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### ► To cite this version:

Sabrina Tartu, Léa Lorrain-Soligon, Marion Cheron, Andréaz Dupoué, François Brischoux. Colouration matters in dull toads: ultraviolet adornment for ladies and agrochemicals fading effects. *Oecologia*, 2023, 203 (3-4), pp.491-502. 10.1007/s00442-023-05482-y . hal-04314052

**HAL Id: hal-04314052**

**<https://hal.science/hal-04314052v1>**

Submitted on 30 Nov 2023

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1 **Colouration matters in dull toads: Ultraviolet adornment for**  
2 **ladies and agrochemicals fading effects**

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18  
19 **AUTHORS CONTRIBUTION**

20 Co-first authors ST and LLS contributed equally to this manuscript, and each has the  
21 right to list themselves first in author order on their CVs. MC carried out fieldwork.  
22 ST and MC carried out the exposure experiment. AD performed the  
23 spectrophotometer measurements, and preliminary statistical analyses. ST and LLS  
24 analysed the data and wrote the first draft of the manuscript; all authors read the  
25 manuscript and provided critical reviews on previous versions. FB conceptualized the  
26 study, acquired the funding and supervised the global project.

28 **Abstract**

29 Integument colouration can influence many aspects of fitness, and is under strong  
30 sexual selection. Amphibians often express sexual dichromatism, and ultra-violet (UV)  
31 colouration is usually biased toward males as a sexual signal. As an honest signal,  
32 colouration is related to several individual traits, but can also be related to  
33 environmental factors such as anthropogenic pollutants, to which amphibians are  
34 highly sensitive. In this study, we investigated sexual dichromatism and UV  
35 reflectance covering a large visual spectrum (wavelength ranging from 300 to 700 nm)  
36 on different body areas (throat, ventral and dorsal areas), in a widespread amphibian  
37 species, the spiny toad (*Bufo spinosus*). Then, we tested the impact of chronic exposure  
38 to two widespread herbicides (glyphosate's primary metabolite [AMPA] and  
39 Nicosulfuron) on their colouration. We found a strong but unexpected sexual  
40 dichromatism with females reflecting more in the UV spectrum (throat and ventral  
41 area) than males, suggesting these body parts might be critical in intra-specific  
42 signalling. Females with higher ventral UV reflectance were in better body condition,  
43 suggesting an honest signal role of UV reflectance which could influence male choice.  
44 Throat colouration was further differentially influenced by agrochemicals according  
45 to sexes. In AMPA-exposed males, throat was more saturated in yellow-orange than  
46 in control males, and Nicosulfuron exposure decreased the throat's reflectance hue in  
47 females, which can bear consequences on mate attractiveness. Future studies need to  
48 investigate the underlying mechanisms that are altered by agrochemical exposure.

49

50 **Keywords:** Amphibians; Honest signal; AMPA; Nicosulfuron; Sexual dichromatism

## 51 INTRODUCTION

52 Integument colouration is a major interface between individuals and their  
53 environment, as it influences many aspects of their biology including anti-predator  
54 defences through crypsis (Caro 2005), thermoregulation (Stuart-Fox and Moussalli  
55 2009), regulation of physiological processes (Caro 2005), immunity (Lifshitz and St  
56 Clair 2016), parasitic exploitation (Côte et al. 2018), UV protection (Jablonski 1998;  
57 Clusella Trullas et al. 2007) and social and sexual signaling in both males and females  
58 (Bradbury and Vehrencamp 1998; Caro 2005; Delhey et al. 2007; Stuart-Fox et al. 2007;  
59 Martín and López 2009; Clutton-Brock 2009; Secondi et al. 2012; Olsson et al. 2013;  
60 Heath et al. 2013; Endler and Mappes 2017). Given the role of body colouration in such  
61 fitness-related functions, the mosaic of colours displayed by an animal can therefore  
62 convey information about individual quality and is considered to be under strong  
63 sexual selection (Hews and Moore 1995; Bradbury and Vehrencamp 1998; Ptacek 2000;  
64 Martín and López 2009; Svensson and Wong 2011; Sever and Staub 2011; Heath et al.  
65 2013; Blévin et al. 2014; Endler and Mappes 2017; Weaver et al. 2018).

66 From vibrant to substrate-matching colours, amphibians show a strong diversity of  
67 colourations and contrasting patterns (Rudh and Qvarnström 2013). Some amphibian  
68 species express sexual dichromatism (i.e. colour difference between the two sexes);  
69 (Lifshitz and St Clair 2016), with adult males being more brightly coloured than adult  
70 females (Bell and Zamudio 2012). Colour signals can facilitate gender recognition,  
71 influence courtship behaviour and mate-choice, even in nocturnal species (Gomez et  
72 al. 2009; Sztatecsny et al. 2010; Secondi et al. 2012). A chromatophore unit (i.e. pigment  
73 containing cells) of amphibians can combine properties of different chromatophores

74 to achieve rapid colour changes (Bagnara et al. 1968; Rudh and Qvarnström 2013).  
75 Across 178 anuran species, males were shown to use rapid colour change (dynamic  
76 dichromatism) to mediate socio-sexual interactions during the breeding season (Bell et  
77 al. 2017). Dynamic dichromatism often precedes explosive breeding, and could  
78 facilitate sexual recognition by helping males distinguish each other from females  
79 (Doucet and Mennill 2010; Sztatecsny et al. 2012; Stückler et al. 2022).

80 Although sexual dichromatism may not be the most prevalent secondary sexual trait  
81 in anurans (Duellman and Trueb 1986; Bell and Zamudio 2012; Bell et al. 2017), features  
82 non-visible to human eye, such as ultra-violet (UV) signalling, may be much more  
83 common than previously thought. Various taxa, including amphibians, have retinal  
84 photopigments that allow for UV vision (Jacobs 1992). Most bright integument colour  
85 patterns reflect light maximally in the UV portion of the spectrum, constituting UV  
86 colour signals (Burkhardt and Finger 1991; Fleishman et al. 1993; Deutschlander and  
87 Phillips 1995; Stoehr and McGraw 2001; Hunt et al. 2001; Pérez i de Lanuza and Font  
88 2007; Martin et al. 2013). This pattern is mainly expressed by males (Stoehr and  
89 McGraw 2001; Pérez i de Lanuza and Font 2007; Martin et al. 2013) and to a lesser  
90 extent by females (Martin et al. 2013). In ectothermic species, iridophores are  
91 chromatophores responsible for these bright reflecting colours, as they contain  
92 reflecting platelets (Rudh and Qvarnström 2013). As observed in reptiles (Pérez i de  
93 Lanuza and Font 2007) and caudates (Secondi et al. 2012), UV reflectance could  
94 increase sexual dichromatism and male conspicuousness in anurans. Additionally,  
95 skin colour is mostly influenced by pigments contained within specialized groups of  
96 cells in the upper epidermal layer of skin. Melanins are the most prevalent pigments,

97 producing many yellow–brownish (pheomelanin) and grey–black (eumelanin) colours  
98 (McGraw 2005). Melanogenesis is controlled genetically and vertebrates synthesize  
99 melanins from internal resources (Lin and Fisher 2007). By contrast to melanin,  
100 carotenoid pigments are acquired through the diet (Schiedt 1989). They produce many  
101 yellow, orange and red patches, which are central to ornamentation (Blount and  
102 McGraw 2008).

