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1                    **High temperature aggravates the effects of pesticides in goldfish**

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28

29 **Abstract**

30 In human-altered rivers, fish are often conjointly exposed to an increase in water temperature due  
31 to global warming and to contamination by organic pollutants such as pesticides, but their  
32 combined effects are still elusive. Thermal and chemical stressors could potentially interact  
33 because high temperature increases metabolism and toxicant uptake, and can alter the ability of  
34 **organisms** to set up adequate stress responses and to maintain homeostasis. These combined  
35 stressors could thus potentially result in higher level of molecular and cellular damage, and  
36 stronger effects on behavior and physiology, but experimental evidence across biological levels  
37 is still scarce. In this study, goldfish *Carassius auratus* were experimentally exposed to an  
38 environmentally realistic cocktail of pesticides (**S-metolachlor, isoproturon, linuron, atrazine-**  
39 **desethyl, aclonifen, pendimethalin and tebuconazol**) commonly found in rivers of South-West  
40 of France at low or high dose in two **different** thermal conditions: a **common** summer  
41 temperature (22°C) or a high temperature recorded during heat waves (32°C). Results showed  
42 that high temperature alone caused behavioral and physiological changes (**increased swimming**  
43 **activity, increased** hepatosomatic index, decreased reproductive index) but limited cellular  
44 damage. However, high temperature aggravated the effects of pesticides at the molecular and  
45 cellular level. Indeed, pesticide exposure resulted in higher genotoxic effects (micronuclei rate)  
46 and irreversible cellular damage of the gills and liver (apoptosis, inflammation, necrosis) at 32°C  
47 compared to 22°C. This suggests potential synergic effects of climate change and pollution, and  
48 highlights the need for multiple stress approaches to better predict the impacts of human  
49 activities on aquatic wildlife.

50

51 **Keywords:** *pesticides, multiple stressors, climate change, freshwater fish, synergic effects,*  
52 *physiology*

## 53 **Introduction**

54

55 In the context of global change, aquatic organisms are exposed to a growing number of  
56 environmental stressors that create new selective pressures affecting their behavior, physiology  
57 and ultimately their fitness (Crozier and Hutchings, 2014; Schinegger et al., 2018; Schulte,  
58 2014). Although a considerable body of literature addressed the isolated effects of different  
59 stressors on aquatic organisms, few studies investigated their combined effects, thereby  
60 hindering our ability to predict the effects of human-induced perturbations in realistic  
61 environmental conditions (Holmstrup et al., 2010; Matthaei and Lange, 2016; Nöges et al., 2016;  
62 Schinegger et al., 2018, 2016). In human-altered rivers, fish are often conjointly exposed to an  
63 increase in water temperature due to global warming, especially during heat **waves**, and to a  
64 concomitant contamination by organic pollutants such as pesticides, which are widely used in  
65 urban and agricultural areas (Page et al., 2014; Polard et al., 2011a). In most cases, rivers were  
66 contaminated by complex **mixtures** of pollutants, including several pesticides, which **can induce**  
67 **different effects** (Hayes et al., 2006; Relyea, 2009; Wang et al., 2017). In addition, thermal and  
68 chemical stressors can potentially interact and alter the ability of **organisms** to set up adequate  
69 defensive stress responses and to maintain homeostasis (Gandar et al., 2017a; Nadal et al., 2015;  
70 Noyes et al., 2009; Schiedek et al., 2007). Indeed, high temperatures can directly increase the  
71 intake and toxicity of organic molecules (Laetz et al., 2014; Nadal et al., 2015). In addition, high  
72 temperature triggers costly stress responses, accelerate metabolism and deplete energy reserves,  
73 which could indirectly affect **ability of organisms to** set up efficient defense responses against  
74 chemicals and to limit cellular damage (Gandar et al., 2017a, 2015; Kennedy and Ross, 2011).  
75 Increased temperature can be particularly deleterious for ectothermic species as temperature  
76 affects most of their behavioral and metabolic processes (Manciocco et al., 2014). As a  
77 consequence, thermal stress might amplify or mask the effects of toxic stressors in fish, making  
78 it difficult to predict their combined effects at different levels of biological organization, from  
79 the cell to the whole organism.

80 **To tackle this question, we used goldfish *Carassius auratus* as a model species**  
81 **because of its extensive use in ecotoxicological tests and for its responsiveness to chemical**  
82 **and natural stressors (Cavas, 2011; Gandar et al., 2017b; Maisano et al., 2013; Parrino et**  
83 **al., 2018). It is non-native species from Asia, that can now be found in a wide range of lentic**  
84 **and lotic systems across Europe, including in the Garonne watershed, South-West of**  
85 **France (Keith et al., 2011).** Goldfish were experimentally exposed to an environmentally

86 realistic cocktail of common pesticides (S-metolachlor, isoproturon, linuron, atrazine-desethyl,  
87 aclonifen, pendimethalin and tebuconazol) at low and high concentrations observed in rivers of  
88 South-West of France (Polard, 2010; Polard et al., 2011a). This experiment was conducted with  
89 a temperature of 22°C, currently registered in **rivers of South-West of France** and  
90 corresponding to the optimal temperature for goldfish, and with a high temperature (32°C),  
91 which is within the upper tolerance limit of goldfish (**0.3°C- 12.6°C** for the lower limit and  
92 **30.8°C- 43.6°C** for the upper limit, Ford and Beitinger, 2005; Fry and Hart, 1948). The chosen  
93 high temperature of 32°C is a temperature observed in the Garonne river (**located in South-**  
94 **West France**) during extreme weather events such as heat waves, which will increase in  
95 intensity and frequency in the future due to climate change (IPCC, 2013). Fish were exposed to a  
96 high temperature or pesticides alone or to both stressors in combination.

97 To understand better the effects of chemical and thermal stress, alone or together, the  
98 behavioral and physiological responses of fish were analyzed at different levels of biological  
99 organization. Pesticides generally induce pathological damage at the molecular, cellular and  
100 individual **levels**, depending on the stress intensity and the duration of exposure. At the  
101 molecular level, genotoxic effects of pesticides are often observed, with alterations of DNA  
102 structure that can result in the production of micronuclei and alter the cell cycle (**Cavalcante et**  
103 **al., 2008; Polard et al., 2011**). Previous proteomic studies on *Carassius auratus* showed that the  
104 cocktail of pesticides used in this study affected proteins involved in cellular inflammatory  
105 responses, oxidative stress, and in cytoskeleton and cell structure (e.g. Gandar et al., 2017b;  
106 Marchand et al., 2006). However, the consequences at the cellular level are not known yet.  
107 **Oxidative stress and proteome changes caused by pesticides are expected to result in**  
108 **apoptosis or accelerated cell cycle, tissue lesions and potentially carcinogenesis, especially**  
109 **in the liver** (Gandar et al., 2017b; **Ratn et al., 2017**; Weber et al., 2013; Wirbisky et al., 2016).  
110 In this study, we thus focused on histopathological modifications of the gills and liver, because  
111 they are the two primary target organs affected by organic pollutants (e.g. Nero et al., 2006).  
112 **Gills are an important interface between the external and internal environment and can be**  
113 **a route of contaminant uptake in fish** (Evans, 1987). Liver is the primary organ for  
114 detoxification and proteomic studies showed differential levels of proteins involved in  
115 hepatocyte apoptosis and cirrhosis in goldfish exposed to pesticides (Gandar et al., 2017b).

116 Given previous proteomic studies (Gandar et al., 2017b), we hypothesized that pesticide  
117 exposure would alter liver and gill structure and result in cellular lesions (such as necrosis,  
118 inflammatory lesions), especially at high temperature. We also hypothesized that pesticides

119 would reduce gonad mass (often measured by Gonadosomatic Index), liver mass (Hepatosomatic  
120 Index) and body condition, because stressful conditions often decrease investment in  
121 reproduction (Schreck, 2010) and lead to reallocation of **energy** reserves (Gandar et al., 2015).  
122 At the individual level, temperature and organic pollution also often trigger immune changes  
123 (here measured as changes in innate immunity) (Fatima et al., 2007) and behavioural changes  
124 (activity, feeding, exploration) (Gandar et al., 2015; Jacquin et al., 2017), although their  
125 combined effects are still elusive. Based on previous metabolomic approaches showing  
126 synergistic effects of temperature and pesticides on goldfish immune proteins, energy allocation  
127 and maintenance (Gandar et al., 2017b, 2017a, 2015), we predicted that pesticides would have  
128 stronger effects at 32°C on immunity and behaviour compared to 22°C.

129

## 130 **Materials and Methods**

131

### 132 *Model species*

133 The goldfish (*Carassius auratus*, Cyprinidae family) was chosen as a biological model for this  
134 study because of its extensive use in ecotoxicological tests (e.g. Bretaud *et al.* 2000, Cavas and  
135 Konen 2007, Feng *et al.* 2013). **Introduced in Europe in the eighteenth century for  
136 ornamentation, it is a non-native species from Asia that can now be found in a wide range  
137 of lentic and lotic systems across Europe, including in the studied Garonne watershed,  
138 South West of France (Keith et al., 2011).** The temperature tolerance of the goldfish is large  
139 (**0.3°C- 12.6°C** for the lower limit and **30.8°C - 43.6°C** for the upper limit) and its optimum  
140 temperature is 25°C. (Ford and Beitinger, 2005; Fry and Hart, 1948). A total of 156 individuals  
141 of 10-12cm **in length** were purchased from the **fish farm** Carpio (Consac, France). They were  
142 acclimatized for two weeks in opaque tanks under controlled conditions (18°C with a 12:12h  
143 light regime). **They were then gradually acclimatized to common (22°C) or high (32°C)  
144 experimental temperatures. The temperature was increased by 0.5°C every 12 hours until  
145 reaching the experimental temperature following previous studies (Gandar et al. 2015).**  
146 Water was aerated and dechlorinated prior to fish introduction. Half of the water was renewed  
147 every day and fish were fed daily with commercial pellets (**Carpio, France**).

