dimension V1.7, Japan)
232 automatically coordinated with the camera for later analysis. Total collagen was calculated as the
233 sum of all connective tissue areas of the coronal sections (type I and type III collagen), divided
234 by the total surface of the section.

235

236 **Lectin histochemistry**

237 To analyze possible changes in the glycosylation pattern of the enterocytes, tissues were evaluated
238 with seven lectins (Lectin Biotinylated BK 1000 Kit, Vector Laboratories Inc., Carpinteria, CA,
239 USA) namely: Con A (*Concanavalia ensiformis*), DBA (*Dolichos biflorus*), SBA (*Glycine max*),
240 PNA (*Arachis hypogaea*), RCA-I (*Ricinus communis-I*), UEA-I (*Ulex europaeus-I*), and WGA
241 (*Triticum vulgare*). Briefly, 9 small intestine sections were deparaffinized with xylene
242 dehydrated with 100% alcohol twice, 10 min each, and then endogenous peroxidase activity was
243 quenched by incubating 5 min with 0.3-3.0% hydrogen peroxide in methanol. They were then
244 hydrated, washed with PBS, and incubated with biotinylated lectins overnight. Sections were then
245 washed with PBS, followed by 10-min incubation with streptavidin-HRP (streptavidin conjugated

246 to horseradish peroxidase in PBS containing stabilizing protein and anti-microbial agents, Vector
247 Laboratories Inc., USA). Finally, the bounded lectins were visualized incubating for 4-10 min
248 with a buffered Tris-HCl solution (0.05 M, pH =6.0) containing 0.02% 3,3'-diaminobenzidine
249 tetrahydrochloride (DAB) and 0.05% H₂O₂ (DAB; Dako, Carpinteria, USA). Sections were
250 counterstain with Maeyer's haematoxilyn. Enterocytes were divided into three zones for analysis:
251 apical zone, stroma and supranuclear zone. Goblet cells were also analyzed. Tissues showing
252 golden brown coloration were considered positive and given a qualitative value according to the
253 intensity of color.

254

255 **Statistical analysis**

256 A Kruskal-Wallis test was used to test morphometric differences. Differences between control,
257 24 h and 48 h post-gavage groups were determined by Dunn's multiple comparison test. For
258 absorptive surface analysis and collagen, one-way ANOVA was performed among control, 24 h
259 and 48 h post-gavage groups. For post hoc comparison, the Tukey's multiple comparison test was
260 used. Statistical analysis was performed using Prism 5.03 (Graphpad Software Inc.). Results were
261 considered significant at the 5% level.

262

263 **RESULTS**

264 **Ingestion of egg PVF affects small intestine morphology of bullfrog**

265 To evaluate the effect of gavage administration of PVF, the morphometry of bullfrog small
266 intestine was analysed (Fig.1A). We found that after 24 h the small intestine mucosa area
267 increased its area fraction with a mean of 0.80 ± 0.05 as compared with the control group (0.62
268 ± 0.08), while at 48h post-gavage the area fraction was reduced to 0.69 ± 0.12 (Fig.1B).
269 Simultaneously, lumen area fraction was significantly reduced after 24 h (0.20 ± 0.08) as
270 compared to controls (0.36 ± 0.08), while at 48 h after gavage values were similar to those of
271 controls (0.31 ± 0.09) (Fig.1C). No changes in the absorptive surface were observed (Fig.1D). The
272 villi of control animals were long with a continuous epithelium containing some goblet cells and
273 well-defined central lymphatic capillaries (lacteal vessel) (Fig. 1E). After 24 h of PVF gavage,
274 villi became wider almost obstructing lumen space, with the tip of the villi showing epithelial
275 disorganization. Besides, the lamina propria was remarkably thickened (Fig.1F). The whole
276 epithelium became discontinuous, with fewer goblet cells and a weak haemorrhage was observed
277 (Fig.2). Forty-eight hours after gavage, the mucosa began to return to its normal morphology,
278 showing narrower villi and wider lumen space than 24 h before. Nevertheless, the lamina propria
279 was thicker than in controls (Fig.1G).