103

104 In amphibians, colouration has been related to several individual traits such as body  
105 size, body condition or hormone levels (Nilsson Sköld et al. 2013; Höbel et al. 2022;  
106 Barzaghi et al. 2022), and can also vary with environmental factors. For instance,  
107 temperature, habitat (e.g. site elevation, productivity, background colour) or food  
108 availability have been related to colour variations in amphibians (Norris and Lowe  
109 1964; Sztatecsny et al. 2010; Mack and Beaty 2021; Barzaghi et al. 2022; Mirč et al. 2023).  
110 Since colouration is thought to be an adaptive trait (Rudh and Qvarnström 2013),  
111 exogenous factors leading to colour modification could affect individual fitness.

112

113 The ubiquity and plasticity of integument colouration make it a powerful indicator of  
114 the competing costs of environmental stressors, such as anthropogenic pollutants  
115 (Lifshitz and St Clair 2016), among which pesticides are dominant in agricultural  
116 habitats where several amphibians persist (McConnell et al. 1998; LeNoir et al. 1999;  
117 Rashid et al. 2010; Guillot et al. 2016). Indeed, pesticides primarily cause substantial  
118 damages to organisms by producing free radicals that overwhelm the antioxidant

119 system (Galván and Alonso-Alvarez 2009; Cheron et al. 2022), and the major groups of  
120 animal pigments exhibit antioxidant activity (McGraw 2005) suggesting a trade-off  
121 between the expression of colouration and resistance to pollutants (Arellano-Aguilar  
122 and Macías Garcia 2008). Pollutants have been related to enhanced traits coloured by  
123 black melanin (Lifshitz and St Clair 2016; Goiran et al. 2017), reduced carotenoid  
124 colouration (Baatrup and Junge 2001; Alonso-Alvarez and Galván 2011; Shenoy 2012),  
125 as well as modified hue and saturation of individuals (Larramendy 2017; Ujhegyi and  
126 Bókony 2020). These impairments could result from negative effects of pollutants on  
127 gonad function (McCoy et al. 2008; Hayes et al. 2010), leading to sex steroid disruption  
128 (Trudeau et al. 2020). Testosterone, for instance, can affect chromatophore expression  
129 and xanthophores dispersion in amphibian (Richards 1982; Tang et al. 2014). Despite  
130 their potential higher susceptibility to contaminants, due to a lack of protective  
131 epidermal structures and permeable skin (Wells 2007), only a handful of studies have  
132 been conducted on the effects of pollutants on colouration in amphibians (Larramendy  
133 2017; Ujhegyi and Bókony 2020). Agrochemicals are mostly sprayed in spring, when  
134 many amphibians' species from temperate areas reproduce (Wells 2007; Berger et al.  
135 2013; Lenhardt et al. 2015), and animals may be exposed to these compounds through  
136 inhalation, skin permeability, and consumption of contaminated food as well as water.

137

138 In this study, we investigated skin colour variations and UV signalling according to  
139 sex and considering different body parts (throat, ventral and dorsal areas) in a  
140 widespread amphibian species, the spiny toad (*Bufo spinosus*), characterized by an  
141 overall dull colouration as compared to other brightly coloured anurans (Rudh and

142 Qvarnström 2013). We also tested the effects of exposure to two heavily used  
143 herbicides (glyphosate's primary metabolite, aminomethylphosphonic acid [AMPA]  
144 and Nicosulfuron) to which free-ranging amphibians are exposed in agricultural areas  
145 (Brühl et al. 2013; Berger et al. 2013; Lenhardt et al. 2015; Adams et al. 2021). To do so,  
146 we captured adults of both sexes in Western France out of the breeding period and  
147 examined their throat, ventral, and dorsal colouration based on spectrophotometric  
148 measures. In amphibians, ventral and throat colours are lighter than the dorsal area  
149 and show clear oxyhaemoglobin absorption peaks (Norris and Lowe 1964). This  
150 species expresses a strong sexual dimorphism (Speybroeck et al. 2018), and sexual  
151 dichromatism has been described in a closely related species, *Bufo bufo*, with males  
152 being yellower-greener (less red) and brighter than females (Ujhegyi and Bókony  
153 2020). We thus expect that colouration will vary with sex in *Bufo spinosus*, and will be  
154 related to individual traits as an honest signal of quality, as demonstrated in males  
155 (Martín and López 2009; Heath et al. 2013). In addition, given the effects of  
156 agrochemicals on gonad function and hormone concentrations (McCoy et al. 2008;  
157 Trudeau et al. 2020), we expect that contaminant exposure may as well affect  
158 individual's colouration, increasing black melanin pigmentation (Lifshitz and St Clair  
159 2016; Goiran et al. 2017), reducing carotenoid colouration (Baatrup and Junge 2001;  
160 Alonso-Alvarez and Galván 2011; Shenoy 2012), and modifying hue and saturation  
161 (Larramendy 2017; Ujhegyi and Bókony 2020).

162



## 163 MATERIAL AND METHODS

### 164 *Study species*

165 The spiny toad is the largest toad species originating from western Europe  
166 (Speybroeck et al. 2018). The species reproduces in ponds during late winter, but is  
167 found migrating both for aestivation and hibernation, respectively in spring and  
168 autumn. Males and females are sexually dimorphic, females being larger than males,  
169 and males presenting nuptial pads on their fingers, allowing them to clasp females  
170 during reproduction (Speybroeck et al. 2018).

171

### 172 *Animal care and housing*

173 Between 21-Sep-2020 and 27-Oct-2020, 81 free-ranging spiny toads (32 males and 49  
174 females) were captured by hand on the roadside to the south of the Deux-Sèvres  
175 department (western France) and brought back to the laboratory. Toads were housed  
176 in a thermally controlled (17°C) room under natural photoperiod (12:12 h cycle), in  
177 individual plastic boxes (80×40×16 cm) lined with paper towels, with a shelter (halved  
178 PVC tubes) and a petri dish for water. Twice a week, in quantities adapted to their size,  
179 toads were fed crickets, meal worms and earth worms dusted with a mixture of CaCO<sub>3</sub>  
180 and multivitamin powder (Repti Calcium, Zoo Med Laboratories, Inc). Once a week,  
181 boxes were cleaned and paper towels were replaced.

### 182 *Agrochemical exposure*

183 AMPA (aminomethylphosphonic acid) is the primary metabolite of glyphosate, and is  
184 one of the main contaminants detected in surface waters worldwide (Grandcoin et al.