148

150 **A total of 156 fish (26 fish in each experimental group) were placed in 30L opaque aquaria (22**  
151 **and 32°C). Temperature regime was controlled using heaters coupled with thermostats.**  
152 **Temperature was monitored every 24h to ensure constant conditions throughout the**  
153 **exposure period. The 22°C temperature was chosen as a control temperature since it is close**  
154 **to goldfish optimal temperature range, while 32°C corresponds to a stressful temperature**  
155 **within the upper tolerance limit of goldfish. The 22°C temperature is a common summer**  
156 **temperature in rivers in South-West of France. The 32°C temperature corresponds to a**  
157 **high river temperature recorded during heat waves ( data from Agence de l'Eau Adour-**  
158 **Garonne, Croze et al., 2007), which are expected to increase in frequency and intensity in**  
159 **the next decades due to climate change (IPCC 2013).**

160 In each temperature group, fish were exposed either to control water (C), or to a low (L)  
161 or high dose (H) of a cocktail of pesticides during 96h, resulting in 6 experimental groups of 26  
162 fish (C22, L22, H22, C32, L32 and H32). The cocktail of pesticides contains seven most  
163 common pesticides: 6 herbicides (S-metolachlor, isoproturon, linuron, atrazine-desethyl,  
164 aclonifen and pendimethalin) and one fungicide (tebuconazol), which are mainly used for  
165 intensive agriculture (corn, wheat and sunflower) (details in Table 1). **This pesticide**  
166 **composition and concentrations were selected according to preexisting data of a typical**  
167 **pesticide contamination in the rivers of the South-West of France (Polard, 2010; Polard et**  
168 **al., 2011a; Taghavi et al. 2011). Two concentrations were chosen: a low dose of 8.4µg.L<sup>-1</sup> (L**  
169 **groups) and a high dose of 42µg.L<sup>-1</sup> (H groups). The Low Dose corresponds to a typical**  
170 **pesticide concentration found after spring flood in French rivers (Debenest et al., 2010;**  
171 **Garmouma et al., 1998; Polard et al., 2011a; Taghavi et al., 2011). For instance Polard et**  
172 **al. (2010, 2011a), Taghavi et al. (2011) and other authors found approximately 2,0 to**  
173 **2,4µg/L of metolachlore, 1,0 to 1,4 µg/L of isoproturon and related molecules, and 0,2 to 0,4**  
174 **µg/L of atrazine and related molecules in the Save river after spring floods. Data from the**  
175 **French national database on water pollution show that these concentrations are in the**  
176 **range of what can be found in other agricultural French rivers (IFEN 2007). The high**  
177 **concentration (H: High dose of 42µg.L<sup>-1</sup>) is five times higher and reflects highest levels of**  
178 **contamination measured in streams and reservoirs of Europe and USA (Battaglin et al.,**  
179 **2000; Graymore et al., 2001; Kreuger, 1998). Details of these concentrations for each**  
180 **pesticide are in Table 1.**

181 Pesticides were obtained from Sigma-Aldrich (St. Louis, MO, USA): S-metolachlor  
182 (CAS-No: 87392-12-9, PESTANAL®, analytical standard), Isoproturon (CAS-No: 34123-59-6,  
183 PESTANAL®, analytical standard), Linuron (CAS-No: 330-55-2, PESTANAL®, analytical  
184 standard), Atrazine-desethyl (CAS-No: 6190-65-4, PESTANAL®, analytical standard),  
185 Aclonifen (CAS-No: 74070-46-5, PESTANAL®, analytical standard), Pendimethalin (CAS-No:  
186 40487-42-1, PROWL®, analytical standard), Tebuconazol (CAS-No:107534-96-3,  
187 PESTANAL®, analytical standard). Pesticides were diluted in acetone (0.3mL.L<sup>-1</sup> of water) and  
188 demineralized water to obtain the two concentrations selected. Acetone (CAS: 67-64-1, Fisher  
189 Chemical, HPLC solvent) was purchased from Fisher Scientific (Illkirch, France). Control  
190 groups (C22 and C32) received only acetone. **The composition of the mixture, characteristics  
191 and concentrations of pesticides are presented in Table 1.**

192 **Prior to fish introduction, aquaria and all equipment were pre-saturated with the**  
193 **mixture of pesticides. Air pumps were placed in each aquarium to supply oxygen in water.**  
194 **Half of water and pesticide solutions was renewed every day to limit concentration variations.**  
195 Previous studies showed that this protocol successfully maintained pesticide concentrations in  
196 the water and that good oxygen and pH conditions across the experiment (Gandar et al. 2015,  
197 2017). No mortality occurred during the exposure. At the beginning of the experiment at T0,  
198 groups did not differ in body mass ( $\chi_2 = 1.51$ ,  $p= 0.91$ ). At the end of the experiment and after  
199 the behavioral tests, fish were **weighed** (to the nearest 10 mg) and measured (fork length to the  
200 nearest mm). Fish were anesthetized and euthanized with benzocaine (80 -120 mg.L<sup>-1</sup>) and  
201 concussion.

202

### 203 *Molecular level: DNA alterations*

204 DNA alterations were estimated on blood smears by counting the **Micronuclei (MN)** rate in  
205 erythrocytes following the protocol described in Polard et al. (2011b) on half of the individuals  
206 of each treatment group (N=12 to 14 randomly chosen slides per group). Micronuclei rate is used  
207 to quantify the genotoxicity of treatments, which might arise through mutagenic or epigenetic  
208 processes (Al-Sabti and Metcalfe, 1995; Luzhna et al., 2013; Torres de Lemos et al., 2007).  
209 **Blood was sampled in the caudal vein using 1mL Terumo syringe with a 26G needle and**  
210 **one drop was fixed on slides.** Blood slides were fixed in pure ethanol for 15 min and dried for  
211 24 hours. All slides were then stained with fluorescent acridine orange AO (0.003% in Dulbecco  
212 PBS) (Polard et al. 2011). Micronuclei yellow-green fluorescence **was counted** under epi-



213 fluorescence with a U-MWB2 filter at 1000 magnification. **Micronuclei rates were calculated**  
214 **as the number of micronuclei per 5000 blood cells.**

215  
216 *Cellular level: histopathological alterations of the gills and liver*  
217 Histopathological lesions were evaluated in the liver (26 fish per experimental group, 3  
218 observations per individual) and in the gills (3 females per experimental group, 30 observations  
219 per individual, 10 measurements per filament).

220 Gill and liver damage was assessed using standard histological techniques (Fernandes and  
221 Mazon, 2003, Bernet et al., 1999; Costa et al., 2011; Cuevas et al., 2015; van Dyk et al., 2007).  
222 Organs were dissected and immediately fixed in Bouin's fluid (picric acid 3V, formaldehyde 1V  
223 and acetic acid 2V) for 24h. They were dehydrated through a graded ethanol series (70–100 %),  
224 following by Butanol-1ol bath, cleared in toluene, and embedded in paraffin. Longitudinal serial  
225 sections of 10 µm (gills) or 6 µm (liver) were mounted on glass slides, stained with hematoxylin-  
226 eosin HE (gills) or with nuclear fast red NR and picro-indigo-carmin PIC (liver), and observed  
227 under Nikon microscope (10X, 40X and 60X). Microphotographies were taken with a light  
228 microscope (Laborlux S – Leitz wetzlar Germany) equipped with a digital camera  
229 (Imagingsource) and analyzed using Archimed software (Version 5.6.0.).

230 Gill remodelling was assessed using the percentage of exchange surface area (ESA),  
231 corresponding to protruding length of secondary lamellae (10 measurements per filament in the  
232 middle of the arch, i.e. 30 measurements per sample, hence, 90 measures per conditions) and was  
233 calculated as:  $ESA (\%) = 100 * (\text{mean SL} - \text{mean ILCM}) / \text{mean SL}$  where: SL= length of  
234 secondary lamellae; ILCM = length of interlamellar cell mass. In addition, five filaments located  
235 in the middle of the arch (i.e. 5 measures per fish, namely 15 measures per condition) were  
236 analysed following Bernet et al. (1999) to calculate the degree of gill damage. Six different types  
237 of alterations were recorded (details in Table S1). A gill pathological index ( $I_{\text{Gill}}$ ) was calculated  
238 as:  $I_{\text{Gill}} = \sum_{\text{alt}} (a_{\text{alt}} \times w_{\text{alt}})$  where:  $a$  = score value (depending on occurrence) for a specific  
239 alteration (i.e. type of lesion), ranging from 0 to 6;  $w$  = importance factor for a specific alteration  
240 (1: reversible lesions with low effects; 2. modified cellular architecture: 3: degenerative  
241 irreversible alterations leading to a loss of gill function),; alt = specific alteration (see Table S1).

242 Six main hepatic alterations were recorded in the liver (on 3 random areas of 25 mm<sup>2</sup>):  
243 lysis-necrosis, mitosis, melano-macrophage aggregates, fibrosis, nuclear alterations, and

244 lipidosis. A global histopathological score was calculated as the mean value of the different  
245 scores (details in Table S1).

246

247 *Organ level: GSI, HSI and body condition*

248 **Sex and maturity were determined by gonadal examination.** The Hepatosomatic Index HSI  
249 representing the energy content of the liver and the Gonadosomatic Index GSI representing the  
250 gonad growth status were calculated as liver or gonad mass/total body mass. **Body condition**  
251 **was measured by the Fulton condition index as (body mass/ body length<sup>3</sup>).**

252

253 *Individual level: immunity*

254 **After centrifugation (10min at 3000 rpm), plasma was frozen for subsequent immune**  
255 **analyses. Innate immunity was assessed by measuring lysozyme activity (which reflects the**  
256 **ability of the individual to eliminate bacteria), and peroxidase activity of the plasma (which**  
257 **is used as a marker of leucocyte activation, Eslamloo et al., 2014; Fatima et al., 2007;**  
258 **Ghafoori et al., 2014; Magnadóttir, 2006).** To measure lysozyme activity, 8 mg of  
259 *Micrococcus lysodeikticus* were suspended in 40 mL of Na<sub>2</sub>HPO<sub>4</sub> buffer/ 50 mM of citric acid  
260 (pH = 5.5). **A total of 15 µL** of plasma were placed in 96 wells microplates, with 150 µL of  
261 revealing solution. Optical density was read at 450 nm from 5 to 60 minutes. The absorbance  
262 was measured at 450 nm using a spectrophotometer at an interval of 30 s (total measuring time  
263 60 min). The unit of lysozyme activity is defined as the **concentration** of sample causing a  
264 decrease in turbidity of 0.001/ min (1 UA = Δ 0.001 A<sub>450</sub>/min).

265 Peroxidase activity was measured following protocols from Quade and Roth, (1997).  
266 Tetramethylbenzidine (TMB) and hydrogen peroxyde **H<sub>2</sub>O<sub>2</sub>** were used as enzymatic substrates.  
267 **A total of 12.5 µL** of plasma was diluted in **0.5 mL** of Hank buffer (HBSS). **A total of 60 µL** of  
268 this dilution were placed in a 96-microplate with a 100 µL solution of TMB, DMSO,  
269 Na<sub>2</sub>HPO<sub>4</sub>/citric acid 50 mM (pH = 5.0) buffer. After 2 minutes of incubation, 50 µL of 2 M  
270 sulfuric acid were added to stop the reaction. The absorbance was measured at 450 nm using a  
271 spectrophotometer, and expressed as UA with: UA = A<sub>450</sub> « sample » - A<sub>450</sub> « control » and used  
272 as a measure of peroxidase activity.

273 Because immunity can differ among individuals before the experiment depending on past  
274 exposure of fish to disease, we also measured their lysozyme and peroxidase levels in the plasma

275 before the experiment to control for individual immune differences before experimental  
276 treatments. Lysozyme or peroxidase variations across the experiment were then calculated as  
277 (value after treatment-value before treatment)/value before treatment.

278

279 *Individual level: behavior*

280 We measured activity, mean speed, trajectory changes, exploration and feeding behaviour at T96  
281 (96 hours post pesticide exposure). **These behavioural traits were chosen because they are**  
282 **often affected by stressors and are tightly linked to fish fitness and functional effects (e.g.**  
283 **Scott and Sloman, 2004, Gandar et al. 2015).** Fish were not fed during the exposure to  
284 pesticides to standardize their physiological state and appetite.