280

281 **Gavage of egg PVF induces inflammatory response in small intestine of bullfrogs**

282 Small intestine showed an increased number of reactive cells 24h and 48h-post-gavage as
283 compared to control group. Eosinophils were found both in the lamina propria and between
284 enterocytes. In control frogs 0 to 2 eosinophils were counted per field (Fig. 3A) while 24h post-
285 gavage an increase in the number of eosinophils (7-14 eosinophils per field) was observed (Fig.
286 3B) decreasing to 1-6 eosinophils per field at 48 h post-gavage (Fig.3C).

287 The number of macrophages in the small intestine was also affected 24h post-gavage, where
288 many immunolabelled macrophages were scattered in the lamina propria or between enterocytes
289 (Fig. 3E). The observed number was much higher than the basal level of macrophages which were

290 barely seen in control sections (Fig. 3D). Again, at 48 h post-gavage less labelled cells were
291 observed than 24 h before, with a counting similar to that of the control group (Fig. 3F).

292 Analysis of the amount of collagen in the lamina propria showed no differences in the amount
293 of total collagen among the three experimental groups (Fig. 3 G). However, changes in the type
294 of collagen were observed 24 h after gavage where there was an increase in collagen I and a
295 reduction in collagen III as compared with the other experimental groups. This resulted in an
296 increase of collagen I: III ratio as compared with the other two groups (Fig 3 H).

297

298 **Ingestion of egg PVF induces changes in carbohydrate glycosylation pattern of enterocytes**

299 Lectin histochemical analysis showed differential reactivity to seven commercial lectins,
300 particularly towards DBA, PNA and SBA (Table 1). Control sections assayed with DBA showed
301 intense staining at the supranuclear and apical regions. Enterocytes and goblet cells were also
302 labelled although less intensely (Fig. 4A). Twenty-four hour after gavage, enterocyte supranuclear
303 zone was clearly negative for DBA as well as goblet cells while the apical region showed no
304 changes as compared to controls (Fig. 4B). In animals euthanized 48 h post-gavage, staining with
305 DBA was strong both at the supranuclear and apical regions while goblet cells display light
306 staining (Fig. 4C). A similar weak reactivity was observed with PNA in supranuclear zone 24 h
307 post-gavage (Fig. 4E) as compared to control group (Fig. 4D), but unlike DBA this zone stayed
308 unstained 48 h after gavage (Fig. 4F). Goblet cells were not stained by PNA neither in control nor
309 in treated intestines. SBA binding showed changes 24 h post-treatment where enterocytes showed
310 stronger reactivity at the supranuclear zone (Fig. 4H) than those of control animals (Fig. 4G).
311 After 48 h of gavage supranuclear and apical regions of enterocytes were strongly stained (Fig
312 4I).

313 Interestingly, PcPV2, the enterotoxic lectin of the PVF, also binds to the intestinal epithelium,
314 as revealed by anti-PcPV2 antibodies. The apical zone of the enterocytes facing the lumen showed
315 clear signs of toxin immunolabelling 24 h and 48 h after gavage especially at the tip of the villi
316 (Fig.5).

317

318 **Intraperitoneal injection of *P. canaliculata* egg PVF is nontoxic to bullfrog**

319 After 96 h of the i.p. injection of *P. canaliculata* PVF no mortality nor behavioural changes among
320 juvenile bullfrogs were observed even though PVF concentration was 50 times higher than LD50
321 reported for mice. Animals were observed for any neurological associated signs, including
322 weakness, lethargy or paralysis of rear limbs. None of these signs or any other behavioural
323 changes were observed.

324

325 **DISCUSSION**

326 It is expected that strong predation would select for characteristics rendering eggs inedible or
327 more difficult to obtain. In this regard, *P. canaliculata* eggs have evolved a suite of unique
328 biochemical defenses to avoid predation well documented in rodents. Here we extend to other
329 taxa the knowledge of potential predators observing that ingestion of the poisonous eggs of *P.*
330 *canaliculata* also adversely affect the digestive system of an amphibian.

331 A work performed on mammals showed that PVF have a lethal neurotoxic effect on mice
332 within hours after i.p. injection (Heras et al. 2008). By contrast, i.p injection of PVF to bullfrogs
333 has no lethal effect, even at a concentration 25 times higher than the concentration that would kill
334 100% of mice. Moreover, while sublethal doses produce rear limbs paralysis, lethargy and pain
335 in mice none of these neurological signs were observed in bullfrogs after injection. These
336 contrasting results between taxa suggest that the target cell or molecule for PcPV2 -the toxic lectin
337 causing these effects on mice (Dreon et al. 2013) might be missing in anurans or, alternatively,
338 that anurans inactivate PcPV2 in their digestive system. This last hypothesis is not supported by
339 the fact that PcPV2 strongly binds to enterocytes *in vivo*.