2017). New herbicides molecules are also regularly developed, such as Sulfonylurea  
Herbicides (e.g. Sulfosulfuron, Rimsulfuron, and Nicosulfuron) which are persistent  
in aquatic environments such as ponds (Cessna et al. 2015). We exposed toads either  
to AMPA or one Sulfonylurea herbicide (Nicosulfuron) and started a chronic  
exposition at environmentally relevant concentrations from 15-Apr-2021 to 01-Jul-  
2021. Individuals were exposed to one of the three treatments: Control (11 males, 20  
females), AMPA (10 males, 17 females), Nicosulfuron (11 males, 12 females). The  
individuals in the three experimental groups did not differ in size, either considering  
males (linear model: Sum Sq=12.352, F-value=0.745, p-value=0.484, Control males:  
58.65 mm  $\pm$  2.08 SE, AMPA males: 59.93 mm  $\pm$  3.87 SE, Nicosulfuron males: 59.98 mm  
 $\pm$  2.49 SE) or females (linear model: Sum Sq=231.43, F-value=1.703, p-value=0.193,  
Control females: 72.81 mm  $\pm$  8.33 SE, AMPA females: 73.81 mm  $\pm$  8.66 SE, Nicosulfuron  
females: 78.22 mm  $\pm$  7.43 SE). AMPA was administered by dissolution in drinking  
water (dechlorinated tap water) at 4  $\mu\text{g}\cdot\text{L}^{-1}$  (crystalline powders, 99% purity, ACROS  
ORGANICS<sup>TM</sup>), and Nicosulfuron was administered by dissolution in drinking water  
(dechlorinated tap water) at 1  $\mu\text{g}\cdot\text{L}^{-1}$  (crystalline powders, 99% purity, ACROS  
ORGANICS<sup>TM</sup>). These concentrations correspond to the concentrations of these  
pollutants found in agricultural environments (Tartu et al. 2022, and data from Agence  
de l'Eau Loire-Bretagne). Concentrations measured in 4 samples of drinking water per  
treatment validated that the actual concentrations were close to the nominal  
concentrations (Qualyse lab, La Rochelle, France). Toads were exposed to their  
treatment through drinking water (petri dish) twice a week.

### 207 *Colouration and morphometrics*

208 On 01-Jul-2021, after a two-and-a-half-month exposure, we obtained reflectance  
209 spectra from the throat, the ventral, and the dorsal area of each individual (two  
210 replicates; reflectance spectrums are given in Appendix A). These measurements were  
211 done using a USB-2000 spectrophotometer (Badiane et al. 2020; Kawamoto et al. 2021).  
212 The probe was hand-held over each area, approximately perpendicular to the patch  
213 surface. We then processed spectral data in R v.4.0.5 (R Core Team 2019) using the  
214 package pavo (Maia et al. 2013). We cropped each spectrum between 300–700 nm,  
215 smoothed them using a loess smooth span of 0.2, and averaged the two replicates  
216 recorded for each body region. For each body part we extracted hue, UV-saturation,  
217 UV-luminance, total brightness (B1 300-700). We then calculated yellow-orange  
218 saturation (B1 575-700 / B1 300-700), violet-blue saturation (B1 400-515 / B1 300-700)  
219 and green saturation (B1 495–570/ B1 300-700) (Badiane et al. 2020). All individuals  
220 were weighed (electronic balance:  $\pm 0.1$  g) and their snout-vent length (SVL) was  
221 measured with an electronic calliper ( $\pm 0.01$  mm) on the same day. We then calculated  
222 a body condition index using residual scores from the linear regression between  
223  $\log(\text{body size})$  and  $\log(\text{body mass})$ . At the end of the experiment, individuals were all  
224 released at their site of capture.

### 225 *Statistical analyses*

226 First, we ran a principal component analysis (PCA) on the different fractions of  
227 reflectance with the ade4 package (Dray and Dufour 2007). We generated principal  
228 components (PCs) for further analyses from the first and second axis of the PCA  
229 (projected inertia > 70%) that we used as a global index of colouration. Correlations  
230 between these PCs and the different fractions of reflectance included in the PCA are

231 presented in Appendix B. Second, we used linear models (LMs) to test 1) the effects of  
232 sex and 2) the effects of body condition on reflectance (PC 1 and PC 2 for each body  
233 part). Sex, body condition and their interaction were defined as explanatory variables  
234 and reflectance PC1 and PC2 for each body part as response variables. We tested these  
235 relationships in control individuals only, to test for this effect without the effect of  
236 contaminant exposure. Third, we tested the effects of the treatment (Control, AMPA,  
237 or Nicosulfuron) on reflectance (PC 1 and PC 2 for each body part) in each sex  
238 separately, as PC1 and PC2 values were highly different between males and females  
239 (LM models: all p-values<0.029, except for the comparisons in PCA 2 throat coloration  
240 between sexes [p-value=0.159], see also **Fig. 1**). Finally, we tested the effects of body  
241 condition, sex and their interaction on reflectance in AMPA and Nicosulfuron exposed  
242 individuals only. These variables were selected by backward stepwise selection, and  
243 only the last retained variables are presented in the final models. All analyses were  
244 performed with R v.4.0.5 (R Core Team 2019).

245

246 **RESULTS**

247 *Reflectance sexual dimorphism in control individuals*

248 In control individuals, we observed a significant sexual dichromatism in reflectance of  
249 different body parts (**Fig. 1, Table 1**). Females reflected in the UV spectrum whereas  
250 males rather reflected in the yellow-orange and green spectra (**Fig. 1**). Ventral PC1  
251 varied according to sex (being higher in males than females,  $0.950 \pm 0.301$ ,  $p=0.004$ ,  
252 values are LM estimates  $\pm$  standard error), body condition ( $-3.639 \pm 1.270$ ,  $p=0.008$ ),  
253 and their interaction ( $6.237 \pm 2.729$ ,  $p=0.030$ ). Indeed, body condition was negatively  
254 related with ventral PC1 in females ( $-3.64 \pm 1.27$ ,  $p=0.008$ , values are Linear Models  
255 estimates  $\pm$  standard error, **Fig. 2**), but not in males ( $1.53 \pm 1.46$ ,  $p=0.302$ , **Fig. 2**), and  
256 body condition did not correlate with the reflectance of other body parts ( $p>0.157$  for  
257 all tests).

258

259 *Effect of agrochemical exposure on reflectance and body condition*

260 Nicosulfuron treatment decreased the hue of throat reflectance in females, whereas  
261 AMPA increased yellow-orange saturation of the throat in males (**Table 2, Fig. 3A**). In  
262 females, AMPA had a slight effect on ventral and dorsal reflectance, with a marginally  
263 higher ventral hue and increased yellow-orange dorsal saturation (**Table 2, Fig. 3A**).  
264 When testing the effects of body condition on reflectance in the exposed groups  
265 (AMPA and Nicosulfuron exposed individuals), we observed no relationship between  
266 body condition and reflectance for any body part, only the effect of sex is found  
267 significant (Appendix C).