285 First, we recorded the “activity” of fish in the home tank as the total time spent  
286 swimming during 5 minutes (i.e., in directed motion and covering a distance > 10% of body  
287 length). Activity was recorded by direct observation in the home tank with a companion fish to  
288 limit isolation stress and reliably reflects the intrinsic activity of the fish. Time of the day of  
289 observation was randomized for each group to avoid any confounding effect of diurnal rhythm.  
290 Second, we recorded more specific features of the trajectory of the fish such as “mean speed”,  
291 “trajectory changes”, and “exploration”, because contaminants can have neurotoxic effects that  
292 can result in erratic behaviours, affect trajectories and exploration patterns (e.g. Jacquin et al.,  
293 2017). For this, we transferred each individual in a test tank (50x30cm with 10cm of water) for  
294 10 minutes with the same conditions as the exposure tank. Each focal fish was placed in a  
295 transparent cylinder in the middle of the tank. After 2 minutes of acclimation the cylinder was  
296 gently removed, using a string on a reel to minimize disturbance. Movements were then recorded  
297 precisely for 5 minutes using a camera (Logitech HD Webcam C270) the VirtualDub software  
298 and the Tracker software (Tracker 5.0, Open Source Physics 2015) to obtain precise coordinates  
299 of the position of the fish at each time point (x, y). This allowed us to obtain the mean speed of  
300 the fish (cm/s). The number of trajectory changes was also recorded as the number of rapid (in  
301 less than 5 sec) and abrupt (180° and > 100 % of body length) changes of direction.

302 Lines were drawn on the bottom of the tank to delimit 15 zones of 10 x 10cm.  
303 Exploration was recorded as the number of different zones reached by the fish during 5 minutes  
304 (Jacquin et al., 2017). The position of the different test tanks was changed every 5 fish to avoid  
305 any confounding effect of a spatial preference. At the end of the behavioural test, we introduced

306 10 food pellets in the test tank and recorded the number of pellets consumed during one minute  
307 and the latency before feeding to compare the feeding behaviour between groups.

308

### 309 *Compliance with ethical standards*

310 All experimental procedures were conducted in compliance with French and European  
311 legislation for animal experimentation (European directive 2010/63/UE). Experimental  
312 procedures were conducted under the French animal handler's certificate (N° 31-103), the  
313 establishment approval for vertebrate experimentation N°A3113002, and were approved by the  
314 ethical committee 073 (authorization N°8538).

315

### 316 *Statistical analyses*

317 Effects of stressors (temperature or pesticides or both) were evaluated by Generalized Linear  
318 Models for parametric data with temperature, pesticide dose, and their interaction as explaining  
319 factors. **Sex was also added in the initial models but was not retained in any model** (all p  
320 values > 0.12), except for Gonadosomatic Index where separate analyses were conducted on  
321 males and females. Best models were chosen using a backward selection and successively  
322 dropping non-significant terms ( $p > 0.10$ ). Posthoc tests were conducted using Wilcoxon tests.  
323 For non-parametric data, Kruskal-Wallis tests were conducted to compare groups. For feeding  
324 behavior, an additional analysis combining feeding latency and feeding status (at least one pellet  
325 eaten or no pellet eaten) was analyzed using survival curve differences (survdiff function,  
326 Kaplan-Meier method).

327

## 328 **Results**

### 329 *DNA alterations and micronuclei*

330 Micronuclei rate significantly differed between experimental groups (Kruskal-Wallis test:  
331  $\text{Chisq}=14.9$ ,  $P=0.011$ ). Fish at 22°C exposed to a **high** dose of pesticides had a marginally  
332 higher micronuclei rate than the control group (C22 vs H22: Wilcoxon test:  $W=47$ ,  $P=0.057$ )  
333 (Fig 1). Fish at 32°C exposed to a high dose of pesticides had a significantly higher rate of  
334 micronuclei compared to the C22 control group (Wilcoxon test, C22 vs H32:  $W=47$ ,  $P=0.029$ )  
335 and marginally higher compared to the C32 group (H32 vs C32:  $W=56$ ,  $P=0.094$ ) (Fig 1).

336

### 337 *Histopathological alterations of the gills*

338 Histopathological alterations of the gills significantly differed between groups (details in Table  
339 S2). Figure 2 shows the different types of changes and lesions observed in the gills of exposed  
340 fish. At 22°C in the control C22 group, no tissue injuries were observed and the lamellae were  
341 embedded in a thick ILCM (C22, Fig 2), resulting in a low Exchange Surface Area ( $ESA = 5.0 \pm$   
342  $11.5$ ; Table S2). At 22°C with pesticides in the L22 group, ILCM length decreased, and partial  
343 fusion of secondary lamellae (Fu) and abundant **mucus** occurred (L22, Fig.2). In the H22 group,  
344 the ILCM was very reduced (H22, Fig 2), resulting in a significant increase of exchange surface  
345 area ESA, that reached 82 % for H22 fish (Table S2).

346 At high temperature in the C32 group, the elevation of temperature to 32°C also induced  
347 a decrease of ILCM in C32 fish (C32, Fig 2) resulting in a significant increase of exchange  
348 surface area ESA ( $77.5 \pm 4.4$  % in C32 versus  $5.0 \pm 11.5\%$  in C22, Table S2), and protective  
349 **mucus** was present. Protective mucus was absent in fish exposed both to 32°C and to pesticide  
350 mixtures (L32, H32 Fig 2). Lamellar hyperplasia (Hy) was present and pesticides induced lifting  
351 of lamellar epithelium (Li), and oedema (Oe) in H22 fish (Fig 2; Table S2). At 32°C with high  
352 dose of pesticides (H32), irreversible lesions appearing frequently, especially necrosis (Ne), and  
353 oedema (Oe) (Fig 2; Table S2).

354 As a result, the integrative gill pathological index ( $I_{Gill}$ ) thus increased in 32°C group  
355 compared to 22°C group and in fish exposed **to a high** dose of pesticides at 22°C and 32°C (Fig  
356 3A). Fish exposed to both stressors (H32) **exhibited a higher** level of gill pathological index as  
357 compared to all other groups of fish, reflecting a highly degraded cellular structure of the gills  
358 (Fig 3A).

359

### 360 *Histopathological alterations of the liver*

361 High temperature alone had no significant effect on fish hepatic structure (C32 vs C22) (Fig 3B).  
362 In contrast, exposure to pesticides induced fibrosis, an increase of the immune response and  
363 hepatocyte nuclear **alterations** typical of an inflammatory syndrome (details in Supplementary  
364 Figure S3 and S4), resulting in a higher histopathological score in L22 and H22 fish compared to  
365 C22 (Fig 3B). At high temperature, most fish exposed to pesticides (L32 and H32) showed  
366 disorganization of some hepatic areas, i.e. an **increase in** the number of hepatocytes involved in

367 mitosis and inflammation, lipid droplets and a proliferation of blood vessels (details in  
368 Supplementary Figure S4). **A total of** 80% of fish exposed to the high concentration of  
369 pesticides and to 32°C (H32) had a significant inflammatory response in the liver (Fig S4). The  
370 global histopathologic index shows that perturbation of the liver due to pesticides was more  
371 severe at 32°C compared to 22°C (Fig 3B).

372

### 373 *HSI, GSI, body mass loss*

374 The Hepatosomatic Index (**HSI**) was affected by temperature only (**Table 2**), with fish exposed  
375 to 32°C having a higher HSI than fish exposed to 22°C ( $t= 2.10$ ,  $P= 0.042$ ). The Gonadosomatic  
376 Index (GSI) of females (but not in males) was significantly affected by temperature and  
377 pesticides (Table 2). More precisely, at 22°C, pesticides decreased female GSI at low dose  
378 ( $W=136$ ,  $P=0.026$ ), and marginally at high dose ( $W=108$ ,  $P=0.10$ ), whereas no effect of  
379 pesticides was detected at 32°C (all p values  $>0.12$ ) (Fig S5). Body mass loss was affected by  
380 temperature only (Table 2), with fish exposed to 32°C losing more body mass than fish exposed  
381 to 22°C ( $t= -2.63$ ,  $P= 0.0093$ ).

382

### 383 *Immunity*

384 Before exposure to pesticides at T0, **there were** no differences in lysozyme ( $\chi_2 = 3.69$ ,  $P= 0.59$ )  
385 or peroxidase activity ( $\chi_2 = 3.37$ ,  $P= 0.64$ ) **among** groups. Lysozyme variations over the  
386 experiment were not affected by treatments (Table 2). In contrast, **peroxidase activity**  
387 **variations** were affected by both temperature and pesticides and their interaction (Table 2).  
388 More precisely, posthoc tests show that 32°C fish exposed to a low dose of pesticides had a  
389 **higher increase** in peroxidase compared to 22°C and 32°C control groups (Fig S6).

390

### 391 *Behaviour*

392 Activity at T96 was affected by pesticide exposure and by temperature (Table 2), with fish  
393 exposed **to a high** dose of pesticides being less active than control fish whatever the temperature  
394 (estimate =  $-46.63 \pm 16.45$ ,  $t= 2.83$ ,  $P= 0.0052$ ), and fish from the 32°C groups being more active  
395 than fish from the 22°C groups (estimate =  $8.34 \pm 1.35$ ,  $t= 6.19$ ,  $P< 0.001$ ). More specifically,

396 posthoc tests show that at 22°C, fish exposed **to a high** dose of pesticides were marginally less  
397 active than control fish (W= 439, P= 0.064), whereas at 32°C, only fish exposed **to a low** dose of  
398 pesticides were significantly less active than control fish (W= 479, P= 0.0038) (Fig 4A). There  
399 was no difference in mean speed, trajectory changes, and exploration between **pesticide** groups  
400 and these behaviours were only affected by temperature (Table 2). More specifically, fish  
401 exposed **to a high** temperature swam faster (estimate =  $0.054 \pm 0.27$ ,  $t= 2.00$ , P= 0.047),  
402 changed trajectory more often (estimate =  $0.52 \pm 0.21$ ,  $t= 2.42$ , P= 0.017), and explored more  
403 zones compared to fish acclimated to 22°C (estimate =  $0.11 \pm 0.040$ ,  $t= 2.59$ , P= 0.010).

404 Feeding behavior (number of pellets eaten) was affected by both temperature and  
405 pesticides (Table 2). Fish at 32°C ate more pellets than 22°C fish (estimate =  $0.029 \pm 0.012$ ,  
406 P=0.017) (Fig 4B). Fish exposed **to a high** dose of pesticides ate marginally more than control  
407 fish (estimate =  $0.25 \pm 0.14$ , P=0.074) (Fig 4B).

408 A complementary analysis integrating both the latency to feed and the feeding status (fish  
409 eating at least one pellet or no pellet) confirmed that feeding differed **among** treatment groups  
410 (survival analysis:  $\chi^2=11.5$ , P=0.043): a higher proportion of fish exposed to a high temperature  
411 only (C32) and to pesticides (H22, L32 and H32) ate at least one pellet and fed more rapidly than  
412 control fish C22 (Supplementary Figure S7).

413

## 414 **Discussion**

415 The trend of results obtained in this study indicates that environmentally relevant  
416 concentrations of pesticides affected goldfish physiology and behaviour, especially at the  
417 molecular and cellular levels. Cellular damage induced by pesticides was more severe at 32°C  
418 compared to 22°C (summary of results in Fig 5) suggesting high deleterious effects of  
419 multiple thermal and toxic stressors.