340 Although apple snail PVF was not lethal to frogs, it severely affects their gastrointestinal tract.
341 After 24 h of PVF gavage, intestinal morphology undergoes a dramatic change such as the
342 increased amount of connective tissue causing an obstruction of the lymphatic vessels, and a
343 marked epithelia disorganization at the tip of the villi. As far as we know, there is no report of

344 similar effects induced by other potential prey on anurans upon ingestion, which limits
345 comparisons. However, some of these morphological changes resemble those exerted by toxic
346 anthropogenic compounds on herpetofauna (Ozelmas and Akay 1995, Çakici 2014; 2016, Çakici
347 and Akat 2012). All changes were temporary and 48 h after gavage treated intestines resembled
348 those of control frogs with thinner villi and the connective tissue almost reduced to the values of
349 the non-exposed group. Moreover, some villi showed normal lymphatic vessels and nearly normal
350 lumen space. Histopathologic effects on anuran small intestine, also observed on rats receiving
351 these egg extracts (Dreon et al. 2014), resemble those exerted by toxic plant lectins (Vasconcelos
352 and Oliveira 2004) that affect gut morphophysiology, at least in mammals and insects (Oliveira et
353 al. 2004; Bardocz et al. 1995). Plant lectins, combined with digestive proteases inhibitors and
354 kinetically stable proteins, provide an effective biochemical defense against predators (Duffey
355 and Stout 1996; Xia et al. 2007, Van Damme 2008). Such mechanism has only been described in
356 animals in *Pomacea* snails (Dreon et al. 2014; Pasquevich et al. 2017, Ituarte et al. 2018). *P.*
357 *canaliculata* PVF protease inhibitors (Ituarte et al. 2019), lectins (Dreon et al. 2014) and
358 enterotoxins (Giglio et al. 2020) are responsible for the changes in the gut of rodents and this may
359 possibly be the case for anurans as well. Indeed, PcPV2 binds to the enterocyte membrane of both
360 frogs (this study) and rats *in vivo* and to Caco-2 cells in culture (Dreon et al. 2013). The fact that
361 the ortholog PmPV2 has enterotoxic properties on mammalian models (Giglio et al. 2020), further
362 support that PV2 lectins from eggs would induce the histopathologic effects on frogs gut.

363 Enterocytes exposed to PVF also showed altered glycosylation patterns, mostly revealed by
364 DBA, SBA and PNA. This study provides one of the few descriptions of the glycan pattern in
365 amphibian intestine. Studies on the lectin histochemistry of the gastrointestinal tract of anurans
366 are scarce and on other developing stages (Kaptan et al. 2013), which limits further comparisons.
367 Considering that glycan pattern can be affected by dietary habits, results suggest that PVF is
368 inducing reversible changes in the surface glycosylation of the enterocytes. Altered reactivity with
369 SBA and PNA were also observed in gut of rats fed with a diet supplemented with *P. canaliculata*
370 PVF (Dreon et al. 2014). Taking into account that dietary lectins can induce changes in

371 glycosylation patterns of gut epithelium (Pusztai et al. 1995) it is tempting to speculate that PVF
372 lectins would be responsible for the observed glycosylation changes in the frog intestine.

373 Inflammation in frogs may be induced by a variety of physiological stressors including habitat
374 perturbation, resource competition, parasites and pollution (Robert et al. 2014). The PVF also
375 induced an inflammatory response 24 h after gavage with eosinophils and macrophages markedly
376 increasing their number. Allergies may induce this cellular response though current results
377 preclude further speculation (McGavin and Zachary 2017). Nonetheless, the response tends to
378 revert to normal by 48 h post-gavage. This cellular reaction and the associated sudden increase of
379 connective tissue may also be related to the enlargement of the villi and lymphatic vessel
380 compression. Inflammatory process was not observed in rats fed with PVF under similar
381 experimental conditions (Dreon et al. 2014; Dreon et al. 2013). Remarkably a few studies on the
382 effect of pesticides on the digestive tract of frogs also report infiltration of eosinophils and other
383 reactive cells (Cengiz and Unlu 2006; Velmurugan et al. 2007, Çakici 2014).