268

269 **DISCUSSION**

270 In this study, we highlighted a strong sexual dichromatism in spiny toads.  
271 Unexpectedly, the throat and ventral areas of females reflected more in the UV  
272 spectrum than those of males, suggesting these body parts might be critical in intra-  
273 specific signalling. In contrast, the throats and ventral areas of males were rather  
274 yellow-orange, with low UV reflectance. The dorsal area spectrum of females was  
275 dominated by yellow-orange, whereas that of males by green. Importantly, females  
276 with higher UV reflectance (ventral area only) were in better body condition.  
277 Moreover, throat colouration of both females and males was influenced by  
278 agrochemicals. The throats of AMPA-exposed males were more saturated in yellow-  
279 orange than that of control males, and Nicosulfuron exposure decreased the  
280 reflectance hue of the throat in females. We also observed a marginal decrease in the  
281 hue of the ventral area of AMPA-exposed females and a marginal increase of the  
282 yellow-orange colour of their back.

283

284 Although sexual dichromatism is not the most prevalent secondary sexual trait in  
285 amphibians (Bell and Zamudio 2012; Bell et al. 2017), adult males of several species are  
286 more brightly coloured than adult females (Bell and Zamudio 2012), this being  
287 considered as a visual signal facilitating gender recognition, enhanced by striking  
288 postures that highlight specific body parts (Sztatecsny et al. 2010). In *Bufo bufo* for  
289 instance, males are yellower-greener and brighter than females (Ujhegyi and Bókonyi  
290 2020). Sexual dichromatism with colouration conspicuousness biased toward males is  
291 common among most vertebrates (Lifshitz and St Clair 2016), but some species of

292 anurans display female-biased colouration conspicuousness, in which females  
293 undergo colour transformation, often resulting in more ornate colourations (Portik et  
294 al. 2019). To our knowledge, our study is the first to report a female-biased UV  
295 reflectance in amphibians, where females expressed throat and ventral reflection in the  
296 UV spectrum, while males did not.

297 UV reflectance has already been shown in males of many species. Diverse taxa possess  
298 retinal photopigments that allow UV vision (Jacobs 1992), and UV sensitivity has been  
299 suggested to be useful in making crucial visual discriminations, for example allowing  
300 to highlight targets against their background and thus aid in their detection (Jacobs  
301 1992). As such, UV colouration in males has been shown to be used in mate choice in  
302 birds (Prum 2006), but also in lizards (Pérez i de Lanuza and Font 2007; Bajer et al.  
303 2010) and amphibians (Secondi et al. 2012), the UV reflection allowing to increase  
304 sexual dichromatism and male conspicuousness (Pérez i de Lanuza and Font 2007).  
305 Here, we highlighted intense UV reflection in female spiny toads, suggesting either  
306 that mate choice can also be performed by males, as it has been found in many species  
307 (Clutton-Brock 2009), or that it allows males to detect the presence of females once they  
308 arrive in the breeding site. As explosive breeders, spiny toad males gather in very large  
309 numbers in the breeding ponds days to weeks before the arrival of females, and often  
310 place themselves either at the bottom of the pond or close to the shore below the water  
311 surface. In both cases they would spot the females from below. The strong ventral and  
312 throat UV reflectance of females could allow them to be easily discriminated from the  
313 background and allow males to visually distinguish them from other males. These two  
314 hypotheses need to be tested.

315 The mate choice hypotheses can be strengthened by the fact that colouration is often  
316 an honest signal of individuals' quality and ability to reproduce (Baatrup and Junge  
317 2001; Clutton-Brock 2009; Shenoy 2012; Blévin et al. 2014). In birds, UV colouration is  
318 already known to reflect individual condition and quality (Prum 2006), and to be  
319 related to age, sex, and morphology in lizards (Martin et al. 2013). In our study, we  
320 show that females with higher UV reflectance were in better body condition, which is  
321 consistent with an honest signal of quality in female spiny toads. The relationship  
322 between colouration and quality is common in various species, as only individuals in  
323 good condition can afford to allocate pigments for trait colouration without  
324 compromising other functions (Hamilton and Zuk 1982). UV colouration might be  
325 costly to produce (Senar 2006), and its production could be traded off with many other  
326 functions. Colouration can be influenced by immunocompetence and parasite load  
327 (Molnár et al. 2012; Olsson et al. 2013), testosterone levels (Cox et al. 2005, 2008), and  
328 oxidative stress (Simons et al. 2012), which could all be influenced by exposure to  
329 agrochemicals (Mann et al. 2009; Brandt et al. 2016; Cheron et al. 2022). Our results  
330 thus suggest that UV colour could be an important female-condition signal for males  
331 in amphibians. To understand the underlying mechanisms, we should further  
332 investigate if UV colouration in this species is linked to immunity or hormone levels  
333 (such as oestrogen), and additionally to fecundity or investment in reproduction, as it  
334 has been shown in lizards (Kopena et al. 2020).

335 We were here able to show that spiny toads are sexually dichromatic, and this  
336 dichromatism can be affected by agrochemicals. Numerous agrochemicals have  
337 enzyme- and endocrine-disrupting capabilities (Colborn et al. 1993; Khan and Law



2005; Lifshitz and St Clair 2016), which could lead to alterations of gonadal form and function (McCoy et al. 2008). As colouration is linked to hormone expression (Richards 1982; Rand 1992; Tang et al. 2014) and reproductive abilities (Clutton-Brock 2009; Kopena et al. 2020), it is also likely to be disrupted by agrochemicals. Pigments involved in trait colouration, such as melanin, may be physiologically costly to produce (Jawor and Breitwisch 2003), and could be traded off with the costs of agrochemical exposure (e.g. reduced growth and increased susceptibility to disease (Baker et al. 2013). Additionally, agrochemicals, including AMPA, can cause damage to organisms by producing free radicals that overwhelm the antioxidant system (Galván and Alonso-Alvarez 2009; Cheron et al. 2022). Thus, pigments may rather be allocated to immune- or antioxidant-system (detoxification mechanisms, (McGraw 2005)), rather than to ornamentation in exposed individuals (Faivre et al. 2003; Arellano-Aguilar and Macías Garcia 2008; Lifshitz and St Clair 2016). Exposure to agrochemicals and other pollutants results in reduced carotenoid colouration (Baatrup and Junge 2001; Alonso-Alvarez and Galván 2011; Shenoy 2012; Lifshitz and St Clair 2016), probably because of the allocation of dietary carotenoids to counteract oxidative stress (Arellano-Aguilar and Macías Garcia 2008), and also to a decrease in the expression of brown melanin and an increase in black melanin (Lifshitz and St Clair 2016).