420

### 421 *Molecular effects of multiple stressors*

422 At the molecular level, DNA damage reflected by micronuclei rate was higher in fish exposed  
423 to both pesticides at high dose and high temperature (H32) compared to control groups. This  
424 confirms previous studies showing that pesticides have genotoxic effects and induce DNA  
425 alterations in various fish species (Cavalcante et al., 2008; Cavas, 2011; Polard et al., 2011a).  
426 Our results further suggest that genotoxic effects of pesticides could be amplified by high

427 temperatures. In general, high temperature itself can increase DNA damage (e.g. Anitha et al.,  
428 2000), but **Heat Shock Proteins** (HSP) and DNA repair mechanisms can often limit such  
429 damage depending on the duration and intensity of stressors (Iwama et al., 1998).  
430 Accordingly, micronuclei rate did not differ between control fish at 22°C and 32°C in our  
431 study. We also observed that a combined exposure to **thermal** and toxic stressors **led** to  
432 important DNA alterations and high micronuclei rate during cell division. This suggests that  
433 fish exposed to multiple stressors probably entered in the exhausted phase of the stress and  
434 became unable to set up DNA protection and/or repair mechanisms, or that these mechanisms  
435 might be not sufficient to repair all damage. However, the underlying mechanisms remain to  
436 be explored.

437

#### 438 *Histopathological effects of multiple stressors on gills*

439 At the cellular level, several histopathological alterations were also visible in fish exposed to  
440 stressors. Increased temperature only had limited effects on gill structure, **and** mostly resulted  
441 in an increase in exchange surface as expected in this species (Sollid et al., 2005). Indeed,  
442 goldfish at low temperature have specific interlamellar cell mass (ILCM) between their gill  
443 lamellae. **This reduces the exchange surface, limits osmoregulation costs and limits the**  
444 **exposure to pathogens and pollutants** (Sollid et al., 2005; Sollid and Nilsson, 2006). **In**  
445 **addition**, goldfish generally show gill remodelling under high temperatures: the ILCM  
446 generally decreases at warmer water **temperatures** ( $\geq 25^\circ \text{C}$ ), thereby increasing the  
447 exchange surface area (ESA) (Sollid et al., 2005; Sollid and Nilsson, 2006), as observed in  
448 our study at 32°C (C32 vs. C22 group). This increase in gill exchange area could in turn  
449 increase their exposure to pollutants. Accordingly, in our study, more severe gill pathological  
450 lesions due to pesticides were observed in fish exposed **to a high pesticide** dose at 32°C  
451 (H32) compared to fish exposed to the same **pesticide dose** at 22°C (H22). Protective mucus  
452 that helps preserving the integrity of the gill epithelium (Fernandes and Mazon, 2003) was  
453 also reduced in fish exposed to pesticides (H22 and H32), which could participate in the  
454 lesions observed.

455 Several lesions were observed, including lamellar hyperplasia, partial lamellar fusion,  
456 lifting of lamellar epithelium and oedema that were very frequent in H32 fish exposed to both  
457 stressors, and that can ultimately lead to necrosis. This is consistent with previous studies in  
458 goldfish exposed to carbaryl and Polycyclic Aromatic Hydrocarbons (**PAHs**), which also  
459 reported partial lamellar fusion and gill epithelial lifting (Nero et al., 2006; Pfeiffer et al.,



460 1997). Interestingly, in our study, L22 and L32 groups had a similar gill pathological index  
461 ( $I_{Gill}$ ) compared to controls, suggesting that fish could maintain their gill functions at the  
462 lowest pesticide concentrations (for 96 hours at least), although results should be interpreted  
463 with caution given the low sample size (Wolf and Maack, 2017). However, at the higher  
464 pesticide concentrations (H22 and H32), the gill pathological index ( $I_{Gill}$ ) was higher,  
465 indicating a higher number and severity of lesions. Fish maintained at 32°C and exposed to a  
466 **high** concentrations of pesticides (H32) exhibited the highest  $I_{Gill}$  (especially a high  
467 occurrence of necrosis), indicating irreversible damage that will impair ionic regulation,  
468 excretory and respiratory functions of the gills. Taken together, these results indicate a limited  
469 impact of single stressors (high temperature or pesticides alone) but a high impact of multiple  
470 stressors (pesticides at high temperature) on gill cellular structure and integrity, with potential  
471 deleterious consequences for gill function and fish survival.

472

#### 473 *Histopathological effects of multiple stressors on the liver*

474 Several histopathological alterations were also visible in the liver of fish exposed to both  
475 stressors. The increase of temperature alone had low effects on the hepatic structure of  
476 goldfish, while the **exposure** to the pesticide induced a pathology characterized by a fibrosis,  
477 melano-macrophage centers and hepatocyte nuclear alterations suggesting an inflammatory  
478 syndrome, as well as an accumulation of lipids in hepatocytes. Most fish also showed  
479 disorganization of some hepatic areas with an increase in the number of hepatocytes involved  
480 in mitosis and a proliferation in blood vessels, which may indicate a carcinogenic process.  
481 The pathology was however moderate probably due to the short duration of the exposure  
482 (96h). The pathology described was more frequent and acute in fish exposed to the highest  
483 pesticide concentration, which indicated a dose-dependent effect. These results are consistent  
484 with previous studies indicating that pesticides can trigger hepatic perturbations (Paris-  
485 Palacios et al., 2003, 2000; Paris-Palacios and Biagianti-Risbourg, 2006) and cause disorders  
486 of fatty acid metabolism in hepatocytes (Liu et al., 2017). This is also consistent with a  
487 previous study reporting proteome disturbances reflecting liver oxidative stress, hepatic  
488 steatosis and necrosis in goldfish exposed to the same cocktail of pesticides (Gandar et al.,  
489 2017b). The sequestration of lipophilic pollutants and the perturbations of liver metabolism  
490 could lead to the **increased** number of intracellular lipid droplets observed in hepatocytes of  
491 H32 fish. This could **indicate** hepatosteatosis and is consistent with the hepatotoxicity and

492 non-alcoholic “fatty liver” syndrome observed in vertebrates exposed to pesticides (e.g.  
493 (Gandar et al., 2017b; Mela et al., 2013; Mesnage et al., 2017; Xing et al., 2012).

494 Interestingly, this study shows that the histopathologic perturbation of the liver **was**  
495 more severe when the pesticide exposure was combined to a rise of temperature, suggesting  
496 that high temperature aggravates the effects of pesticides on hepatocytes and liver integrity.  
497 Although histopathological data need to be analyzed with caution (Wolf and Wheeler, 2018),  
498 our results are consistent with a previous study showing different liver proteome **profiles** in  
499 goldfish under multiple stressors compared **to a single** stressor (Gandar et al., 2017b). This  
500 previous study outlined that pesticides at high temperature modified the amount of proteins  
501 involved in regulation of oxidative stress, cell cycle and cell death control (Gandar et al.,  
502 2017b), which were predicted to cause the observed liver lesions observed in this study.

503

#### 504 *Multiple stressors effects at the individual level*

505 At higher levels of biological organization (organ and individual level), gonad mass was  
506 reduced in females exposed to a high temperature or pesticides, which is consistent with a  
507 reduction in reproductive investment in stressed fish (Matthaei and Lange, 2016; Pankhurst  
508 and van der Kraak, 2011; Schreck, 2010). Goldfish exposed to both stressors (L32 and H32)  
509 did not have a higher reduction in gonad mass than controls C32, likely because temperature  
510 already caused a **high reduction of gonad mass**. At the individual level, behavioral and  
511 immune changes were observed. Activity (as well as speed, trajectory changes and  
512 exploration) were increased by temperature as expected by an increase in metabolism and  
513 behavioral changes caused by enhanced temperatures (Canal et al., 2015; López-Olmeda and  
514 Sánchez-Vázquez, 2011). This is likely due to increased metabolism and higher energy  
515 mobilization at high temperature, as expected from previous studies (Gandar et al., 2015).  
516 Accordingly, increases in feeding behavior, body condition and HSI were observed in fish  
517 exposed to a high temperature.

518 Interestingly, activity was reduced by pesticides, which is consistent with the  
519 hypoactivity syndrome that is often observed in freshwater fish exposed to herbicides ((Scott  
520 and Sloman, 2004; Shinn et al., 2015), and could explain the reduction in **sediment surface**  
521 reworking observed in goldfish exposed to the same cocktail of pesticides in a previous study  
522 (Gandar et al., 2015). However, no other behavioral changes (speed, trajectory, exploration)

523 were observed in response to pesticides. **It is not clear yet whether the environmental**  
524 **concentrations chosen have neurotoxic effects and further studies are needed to test**  
525 **neurotoxic effects.** However, no decrease in body condition or Hepatosomatic Index were  
526 observed under pesticide exposure, which could be **explained** by the short duration of the  
527 exposure (96h). Alternatively, **lipids might have been reallocated from other life-history**  
528 **traits (growth, reproduction) and/or organs (for instance gonads) to maintain liver mass.**  
529 In addition, pesticides did not trigger a stronger change in behavior under high temperature  
530 than under normal temperature, maybe because temperature effects masked the effects of  
531 pesticides, or because different metabolic strategies were set up under different temperatures.  
532 Further studies specifically testing the allocation of energy in different tissues and function  
533 would be useful to **identify** the metabolic strategy used by goldfish exposed to multiple  
534 stressors.

535

### 536 *Conclusions*

537 To conclude, a single thermal stress (increased temperature alone) caused behavioral and  
538 allocation adjustments (increased activity, feeding and reduced reproductive investment) but  
539 did not result in deleterious **effects** at the cellular level (no deleterious effect on DNA, gills or  
540 liver structure). However, high temperature aggravated the effects of pesticides on fish gill  
541 and liver. Indeed, pesticides at low temperature (22°C) had limited deleterious effects on  
542 behavior, gills and liver structure, but stronger deleterious effects at 32°C. More specifically,  
543 combined thermal and toxic stressors resulted in high level of irreversible damage of the gills  
544 and liver (apoptosis, inflammation, necrosis), with potential consequences for fish  
545 osmoregulation, detoxification and survival. This suggests that high water temperatures could  
546 enhance the negative effects of pesticides on freshwater fish. **Although the chosen model**  
547 **species *Carassius auratus* is a non-native species in Europe, it is now widely found in**  
548 **Europeans rivers and lakes, and has a higher range of temperature tolerance than**  
549 **several native European fish species. It is thus likely that several other wild freshwater**  
550 **fish species might be severely impacted by combined thermal and pesticide stressors.**  
551 Given that climate change will likely increase the duration and intensity of heat waves in the  
552 next decades (IPCC 2014), this raises concerns about potential synergic effects of climate  
553 change and pollution, and highlights the need for multiple stress approaches to better predict  
554 the impacts of human activities on aquatic wildlife.