384 As a whole, our results indicate that 48 h after the ingestion of the PVF bullfrogs adapt to the
385 exposure to poisonous apple snail eggs extracts. Similar temporary effects were observed in rats
386 ingesting the same *Pomacea* egg extracts, and in rats and pigs ingesting plant lectins. It is known
387 that the anatomy and function of the digestive tract of many vertebrate species are flexible, and
388 can change in response to variations in environmental conditions (Karasov and Douglas 2013).
389 Considering digestive flexibility (or plasticity) as the ability to adaptively modulate the
390 physiology of the gut to digest different food types (Brzęk et al. 2011) we can suggest that
391 bullfrogs have a fast-adaptive response. This is possible because the small intestine has the
392 shortest turnover rate of all tissues in the body and, at least in mammals, it takes only 3 days to
393 cover the entire surface with new cells. Likewise, studies on gut plasticity in amphibian, mostly
394 performed in tadpoles (Naya et al. 2005; Ruthsatz et al. 2019), show that anurans have adaptive
395 mechanisms that modify the activity of gastrointestinal tract (Cramp and Franklin 2005;
396 Seliverstova and Prutskova 2012). Changes in the relative amount of collagen fibers may be
397 related to gut reaction after ingestion of snail PVF and associated with a remodeling process
398 although more work is needed to fully understand this issue. Our findings are consistent with the

399 notion that bullfrogs have an adaptive response towards toxic eggs ingestion that includes a fast
400 remodeling of the gut. To the best of our knowledge, there is no report for such response
401 mechanism towards a potential prey in amphibians. The recently sequenced genomes of both
402 species, the American bullfrog (Hammond et al. 2017) and *P. canaliculata* (Sun et al. 2019)
403 provide an unprecedented model to study predator-prey interactions of two invasive aquatic
404 organisms at a molecular level, and may suggest interesting directions for future research.

405

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575

576 **FIGURE LEGENDS**

577 **Figure 1. Changes in bullfrog small intestine after 24 h and 48 h gavage with apple snail egg**

578 **PVF.**

579 **A.** Small intestine schemes highlighting parameters determined for morphometry. Left image:
580 Section area (SA) defined as the region of interest (ROI) within the green line that includes
581 mucosa and lumen space. Middle image: Tissue area (TA) shown in red within the ROI including
582 only mucosa. Right image: ROI (in green) delimiting the lumen area. **B.** Changes in relative tissue
583 area (TA). **C.** Changes in the lumen space ratio (lumen area/SA). 24 h after ingestion of *P.*
584 *canaliculata* PVF, an increment in TA as well as a decrease in lumen space when compared to
585 control group was observed. Note that these changes are partially reverted 48h after treatment
586 where TA and lumen space begin to return to control levels. **D.** Absorptive surface (perimeter/SA)
587 displays no changes regardless the time assayed after treatment. **E.** Typical small intestine of
588 controls with a large lumen space and long and thin villi (arrow indicates lymphatic capillaries).
589 **F.** Representative 24 h post-treatment section displaying a reduced lumen space with wider villi.
590 Lymphatic capillaries (arrow) are reduced due to an increased connective tissue. **G.** Small
591 intestine 48 h post-gavage showing villi gradually returning to control levels though its chorion
592 still shows reduced lymphatic capillaries due to thickening of the connective tissue. Scale bar 100
593 μm .

594

595 **Fig 2. Bullfrog small intestine hemorrhage induced by *Pomacea canaliculata* eggs PVF**
596 **ingestion.**

597 **A.** Representative control villi section. Inset: detail of villi chorion. **B.** 24h post-treatment
598 showing signs of weak hemorrhages (rectangles) and epithelial disorganization at the tips of villi
599 (arrows). Inset: detail of the weak hemorrhage (rectangle). Scale bar 100 μm .

600

601 **Fig 3. Presence of eosinophils and macrophages in bull frogs' small intestine after gavage of**
602 ***Pomacea canaliculata* eggs PVF.**

603 A-C Eosinophils (HE), D-F Macrophages (IHC). **A.** Control sections, eosinophils are marked
604 with an arrow. Inset: eosinophil showing typical cytoplasmic granules (scale bar 10 μm); **B.** 24h
605 post-gavage; **C.** 48h post-gavage intestines. D-F. Representative images showing intestine
606 macrophages (arrows) which were more prevalent 24 h after gavage with egg PVF. **D.** Control
607 intestines showing immunolabelled macrophages with CD⁶⁸⁺. Inset: detail of a macrophage (scale
608 bar 10 μm); **E.** 24 h after-gavage; **F.** 48h after-gavage. **G.** Percentage of total collagen in the
609 mucosa after 24 h and 48 h post gavage. H Ratio between collagen type I and III. A-F scale bar
610 40 μm .