357

Interestingly, we show that, in AMPA-exposed males, throat yellow-orange colouration was more vivid than that of control males, probably indicating higher levels of carotenoids (Blount and McGraw 2008). This was unexpected given previous

361 evidence that agrochemicals lead to reduced carotenoid colouration (Baatrup and  
362 Junge 2001; Alonso-Alvarez and Galván 2011; Shenoy 2012; Lifshitz and St Clair 2016).  
363 This effect either suggests that 1) AMPA concentrations used in our study were too  
364 low to induce oxidative damages in adults, or 2) males are not able to mobilize  
365 carotenoids to counteract oxidative stress (because carotenoids may have low  
366 antioxidant effects as shown in other species [Costantini and Møller, 2008]). These two  
367 hypotheses need to be untangled by evaluating oxidative stress concomitantly with  
368 colouration. One additional hypothesis could be that the observed pattern results from  
369 endocrine disruption. *In vitro* and *in vivo* studies have shown that glyphosate-based  
370 herbicides and AMPA exhibit estrogen-like properties in various taxa (Uren Webster  
371 et al. 2014; Jarrell et al. 2020; Milesi et al. 2021). In zebra fish (*Danio rerio*) for instance,  
372 exposure to glyphosate-based herbicide increases the expression of ovarian aromatase,  
373 an enzyme which catalyses the conversion of testosterone to estradiol in the gonads of  
374 females (Uren Webster et al. 2014). Estradiol exposition in male African clawed frogs  
375 *Xenopus laevis* led to sub-cellular events indicative of disrupted testicular development  
376 and decreased testosterone concentrations (Hecker et al. 2005). Consequently, AMPA  
377 exposure could decrease testosterone concentrations in male toads through estrogenic  
378 effects. Because a trade-off exists between testosterone and immunity (Peters 2007;  
379 Schroderus et al. 2010), if AMPA is related to lower testosterone concentrations,  
380 polluted males could afford mobilizing enough carotenoids to counteract oxidative  
381 stress and in parallel display a more yellow-orange throat colour. Estradiol, in addition  
382 to testosterone levels, need to be further assessed in exposed individuals to test for this  
383 last hypothesis and understand these variations.

384

385 AMPA and Nicosulfuron were also shown to respectively increase ventral and  
386 decrease throat hue in females. Hue represents the colour shade and is produced by a  
387 combination of dermal chromatophores, epidermal melanocytes, and epidermal  
388 diffraction gratings (McNamara et al. 2016). In toads, throat hue positively correlates  
389 with age, body mass and body size, and thus represents an honest signal of condition  
390 and age (Zamora-Camacho and Comas 2019). As all experimental groups were  
391 balanced in size, this suggests that exposed females have lost this honest signal.  
392 Additionally, older toads are darker (Zamora-Camacho and Comas 2019). In this  
393 study, we did not evaluate individual's age, which could have been done via  
394 skeletochronology. Since age correlates with size in amphibians (Shine 1979), even  
395 with some variance (Olsson and Shine 1996; Moreno-Rueda et al. 2021), we  
396 hypothesized that age-class might be similar between treatments. This suggests  
397 Nicosulfuron, and to a lesser extent AMPA, exposure could mimic or accelerate  
398 integument aging in exposed females, probably linked to telomere attrition, which  
399 might be a cost for maintaining colouration (Giraudeau et al. 2016). Telomere length  
400 should be analysed to test this hypothesis. Lastly, in another toad species, hue has been  
401 shown to decrease in intersex individuals (individuals with mixed-sex gonads or  
402 gonadal abnormalities; Ujhegyi and Bókony 2020), which effect is often found in  
403 response to herbicide (Howe et al. 2004; Lanctôt et al. 2014; Ujhegyi and Bókony 2020).  
404 This could indicate that Nicosulfuron has caused gonadal abnormalities in females,  
405 which effect might also be found with the exposition to AMPA, leading to a  
406 masculinisation of their dorsal reflectance (more saturated in yellow-orange in

407 comparison to control females). Interestingly, the effect of body condition on UV  
408 reflectance was only found in control females and disappeared when considering  
409 exposed females. This suggests that in female spiny toads, agrochemicals could fade  
410 the honest signal carried by UV reflectance. Our results highlight sex-dependent  
411 responses of spiny toads to environmentally-relevant agrochemical exposure. By  
412 influencing mate attractiveness, these sex-dependent effects are likely to lead to  
413 reduced reproductive abilities and breeding success (Shenoy 2012; Ujhegyi and  
414 Bókony 2020; Yang et al. 2021).

415

#### 416 *Conclusion*

417 We here provide the first evidence of UV reflectance in female spiny toads as a  
418 significant honest signal of quality for their mates. To go further, we would need to  
419 investigate whether male choice toward females does depend on UV colouration, and  
420 if this feature correlates with increased fecundity or investment in reproduction.  
421 Additionally, we highlighted that AMPA and Nicosulfuron exposure affected throat  
422 colouration in males and females, but in a sex-dependent manner, which can bear  
423 consequences on mate attractiveness, and thus individuals' fitness in agricultural  
424 landscapes. Further investigations need to be conducted to better understand the  
425 underlying mechanisms disrupted by agrochemical exposure.

426

427 **ACKNOWLEDGEMENTS**

428 The authors thank JF Le Galliard for providing USB-2000 spectrophotometer. Funding  
429 was provided by the CNRS, the Agence de l'Eau Loire-Bretagne, the Agence de l'Eau  
430 Adour-Garonne, the Région Nouvelle-Aquitaine (Aquastress 2018-1R20214,  
431 Amphitox 2019-1R20216), the ANSES (BiodiTox project # 2019/1/031), The Plan  
432 d'Action National ECOPHYTO (n°OFB-21-0941), and the Conseil Départemental des  
433 Deux-Sèvres.

434

435 **ETHICS STATEMENT**

436 All applicable institutional and/or national guidelines for the care and use of animals  
437 were followed. This work was approved by the French authorities (COMETHEA ethic  
438 committee and Ministère de L'Enseignement Supérieur, de la Recherche et de  
439 L'innovation) under permits APAFIS#29265-2021012014416948 and  
440 DREAL/2020D/8041.

441

442 **DATA AVAILABILITY STATEMENT**

443 The data that supports the findings of this study will be deposit on a public repository  
444 upon acceptance, for now they are available in the supplementary material of this  
445 article.