555

556

557

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567

568

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811

812

813 **Table 1:** *Composition of the pesticide cocktail*

<b>Chemicals</b>	<b>Family</b>	<b>Use</b>	<b>Action mode</b>	<b>Low Dose µg/L</b>	<b>High Dose µg/L</b>
S-metolachlor	Chloroacetanilide	Herbicide	<i>Mitosis perturbation</i>	2.4	12.0
Linuron	Urea	Herbicide	<i>Photosystem II inhibitor</i>	2.0	10.0
Isoproturon	Sustituted urea	Herbicide	<i>Photosystem II inhibitor</i>	1.2	6.0
Tebuconazole	Triazole	Fungicide	<i>Sterol synthesis inhibitor</i>	1.2	6.0
Aclonifen	Diphenyl ether	Herbicide	<i>Carotenoid inhibitor</i>	0.8	4.0
Atrazin-desethyl	Triazine	Herbicide	<i>Photosystem II inhibitor</i>	0.4	2.0
Pendimethalin	Dinitroaniline	Herbicide	<i>Mitosis perturbation</i>	0.4	2.0
<b>Total</b>				<b>8.4 µg/L</b>	<b>42 µg/L</b>

814

816 **Table 2.** *Best Generalized Linear Models (GLM) explaining physiological and behavioural*  
 817 *traits in goldfish exposed to temperature increase and/or pesticides. Sex was included as*  
 818 *explanatory variable but was not retained in any model (see results). Separate models on*  
 819 *males and females were run for GSI. Non parametric analyses were run on other traits and*  
 820 *presented in the text.*

	Temperature		Pesticides		Temp*Pesticides	
	LR $\chi^2$	P	LR $\chi^2$	P	LR $\chi^2$	P
<i>Organs and body condition</i>						
Hepatosomatix Index HSI	4.41	0.036	-	-	-	-
Male Gonadosomatic Index GSI	-	-	-	-	-	-
Female Gonadosomatic Index GSI	0.30	0.58	9.72	0.0078	9.46	0.0088
Body mass loss	6.91	0.0086	-	-	-	-
<i>Immunity</i>						
Lysozyme activity	-	-	-	-	-	-
Peroxidase activity	0.0002	0.98	5.68	0.058	6.67	0.035
<i>Behavior</i>						
Activity	38.31	<0.001	10.47	0.0053	-	-
Speed	4.00	0.045	-	-	-	-
Trajectory changes	5.86	0.015	-	-	-	-
Exploration	9.29	0.0022	-	-	-	-
Feeding	5.84	0.016	9.27	0.0097	-	-

822 **Figure 1.** Level of DNA damage estimated by micronuclei rate (mean  $\pm$  SE) after a 96h exposure at  
823 22°C (left) or 32°C (right) to control water (dark grey), a low dose of pesticides (medium grey), or a  
824 high dose of pesticide (light grey) Different letters above bars indicate significant differences between  
825 groups ( $P < 0.05$ ).

826 **Figure 2.** Gill histological longitudinal sections (**C22**) Control at 22°C. White arrowheads indicate  
827 secondary lamellae embedded in thick InterLamellar Cell Mass (ILCM, white double arrow) in control  
828 fish. (**L22**) Low Dose of pesticides at 22°C. Mucus is present, and ILCM between the gill filaments are  
829 reduced and lesions such as fusions of secondary lamellae (Fu) appear. (**H22**) High Dose at 22°C.  
830 More types of lesions are observable, including Oedema (Oe). (**C32**) Control at 32°C. The ILCM is  
831 very reduced, increasing the Exchange Surface Area (ESA). (**L32**) Low Dose of pesticides at 32°C.  
832 Hyperplasia (Hy) and irreversible lesions such as necrosis (Ne) occur. (**H32**) High Dose of pesticides  
833 at 32°C. Secondary lamellae are shorter and most types of lesions are present and frequent: necrosis  
834 (Ne) is abundant and all gill filaments show severe tissue lesions. Fu: partial fusion of secondary  
835 lamellae; Hy: lamellar Hyperplasia; ILCM: interlamellar cell mass, Li: Lifting of lamellar epithelium,  
836 Ne: Necrosis, Oe: intercellular Oedema

837 **Figure 3.** A. Gill pathological index ( $I_{Gill}$ , mean  $\pm$  SE) and B. global score of histopathologic  
838 alterations (mean  $\pm$  SE) in *Carassius auratus* exposed during 96 h to pesticides at different  
839 concentrations (Control in white, Low Dose in grey, and High Dose in black) and at two temperatures  
840 (22°C on the left and 32°C on the right). A high index indicates a high degree of cellular damage.  
841 Data are mean  $\pm$  SEM. Different letters above bars indicate significant differences between groups

842 **Figure 4.** Routine behaviours, i.e. A. activity (time spent swimming) and B. feeding (number of pellets  
843 eaten) of goldfish exposed for 96h at 22°C (left) or 32°C (right) to control water (dark grey), a low  
844 dose of pesticides (medium grey), or a high dose of pesticide (light grey) Different letters above bars  
845 indicate significant differences between groups.

846 **Figure 5:** Summary of the main effects of temperature and pesticides at 22°C or 32°C on goldfish  
847 across levels of biological organization (0: no effect, -: negative effect, +: positive effect) . Taken  
848 together, results suggest that pesticides induced more severe molecular and cellular damage on DNA  
849 structure, gills and liver cells at 32°C compared to 22°C, while other traits were mainly affected by  
850 temperature only. This suggests that high temperature aggravates the deleterious non reversible  
851 effects of pesticides on cellular and organ structure in a multiple stressor context

852

853

Figure

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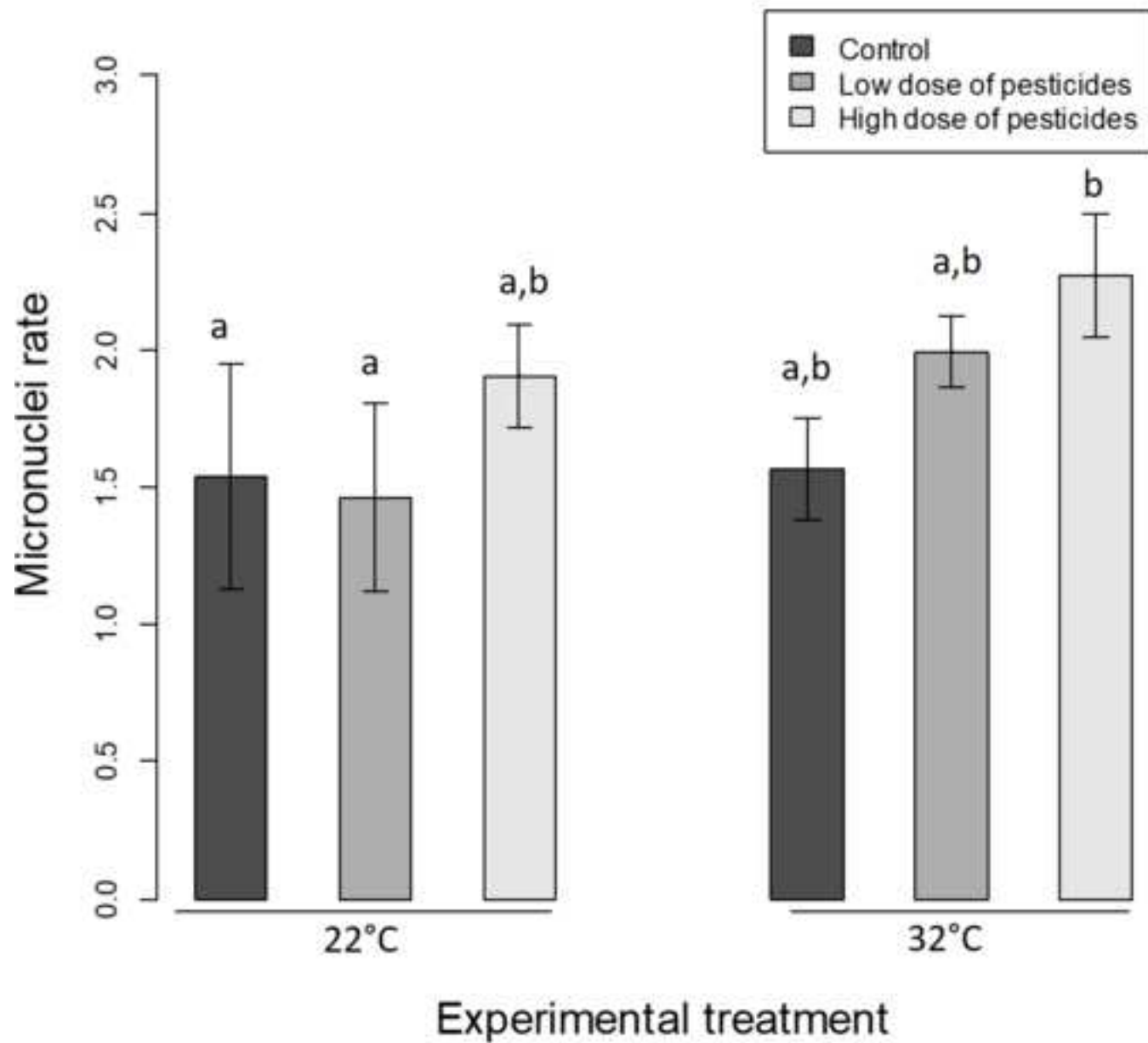


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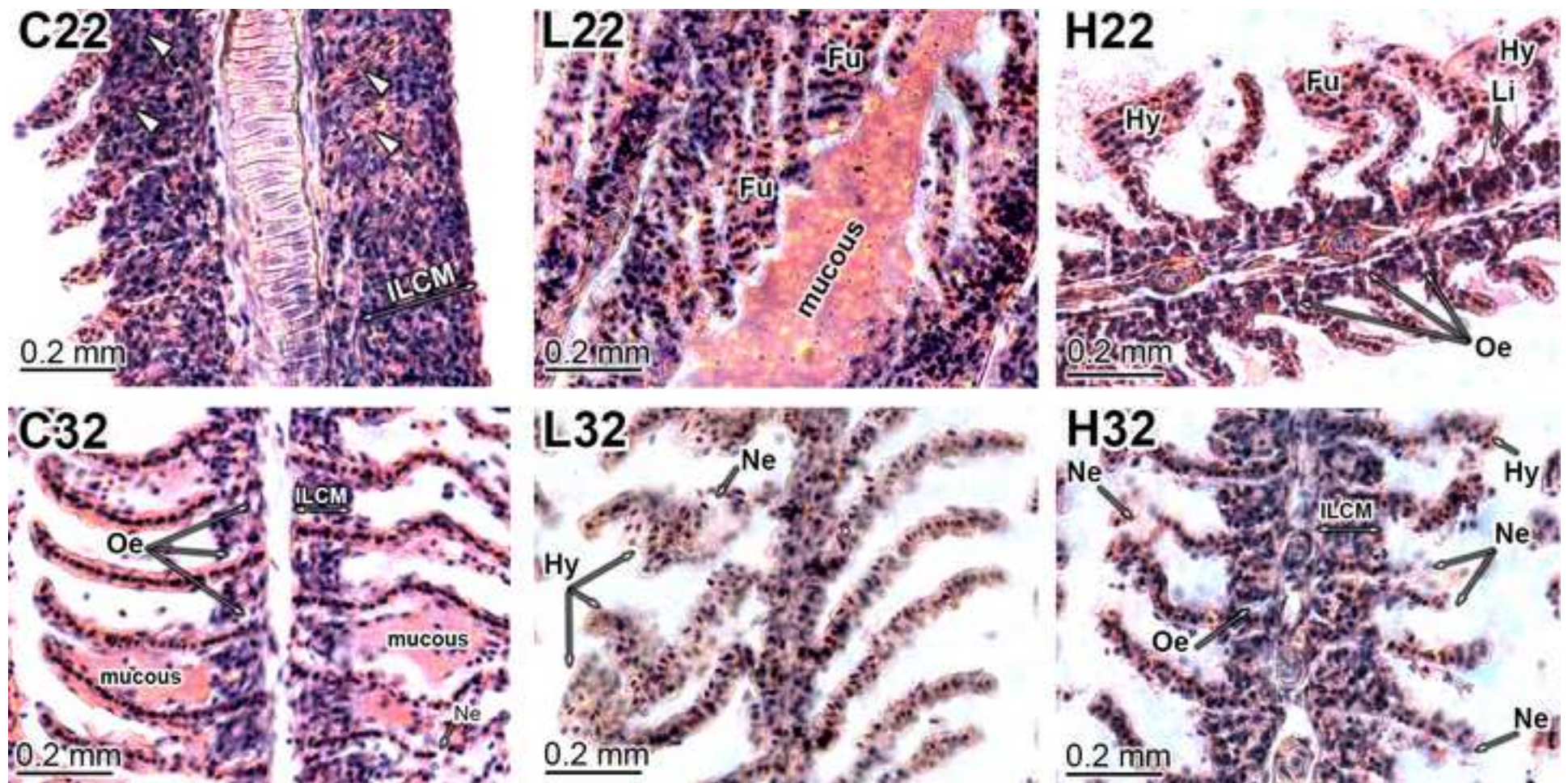