611

612 **Figure 4. Effect of snail egg PVF ingestion on bullfrog small intestine glycosylation**
613 **pattern.**

614 Lectin histochemistry using DBA, PNA, SBA. **DBA** does not stain supranuclear zone (arrows)
615 and Goblet cells (arrow heads) 24 h after-gavage when compared to control. These effects are
616 reverted after 48 h post-gavage. **PNA** strongly stains supranuclear zone (arrows) in control
617 sections although this is not seen in experimental intestines. **SBA** shows light staining in
618 enterocytes of control animals (arrows) but a strong staining at the supranuclear zone is observed
619 in 24 h after-gavage of experimental frogs. Staining with SBA of 48h after gavage intestines is
620 observed not only at the supranuclear zone but also in the apical zone. Bar = 40 μm . For the
621 complete glycosylation pattern refer to Table 1.

622

623 **Fig 5. Binding of PcPV2 to bullfrog small intestine.**

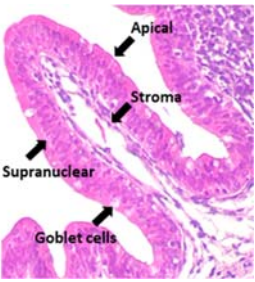
624 Immunolocalization of PcPV2 at the brush border of frog enterocytes. Control group (Control)
625 show no anti-PcPV2 antibody binding (arrow) while at 24 h post-gavage of *P. canaliculata* PVF
626 containing the equivalent of 200 μg PcPV2, a strong labelled epithelium was observed, especially

- 627 at the glycocalyx of the villi (brown colour, arrows) (24 h). The same binding pattern is observed
- 628 at 48 h post-gavage (brown colour, arrows) (48 h). Scale bar 40 μm .

629 **Table 1. Glycosylation pattern of bullfrog small intestine villi after snail egg PVF gavage.**

630 Glycosylation changes in villi regions (see left image) were assayed by lectin histochemistry 24
 631 h and 48 h after gavage and compared to control. No changes are symbolized with (=), and up or
 632 down arrows indicate increased or decreased reactivity, respectively. Thin arrows indicate less
 633 change (see text and Fig. 4 for details on DBA, PNA and SBA). The acronym of each lectin and
 634 their relative glycan specificity are indicated in the first two columns.