446 **CONFLICT OF INTEREST STATEMENT**

447 The authors declare that they have no conflict of interest.

448

449 **CONSENT TO PARTICIPATE**

450 Not applicable

451

452 **CONSENT FOR PUBLICATION**

453 Not applicable

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795

## 796 **Caption to figures**

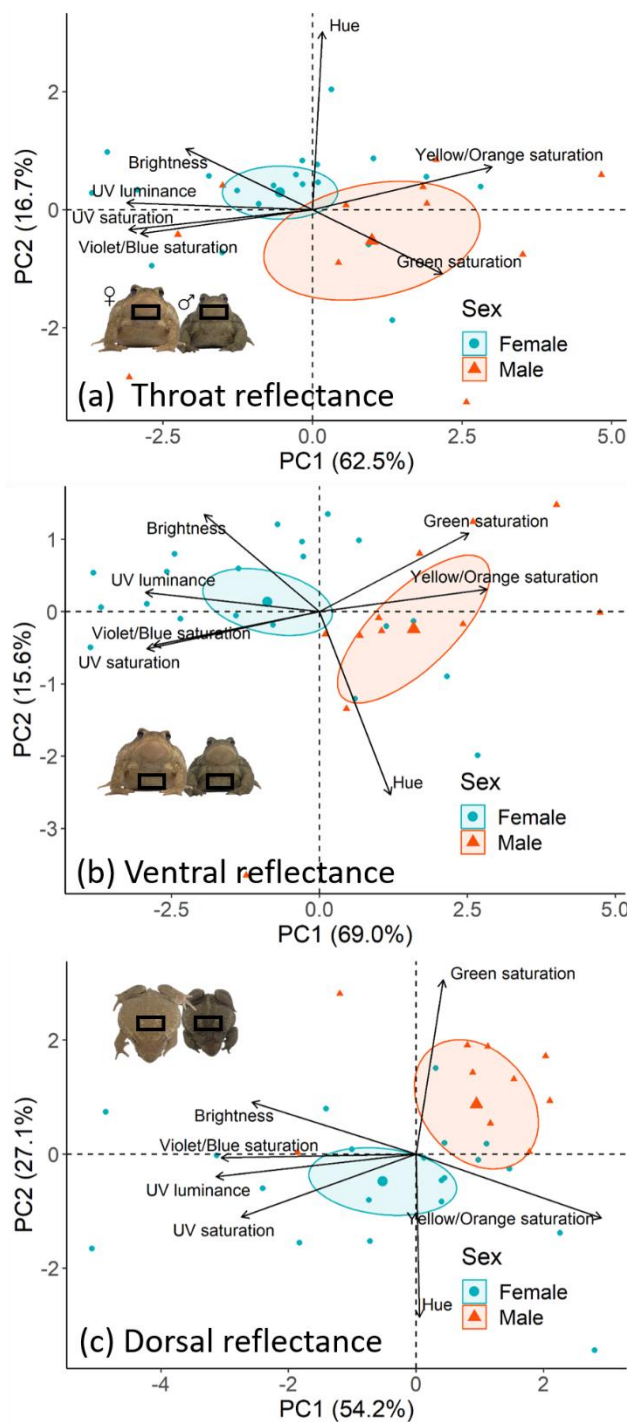
797 **Fig. 1: Ordination plot from reflectance principal component analysis (PCA) scores**  
798 **grouped by sex in non-exposed spiny toads *Bufo spinosus*, for (a) throat reflectance,**  
799 **(b) ventral reflectance and (c) dorsal reflectance.** PC1 and PC2 values show the  
800 contribution of the axes to the total variation. Each dot represents an individual.  
801 Individuals with similar reflectance (PCA scores) are near each other and individuals  
802 with dissimilar reflectance are farther from each other. Circles represent females and  
803 triangles represent males. The circle and triangle at the center of each ellipse represent  
804 the centroid of PCA scores for that group.

805 **Fig. 2: Relationship between ventral reflectance and body condition index in male and**  
806 **female spiny toads *Bufo spinosus*.** More negative values on the PC1 axis represent  
807 stronger reflectance in the UV. Females and males are represented by turquoise and  
808 orange dots, respectively. The full line represents a significant relationship, the dashed  
809 line a non-significant relationship.

810 **Fig. 3: Ordination plot from reflectance principal component analysis (PCA) scores**  
811 **grouped by sex and agrochemical treatment in spiny toads *Bufo spinosus*, for (a)**  
812 **throat reflectance, (b) ventral reflectance and (c) dorsal reflectance.** PC1 and PC2  
813 values show the contribution of the axes to the total variation. Each combination of sex  
814 and treatment is represented by a different symbol. Each dot represents an individual.  
815 In each sex, individuals were exposed either to tap water (control, FC= female control,  
816 MC= male control), Nicosulfuron (FN= female Nicosulfuron, MN= Male  
817 Nicosulfuron) or AMPA (FA= female AMPA, MA= male AMPA). The symbol at the  
818 centre of each ellipse represents the centroid of PCA scores for that group.

819

820 **Fig. 1: Ordination plot from reflectance principal component analysis (PCA) scores**  
 821 **grouped by sex in non-exposed spiny toads *Bufo spinosus*, for (a) throat reflectance,**  
 822 **(b) ventral reflectance and (c) dorsal reflectance.** PC1 and PC2 values show the  
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 825 with dissimilar reflectance are farther from each other. Circles represent females and  
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 827 the centroid of PCA scores for that group.

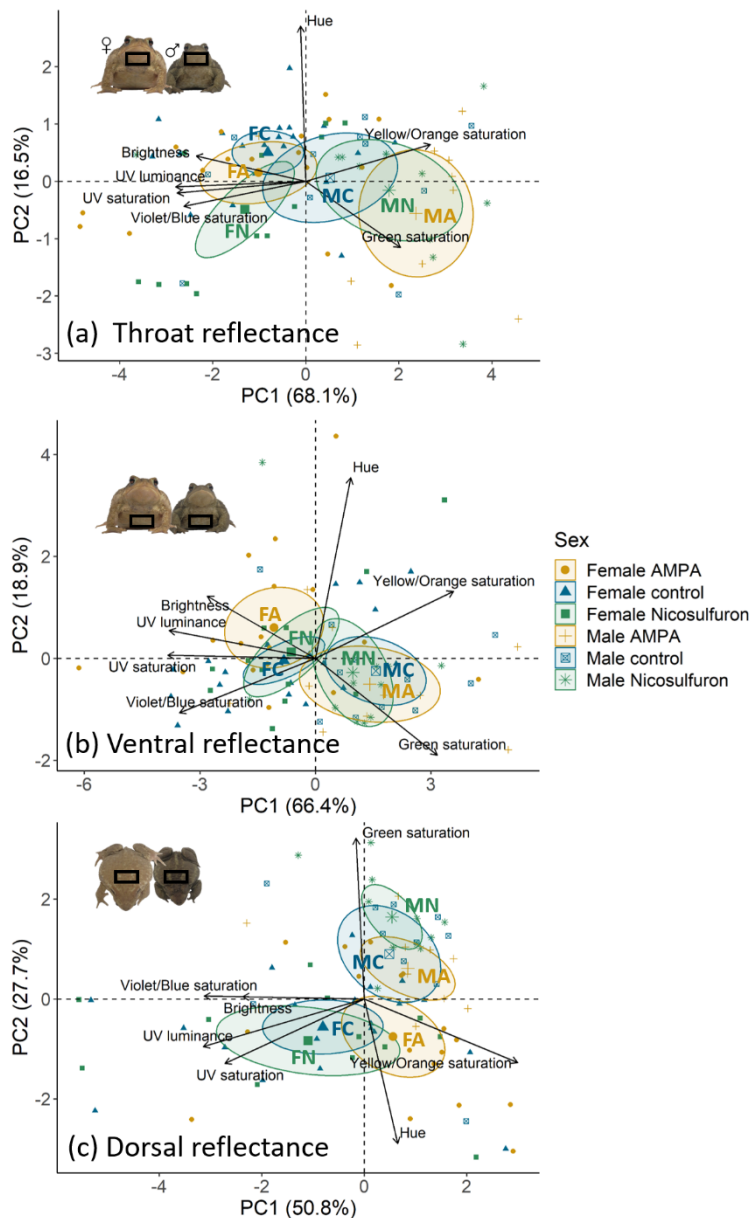


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835 **Fig. 3: Ordination plot from reflectance principal component analysis (PCA) scores**  
 836 **grouped by sex and agrochemical treatment in spiny toads *Bufo spinosus*, for (a)**  
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 842 Nicosulfuron) or AMPA (FA= female AMPA, MA= male AMPA). The symbol at the  
 843 centre of each ellipse represents the centroid of PCA scores for that group.