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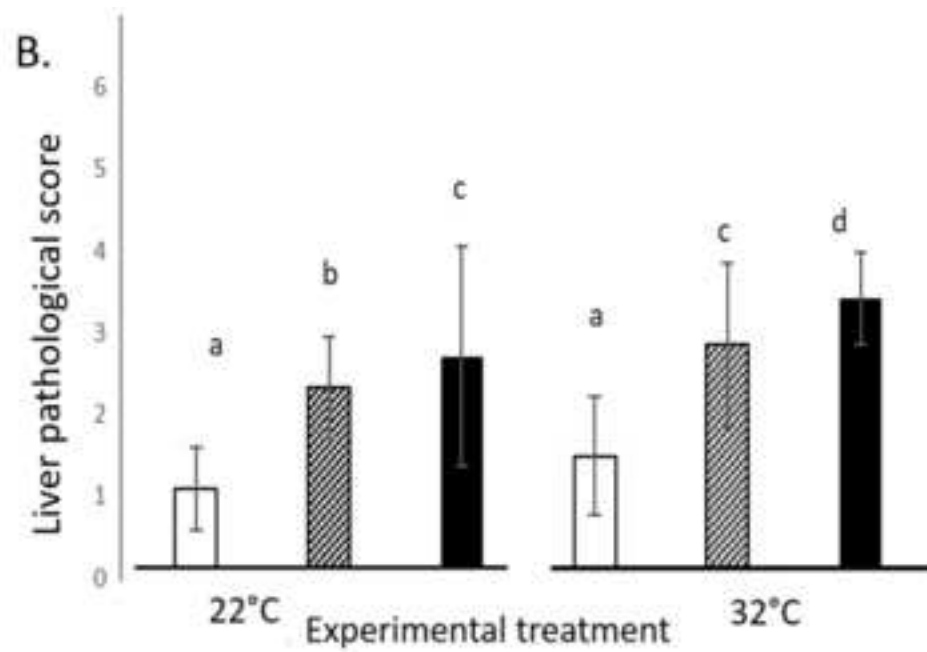
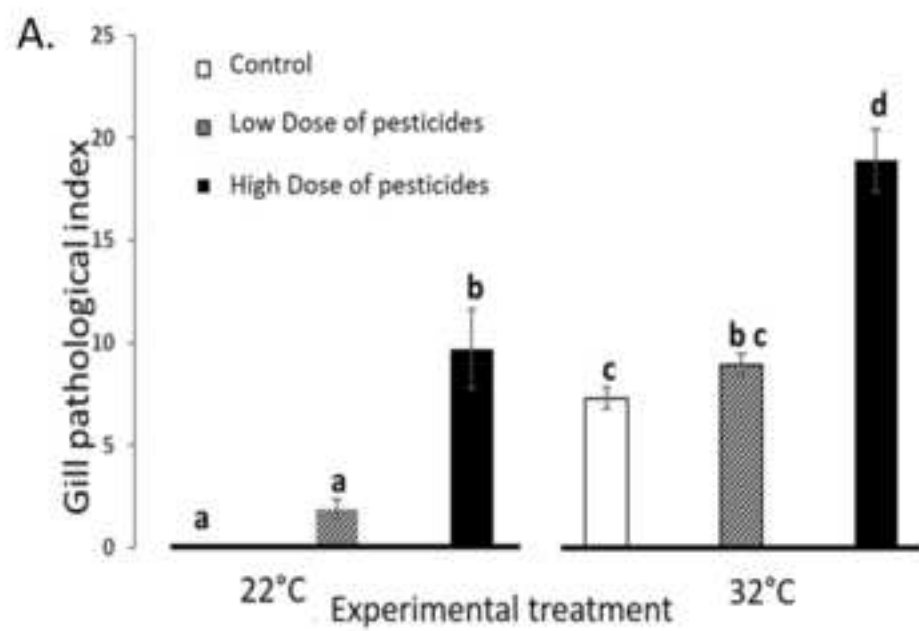
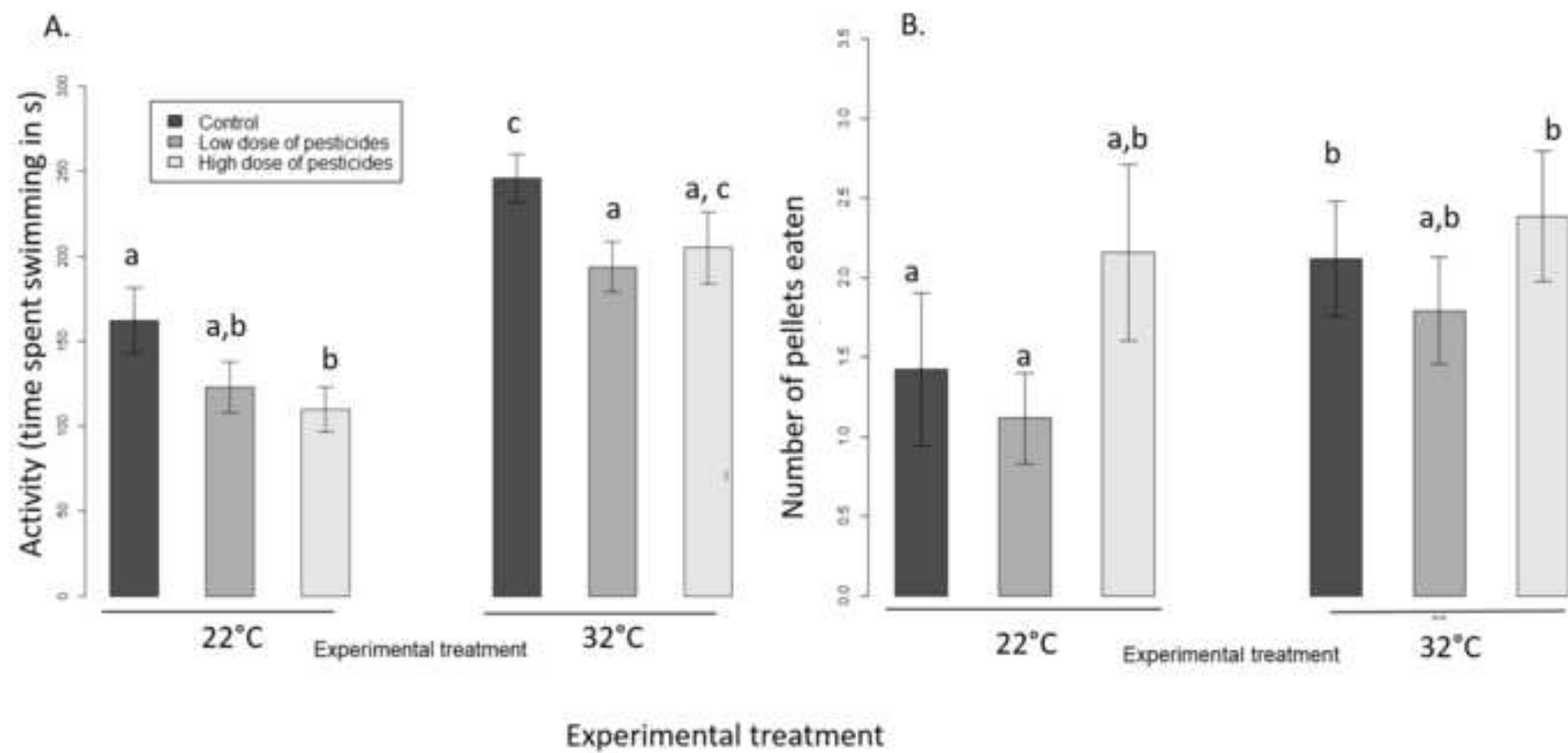
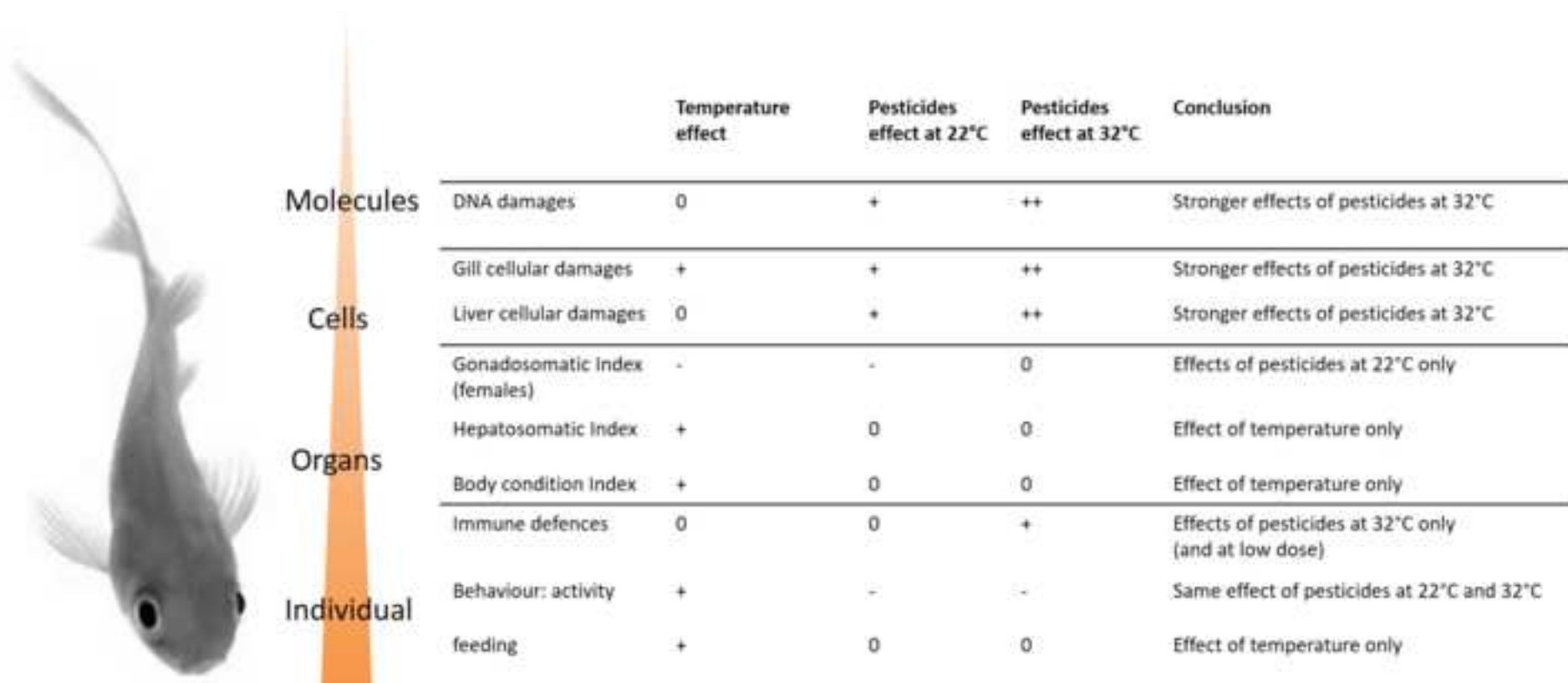