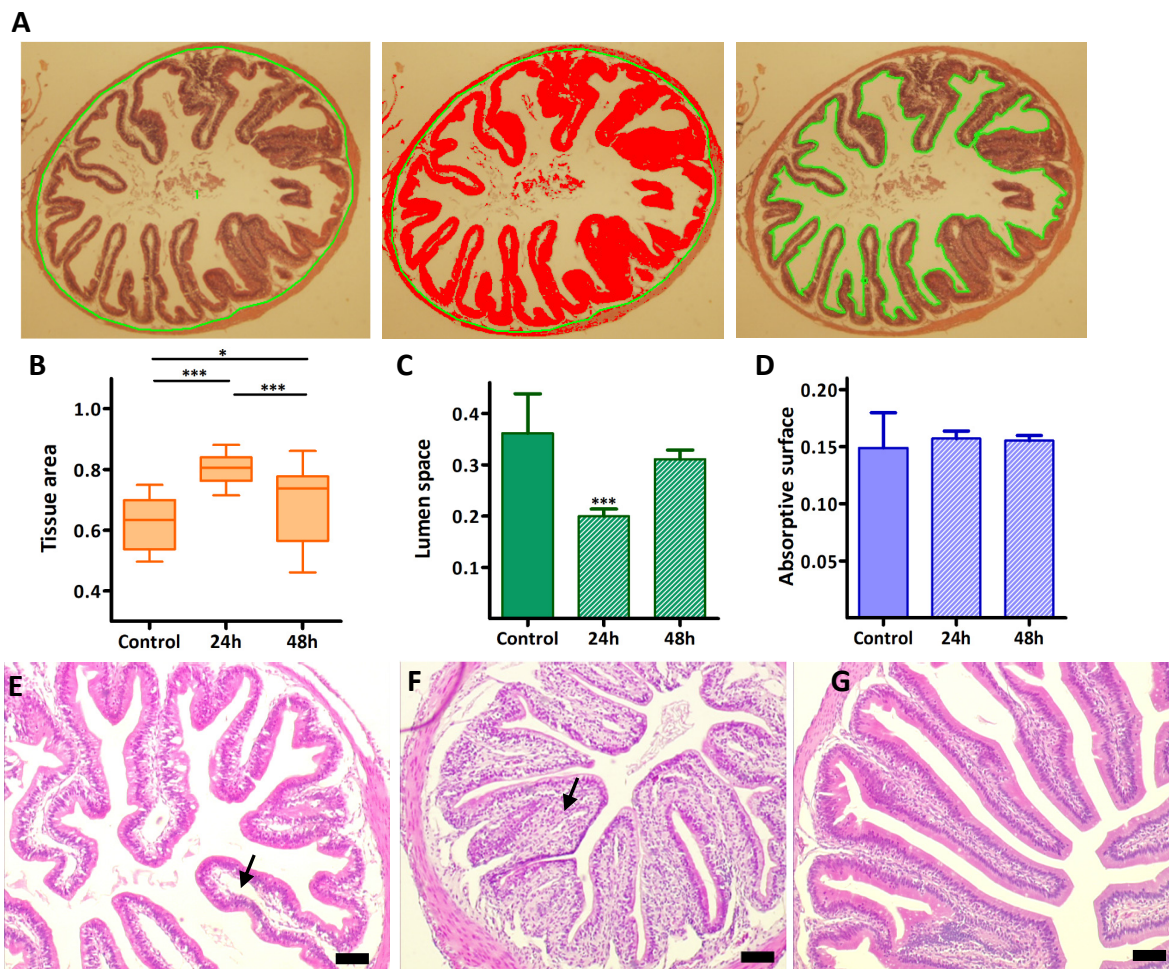
635



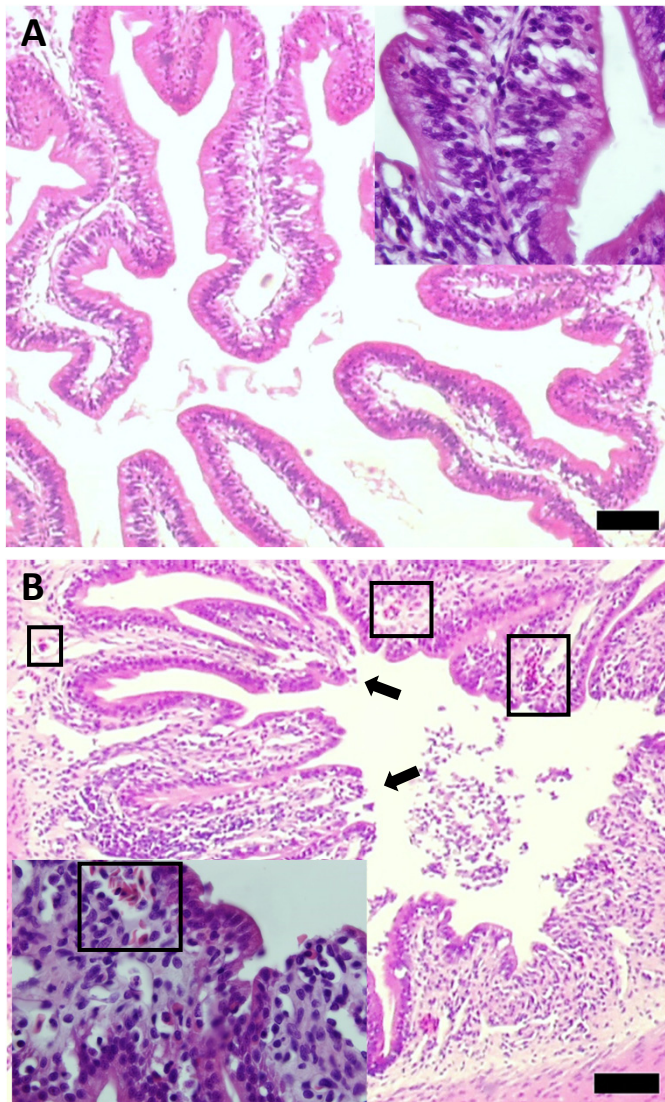
Lectin	Specificity	Apical		Supranuclear		Stroma		Goblet	
		24	48	24	48	24	48	24	48
Con A	α -D-Man; α -D-Glc	=	=	=	=	=	=	=	=
DBA	α -D-GalNAc	=	↑	=	↑	=	=	=	↑
WGA	β -D-GlcNAc; NeuNA	=	=	=	=	=	=	=	=
PNA	β -D-Gal (β 1-3) D-GalNAc	=	=	↓	↓	↓	↓	=	=
UEA I	α -L-Fuc	=	=	=	=	=	=	=	=
SBA	α -D-GalNAc; β -D-GalNAc	↑	↑	↑	↑	=	=	=	=
RCA I	β -Gal	=	=	↓	=	=	=	=	=