844

845 **Caption to tables**

846 **Table 1: Relationships between reflectance PCA scores and sex in spiny toads (*Bufo***  
847 ***spinosus*).** Values are estimates obtained from linear regressions with female as a  
848 reference level. PC scores were obtained from a PCA including hue, UV-saturation,  
849 UV-luminance, total brightness (B1 300-700), yellow-orange saturation, violet-blue  
850 saturation, and green saturation. The variables that correlated the most with axis 1  
851 (PCA 1) and axis 2 (PCA 2) are identifiable in Fig. 1 and Appendix B. Achromatic  
852 components (UV, luminance) are mainly correlated with PCA 1, while chromatic  
853 components (e.g. hue) are mostly correlated with PCA 2. Values in bold are significant  
854 at  $\alpha=0.05$ .

855 **Table 2: Relationships between reflectance PCA scores and pesticide exposure**  
856 **according to sex in spiny toads (*Bufo spinosus*).** Values are estimates obtained from  
857 linear regressions with control as a reference level. PC scores were obtained from a  
858 PCA including hue, UV-saturation, UV-luminance, total brightness (B1 300-700),  
859 yellow-orange saturation, violet-blue saturation, and green saturation. The variables  
860 that correlated the most with axis 1 (PCA 1) and axis 2 (PCA 2) are identifiable in Fig.  
861 2 and Appendix B. Achromatic components (UV, luminance) are mainly correlated  
862 with PCA 1, while chromatic components (e.g. hue) are mostly correlated with PCA 2.  
863 Values in bold are significant at  $\alpha=0.05$ . \*Represents relationships close to statistical  
864 significance ( $p<0.10$ ).

865

866 **Table 1: Relationships between reflectance PCA scores and sex in spiny toads (*Bufo***  
867 ***spinosus*).** Values are estimates obtained from linear regressions with female as a  
868 reference level. PC scores were obtained from a PCA including hue, UV-saturation,  
869 UV-luminance, total brightness (B1 300-700), yellow-orange saturation, violet-blue  
870 saturation, and green saturation. The variables that correlated the most with axis 1  
871 (PCA 1) and axis 2 (PCA 2) are identifiable in Fig. 1 and Appendix B. Achromatic  
872 components (UV, luminance) are mainly correlated with PCA 1, while chromatic  
873 components (e.g. hue) are mostly correlated with PCA 2. Values in bold are significant  
874 at  $\alpha=0.05$ .

Response variables	Explanatory variable	Estimate $\pm$ SE	p-value
<i>Throat reflectance</i>			
PCA 1	Sex	<b>0.61 <math>\pm</math> 0.28</b>	<b>0.036</b>
PCA 2	Sex	-0.41 $\pm$ 0.29	0.174
<i>Ventral reflectance</i>			
PCA 1	Sex	<b>1.11 <math>\pm</math> 0.33</b>	<b>0.002</b>
PCA 2	Sex	-0.18 $\pm$ 0.29	0.539
<i>Dorsal reflectance</i>			
PCA 1	Sex	0.69 $\pm$ 0.38	0.082
PCA 2	Sex	<b>1.05 <math>\pm</math> 0.3</b>	<b>0.002</b>

875

876 **Table 2: Relationships between reflectance PCA scores and pesticide exposure**  
877 **according to sex in spiny toads (*Bufo spinosus*).** Values are estimates obtained from  
878 linear regressions with control as a reference level. PC scores were obtained from a  
879 PCA including hue, UV-saturation, UV-luminance, total brightness (B1 300-700),  
880 yellow-orange saturation, violet-blue saturation, and green saturation. The variables  
881 that correlated the most with axis 1 (PCA 1) and axis 2 (PCA 2) are identifiable in Fig.  
882 2 and Appendix B. Achromatic components (UV, luminance) are mainly correlated  
883 with PCA 1, while chromatic components (e.g. hue) are mostly correlated with PCA 2.  
884 Values in bold are significant at  $\alpha=0.05$ . \*Represents relationships close to statistical  
885 significance ( $p<0.10$ ).

Response variables	Explanatory variables	Females		Males	
		Estimate $\pm$ SE	p-value	Estimate $\pm$ SE	p-value
<i>Throat reflectance</i>					
PCA 1	AMPA	-0.09 $\pm$ 0.26	0.724	<b>0.84 <math>\pm</math> 0.38</b>	<b>0.036</b>
	Nicosulfuron	-0.23 $\pm$ 0.29	0.432	0.58 $\pm$ 0.37	0.132
PCA 2	AMPA	-0.33 $\pm$ 0.28	0.246	-0.58 $\pm$ 0.5	0.257
	Nicosulfuron	<b>-0.92 <math>\pm</math> 0.31</b>	<b>0.004</b>	-0.2 $\pm$ 0.49	0.680
<i>Ventral reflectance</i>					
PCA 1	AMPA	-0.38 $\pm$ 0.2	0.699	-0.07 $\pm$ 0.38	0.850
	Nicosulfuron	-0.12 $\pm$ 0.3	0.789	-0.28 $\pm$ 0.37	0.459
PCA 2	AMPA	0.56 $\pm$ 0.33	0.093*	-0.23 $\pm$ 0.43	0.605
	Nicosulfuron	0.14 $\pm$ 0.36	0.692	-0.03 $\pm$ 0.42	0.950
<i>Dorsal reflectance</i>					
PCA 1	AMPA	0.73 $\pm$ 0.37	0.055*	0.19 $\pm$ 0.26	0.474
	Nicosulfuron	-0.15 $\pm$ 0.41	0.712	0.03 $\pm$ 0.26	0.913
PCA 2	AMPA	-0.14 $\pm$ 0.27	0.608	-0.21 $\pm$ 0.32	0.530
	Nicosulfuron	-0.2 $\pm$ 0.3	0.510	0.53 $\pm$ 0.32	0.103