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*Gill histopathology*

<b>Type of gill alteration (alt)</b>	Oe: intercellular Oedema	Li: lifting of lamellar epithelium	Hy: lamellar Hyperplasia	Fu: partial lamellar Fusion	At: Atrophy of secondary lamellae	Ne: Necrosis,
<b>Score value</b>	1: 0 % to 16.66 %	2: 16.66 % to 33.33 %	3: 33.33 % to 50 %	4: 50 % to 83.33 %	5: 83.33 % to 100 %	6: 100 %
<b>(% of occurrence)</b>						
<b>Importance factor (severity)</b>	1 (reversible alteration)	1(reversible alteration)	2 (sometimes irreversible)	2 (sometimes irreversible)	3 (irreversible)	3 (irreversible)
<b>Gill histopathological index <math>I_{Gill}</math></b>	$I_{Gill} = \sum_{alt} (a_{alt} \times w_{alt})$ where: $a$ = score value for each alteration (from % of occurrence)					
	$w$ = importance factor for a specific alteration (w=1 for Oe and Li; w=2 for Hy and Fu; w=3 for At and Ne) alt = type of alteration					

<b>Type of liver alteration</b>	<b>Scores used to quantify the occurrence and severity of alterations</b>				
<b>Lysis-necrosis</b> (hepatocyte death)	0 : less than 10 % of dead hepatocytes	2.5 : 10 to 25 %	5 : 25 to 50 %	7.5 : 50 to 75 %	10 : more than 75 %
<b>Mitosis</b> (hepatocytes involved in mitosis)	0 : < 5 % in mitosis	2 : 5 to 10 %	4 : 10 to 20 %	6 : 20 to 40 %	10 : more than 40 %
<b>Melano-macrophage aggregates</b> (inflammation and immune cells on 0.25 cm <sup>2</sup> )	0: no immune cell	1: low density	2: middle density		3: high density
<b>Fibrosis</b> (collagen deposit on blood vessels and bile ducts)	0: only on big blood vessel	2.5 : some vessels and bile duct	5: most blood vessels	7.5: venules and small bile ducts	10: throughout all the parenchyma
<b>Nuclear alterations</b> (heterochromatin, abnormal stain, shape and/or size on 40 nuclei)	0: no altered nucleus	0,25: 10 altered nuclei	0,5: 20 altered nuclei	0,75: 30 altered nuclei	1: 40 altered nuclei
<b>Lipidosis</b> (size and the amount of lipid droplets in hepatocytes)	0 : no droplet	2.5 : few droplets	5: some little droplets	7.5 : 50% large cells with droplets	10: 100% with droplets and doubled size.
<b>Global histopathological score</b>	Mean score calculated from the value of all scores				

1 **Supplementary Table S1:** details of alterations and scores of gill and liver histopathological damages

2

3 **Supplementary Table S2:** Gill remodelling and histopathological alterations in *Carassius auratus* exposed during 96 h to pesticides at different  
4 concentrations (Control C, L Low Dose /4.8 µg.L<sup>-1</sup> and H High Dose /42 µg.L<sup>-1</sup>) and at two temperatures (22°C and 32°C).

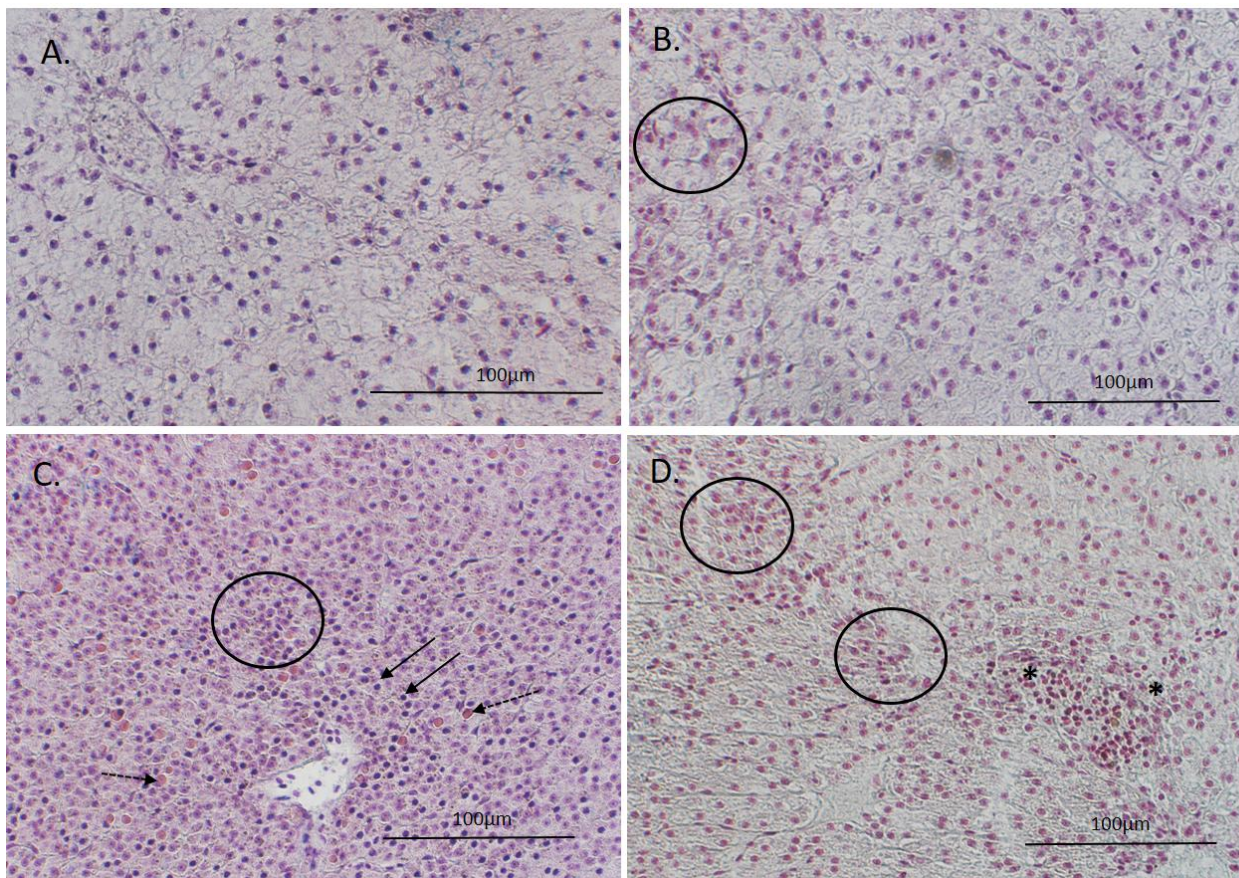
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Exposure conditions	Gill remodelling			Gill lesions (%)					
	SL (mm)	ILCM (mm)	ESA (%)	Importance Factor 1 (Circulatory disturbances)		Importance Factor 2 (Progressive changes)		Importance Factor 3 (Regressive changes)	
				Oe	Li	Hy	Fu	At	Ne
<b>C22</b>	0.28±0.04	0.26±0.03	5.0±11.5 <b>a</b>	0	0	0	0	0	0
<b>L22</b>	0.47±0.04	0.11±0.02	75.4±5.1 <b>b</b>	0	0	3.0±4.5	5.6±7.1	0	0
<b>H22</b>	0.29±0.08	0.05±0.01	82.0±5.3 <b>c</b>	33.0±21.0	0.25±0.52	34.2±20.3	4.3±2.8	0.6±0.8	0
<b>C32</b>	0.53±0.05	0.12±0.03	77.5±4.4 <b>b</b>	19.0±10.1	1.1±0.9	1.1±1.4	0	0	7.7±3.1
<b>L32</b>	0.42±0.08	0.03±0.01	93.3±2.7 <b>d</b>	0	0	16.1±2.7	0	0	19.8±2.1
<b>H32</b>	0.29±0.03	0.12±0.01	57.7±4.6 <b>e</b>	37.4±15.5	5.6±14.9	20.5±25.8	0.5±1.3	0	66.0±7.3

At: Atrophy of secondary lamellae, Fu: partial lamellar Fusion, Hy: lamellar Hyperplasia, ILCM:

15 length of interlamellar cell mass, Li: lifting of lamellar epithelium, Ne: Necrosis, Oe: intercellular Oedema, ESA: percentage of exchange surface area, SL:  
16 length of secondary lamellae. Data are mean ± SEM

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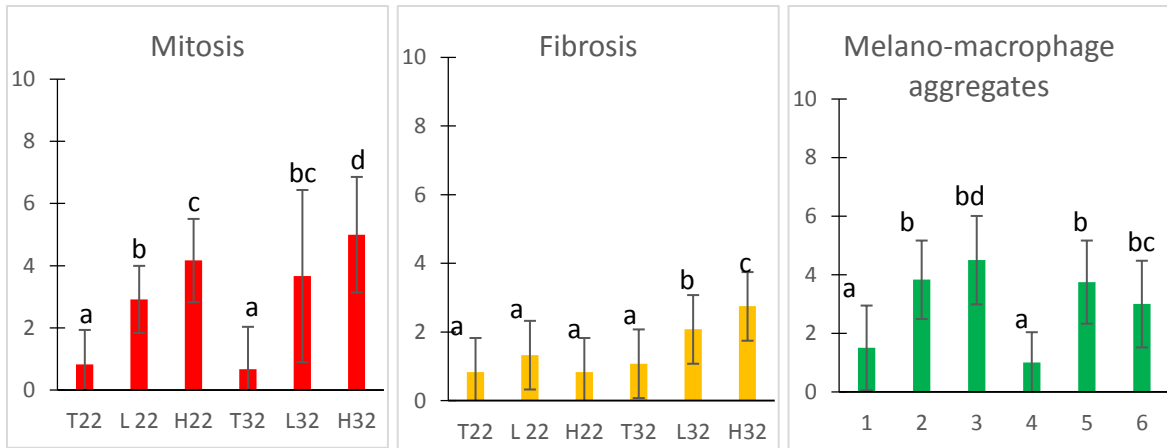


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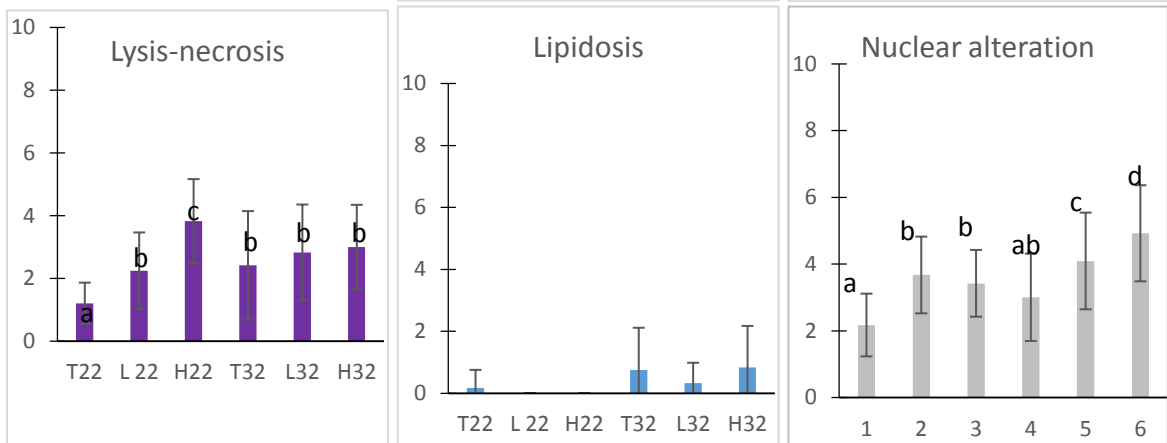
20 **Supplementary Figure S3.** A. Hepatic parenchyma of control fish (C22 group) showing no  
21 cellular alteration. B. Hepatic parenchyma of fish exposed to low dose of pesticides at 22°C  
22 (L22 group) showing limited alterations such as some hepatocytes involved in mitosis (circle).  
23 C. and D Hepatic parenchyma of fish exposed to high temperature (32°C) and to high  
24 pesticide concentrations (H32 group) showing important cellular alterations such as  
25 numerous hepatocytes involved in mitosis (circles), Melano-Macrophage Centers MMC  
26 formation indicating inflammation (\*), nuclear alterations, i.e. altered pycnotic nuclei (plain  
27 arrow) and lipidosis with intra-hepatocyte lipid droplets (dashed arrows). This resulted in  
28 high global histopathological scores (Fig 4C: score of 6, Fig 4D: score of 4) reflecting high  
29 levels of cellular alterations in fish exposed to pesticides at 32°C.