636

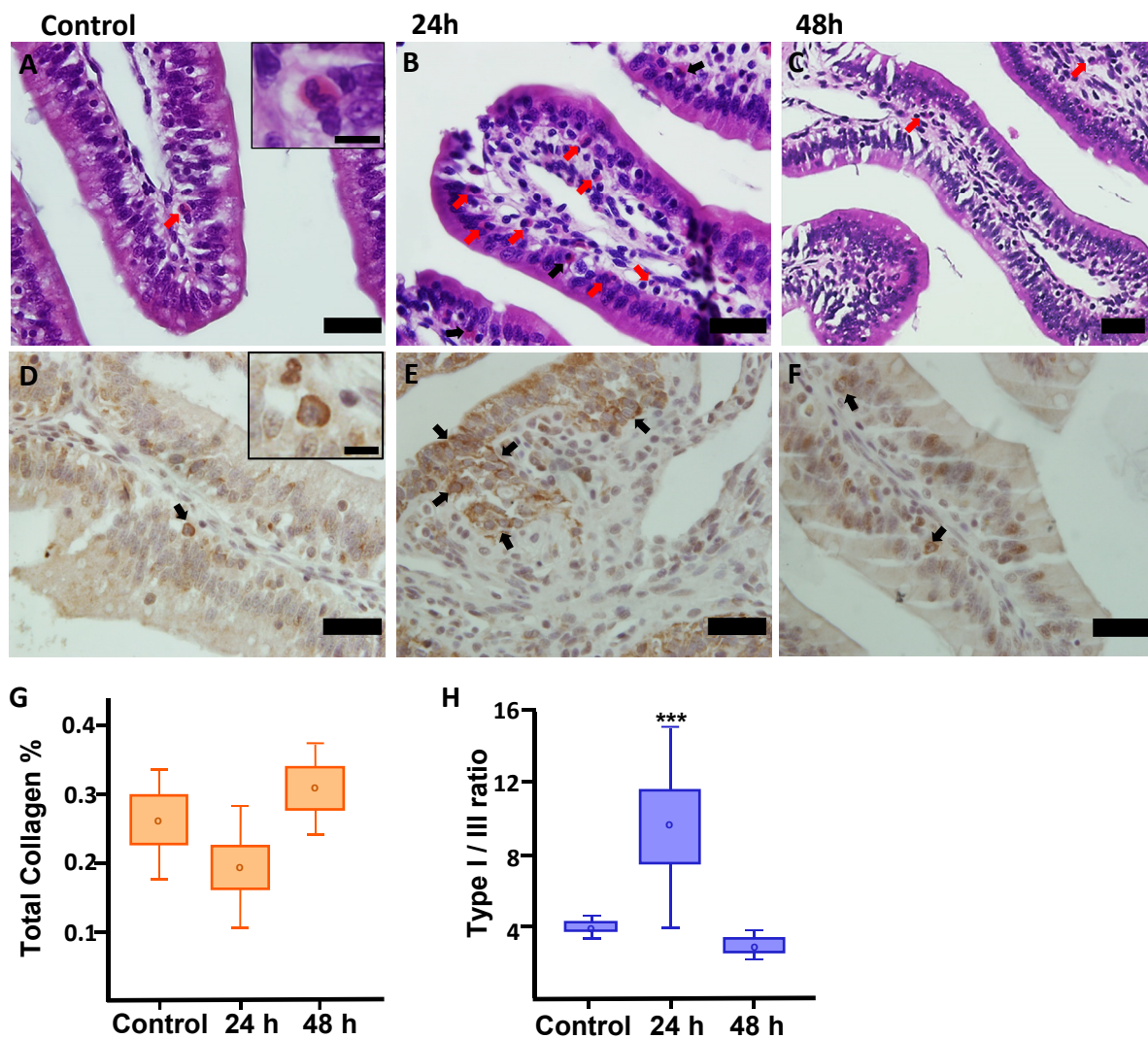
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