886

887 **Caption to Appendices**

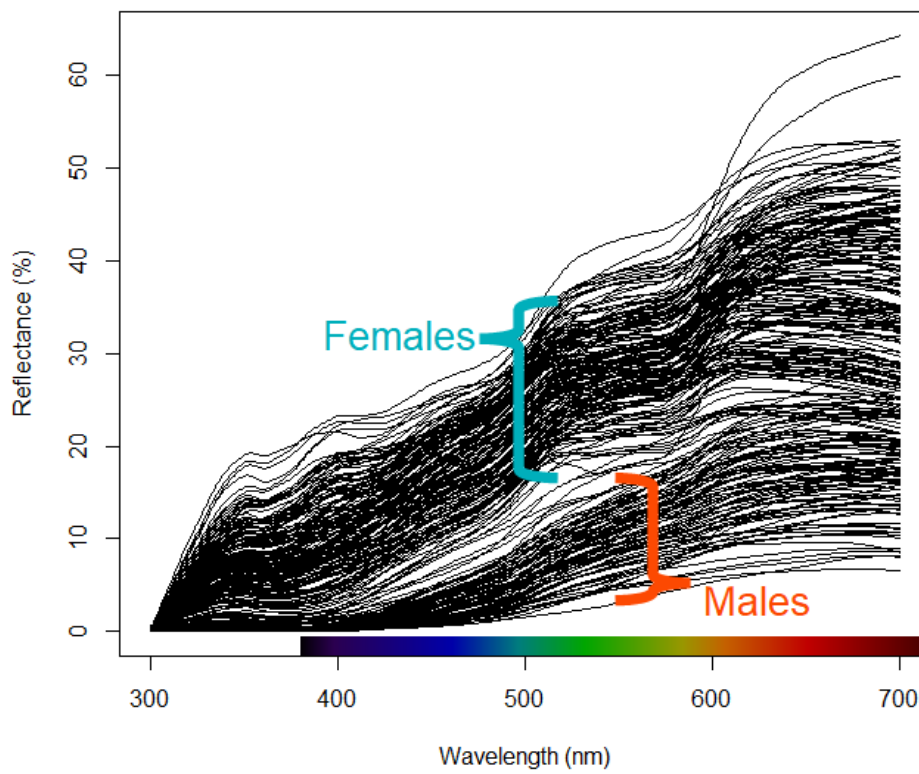
888 **Appendix A : Reflectance spectrum in female (turquoise bracket) and male (coral**  
889 **bracket) spiny toads (*Bufo spinosus*, n=81).** The reflectance was measured in  
890 duplicates on three body parts (throat, ventral and dorsal areas) by using a USB-2000  
891 spectrophotometer.

892 **Appendix B: Correlations between the Principale Components (PCs) 1 and 2, and**  
893 **the different fractions of reflectance included in the PCA:** hue, UV-saturation, UV-  
894 luminance, total brightness (B1 300-700), yellow-orange saturation, violet-blue  
895 saturation, and green saturation; for the different body areas, and for PCA realized for  
896 differences between sexes, and between treatments and sexes.

897 **Appendix C : Relationships between reflectance PCA scores and body condition or**  
898 **sex in spiny toads (*Bufo spinosus*).** Values are estimates obtained from linear  
899 regressions. Note that model included the interaction between body condition and sex,  
900 but the final model was selected using a top-down selection procedure, and only the  
901 last retained variable is represented (the interaction or additive effect of body  
902 condition and sex was never selected). PC scores were obtained from a PCA including  
903 hue, UV-saturation, UV-luminance, total brightness (B1 300-700), yellow-orange  
904 saturation, violet-blue saturation, and green saturation. The variables that correlated  
905 the most with axis 1 (PCA 1) and axis 2 (PCA 2) are identifiable in Fig. 2 and Appendix  
906 B. Achromatic components (UV, luminance) are mainly correlated with PCA 1, while  
907 chromatic components (e.g. hue) are mostly correlated with PCA 2.

908

909 **Appendix A : Reflectance spectrum in female (turquoise bracket) and male (coral**  
910 **bracket) spiny toads (*Bufo spinosus*, n=81).** The reflectance was measured in  
911 duplicates on three body parts (throat, ventral and dorsal areas) by using a USB-2000  
912 spectrophotometer.



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914

915 **Appendix B: Correlations between the Principale Components (PCs) 1 and 2, and**  
 916 **the different fractions of reflectance included in the PCA:** hue, UV-saturation, UV-  
 917 luminance, total brightness (B1 300-700), yellow-orange saturation, violet-blue  
 918 saturation, and green saturation; for the different body areas, and for PCA realized for  
 919 differences between sexes, and between treatments and sexes.

920

921

Body area	Variable included in the PCA	PCA sex		PCA treatment	
		PCA1	PCA2	PCA1	PCA2
Dorsal	Brightness	-0.774	0.274	-0.689	0.014
	Green saturation	0.128	0.920	-0.047	0.936
	Hue	0.017	-0.860	0.19	-0.841
	UV luminance	-0.943	-0.120	-0.913	-0.277
	UV saturation	-0.824	-0.332	-0.790	-0.376
	Violet/Blue saturation	-0.92	-0.019	-0.908	0.017
	Yellow/Orange saturation	0.876	-0.337	0.870	-0.371
Throat	Brightness	-0.657	0.319	-0.821	0.155
	Green saturation	0.671	-0.337	0.711	-0.402
	Hue	0.051	0.935	-0.038	0.945
	UV luminance	-0.962	0.035	-0.975	-0.033
	UV saturation	-0.952	-0.106	-0.964	-0.072
	Violet/Blue saturation	-0.892	-0.126	-0.913	-0.151
	Yellow/Orange saturation	0.930	0.224	0.936	0.224
Ventral	Brightness	-0.64	0.442	-0.707	0.308
	Green saturation	0.831	0.357	0.797	-0.478
	Hue	0.395	-0.834	0.232	0.893
	UV luminance	-0.966	0.088	-0.956	0.138
	UV saturation	-0.96	-0.17	-0.967	0.015
	Violet/Blue saturation	-0.922	-0.154	-0.889	-0.269
	Yellow/Orange saturation	0.931	0.102	0.904	0.331

922

923



924 **Appendix C : Relationships between reflectance PCA scores and body condition or**  
 925 **sex in spiny toads (*Bufo spinosus*).** Values are estimates obtained from linear  
 926 regressions. Note that model included the interaction between body condition and sex,  
 927 but the final model was selected using a top-down selection procedure, and only the  
 928 last retained variable is represented (the interaction or additive effect of body  
 929 condition and sex was never selected). PC scores were obtained from a PCA including  
 930 hue, UV-saturation, UV-luminance, total brightness (B1 300-700), yellow-orange  
 931 saturation, violet-blue saturation, and green saturation. The variables that correlated  
 932 the most with axis 1 (PCA 1) and axis 2 (PCA 2) are identifiable in Fig. 2 and Appendix  
 933 B. Achromatic components (UV, luminance) are mainly correlated with PCA 1, while  
 934 chromatic components (e.g. hue) are mostly correlated with PCA 2.

Treatment	Response variable	Dependant variable	Estimate	SE	p-value
AMPA	Throat reflectance				
	PCA1	Sex (Males-Females)	1.549	0.354	<0.001
	PCA2	Sex (Males-Females)	-0.658	0.418	0.128
	Belly reflectance				
	PCA1	Sex (Males-Females)	1.152	0.414	0.010
	PCA2	Sex (Males-Females)	-0.963	0.421	0.031
	Dorsal reflectance				
	PCA1	BCI	-0.848	1.189	0.482
	PCA2	Sex (Males-Females)	0.981	0.343	0.008
Nicosulfuron	Throat reflectance				
	PCA1	Sex (Males-Females)	1.42	0.350	<0.001
	PCA2	Sex (Males-Females)	0.312	0.463	0.507
	Belly reflectance				
	PCA1	Sex (Males-Females)	0.739	0.311	0.027
	PCA2	Sex (Males-Females)	-0.349	0.485	0.480
	Dorsal reflectance				
	PCA1	Sex (Males-Females)	0.867	0.423	0.053
	PCA2	Sex (Males-Females)	1.78	0.281	<0.001

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936