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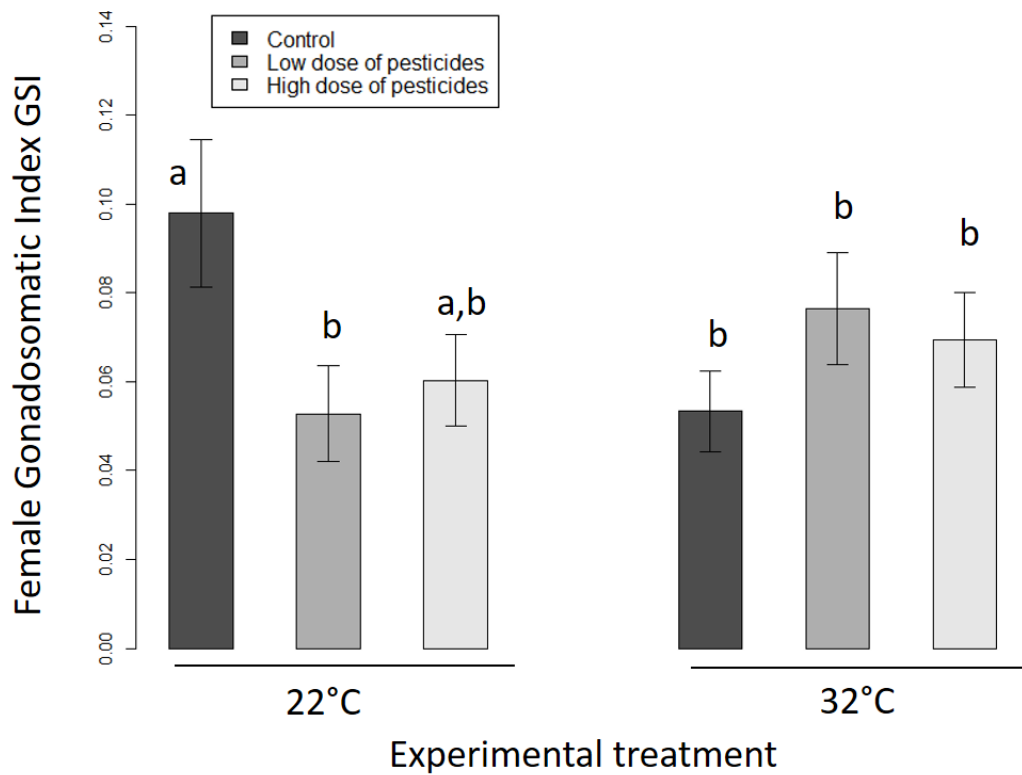
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34 **Supplementary Figure S4:** Details of histopathologic alterations (mean  $\pm$  SE) observed in the liver  
35 of goldfish exposed at low (L) or high concentration of pesticides (H) at a temperature of 22°C or  
36 32°C. C22: control 22°C; L22: low dose of pesticides at 22°C; H22: high dose of pesticides at 22°C;  
37 C32: control 32°C; L32: low dose of pesticides at 32°C; H32: high dose of pesticides at 32°C.  
38 Different letters above bars represent significant differences after Wilcoxon tests.

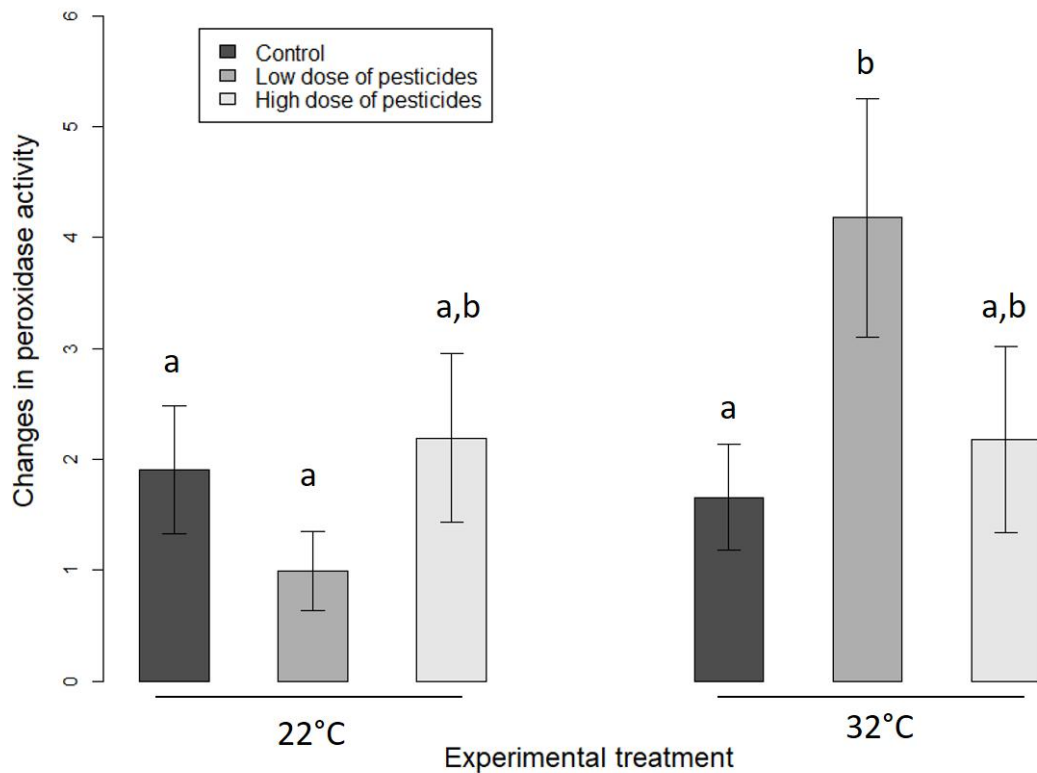
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42 **Figure S5.** Gonadosomatic index (GSI, representing the investment in reproduction) of female goldfish  
43 after a 96h exposure at 22°C (left) or 32°C (right) to control water (dark grey), a low dose of  
44 pesticides (medium grey), or a high dose of pesticide (light grey) Different letters above bars indicate  
45 significant differences between groups.

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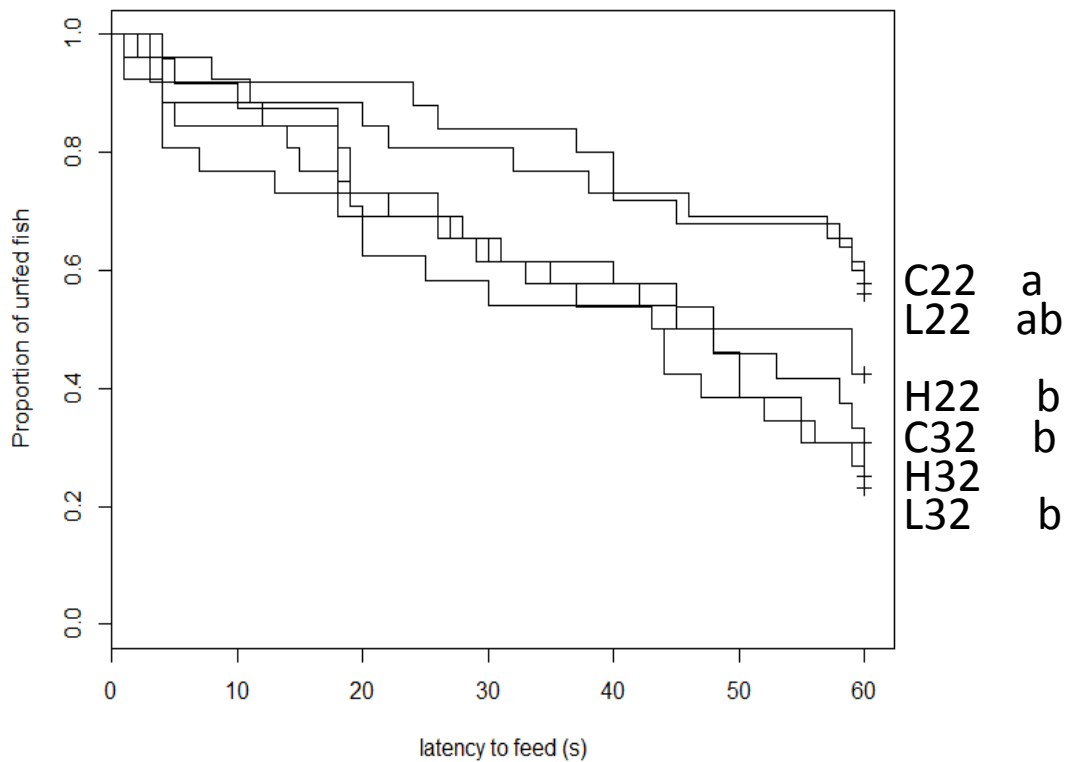
48 **Supplementary Figure S6.** *Changes in peroxidase activity in the plasma (proxy of innate*  
 49 *immune defence) of goldfish after a 96h exposure at 22°C (left) or 32°C (right) to control*  
 50 *water (dark grey), a low dose of pesticides (medium grey), or a high dose of pesticide (light*  
 51 *grey) Different letters above bars indicate significant differences between groups.*

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56 **Supplementary Figure S7.** *Proportion of unfed fish over time after a 96h exposure at 22°C*  
 57 *to control water (C22), a low dose of pesticides (L22), or a high dose of pesticide (H22), or at*  
 58 *32°C to control water (C32), a low dose of pesticides (L32) or a high dose of pesticides (H32)*  
 59 *Different letters indicate significant differences between groups after posthoc tests. Results*  
 60 *show that a higher proportion of fish exposed to high temperature only (C32) and to*  
 61 *pesticides (H22, L32 and H32) ate at least one pellet and fed more rapidly than control fish*  
 62 *C22.*

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