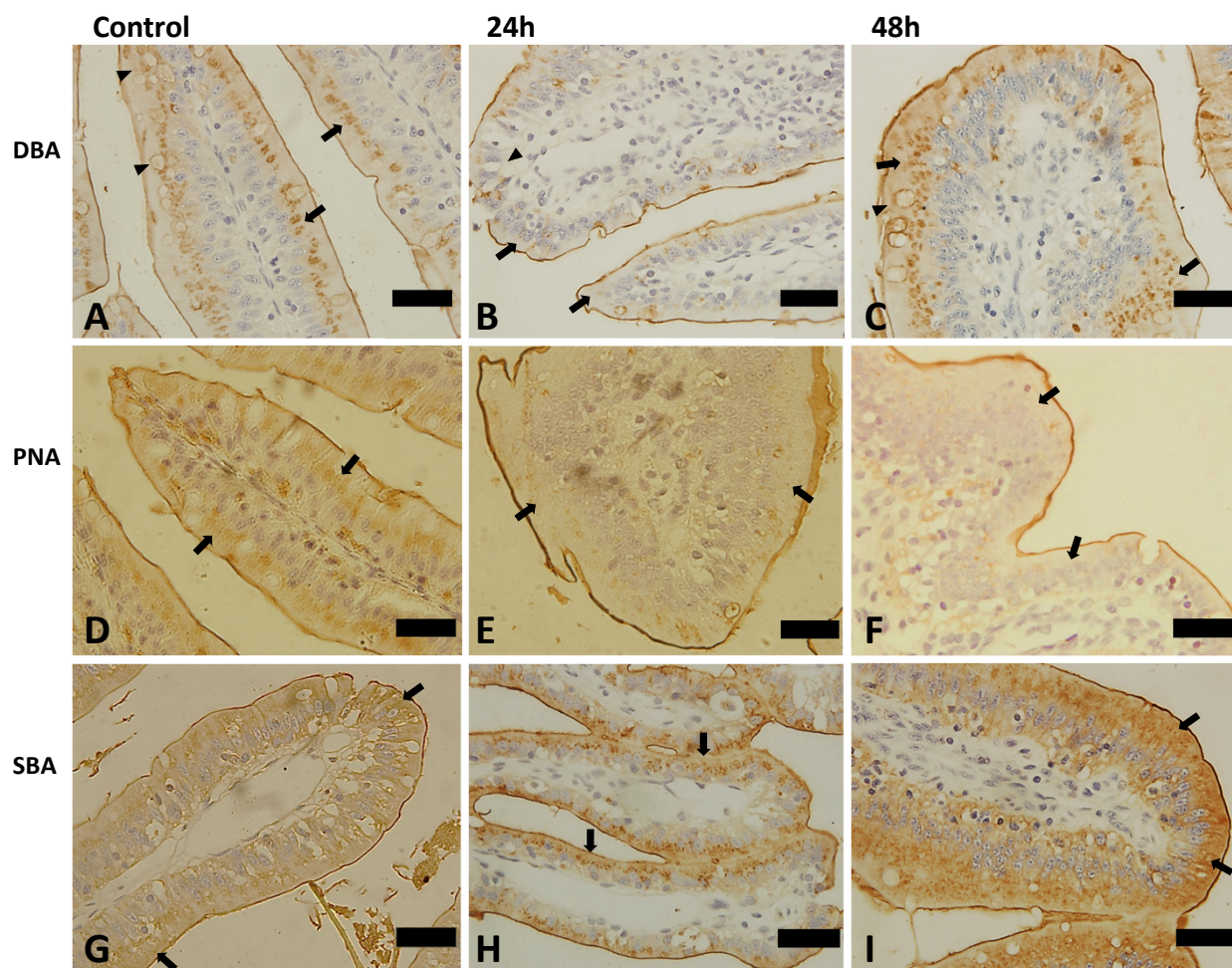
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