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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**

3 **Sabrina Tartu^{1*}, Léa Lorrain-Soligon^{1*}, Marion Cheron¹, Andréaz Dupoué² and**
4 **François Brischoux¹**

5 ¹ Centre d'Etudes Biologiques de Chizé (CEBC), UMR 7372 CNRS- Université de la
6 Rochelle, 79360 Villiers-en-Bois, France

7 ² Ifremer, Univ Brest, CNRS, IRD, UMR 6539, LEMAR, Plouzane, France

8
9 *Joint first authors, *corresponding authors: llorrain.lea@gmail.com;
10 tartu.sabrina@gmail.com

11
12 **ORCID IDs:**

13 **ST: 0000-0002-4257-7495**

14 **LLS: 0000-0002-8723-7478**

15 **MC: 0000-0001-6416-6374**

16 **AD: 0000-0002-2501-464X**

17 **FB: 0000-0002-5788-1326**

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 CaCO₃
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
ORGANICSTM), 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
ORGANICSTM). 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: ± 0.1 g) and their snout-vent length (SVL) was
221 measured with an electronic calliper (± 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 ± 0.301 , $p=0.004$,
252 values are LM estimates \pm standard error), body condition (-3.639 ± 1.270 , $p=0.008$),
253 and their interaction (6.237 ± 2.729 , $p=0.030$). Indeed, body condition was negatively
254 related with ventral PC1 in females (-3.64 ± 1.27 , $p=0.008$, values are Linear Models
255 estimates \pm standard error, **Fig. 2**), but not in males (1.53 ± 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

338 2005; Lifshitz and St Clair 2016), which could lead to alterations of gonadal form and
339 function (McCoy et al. 2008). As colouration is linked to hormone expression (Richards
340 1982; Rand 1992; Tang et al. 2014) and reproductive abilities (Clutton-Brock 2009;
341 Kopena et al. 2020), it is also likely to be disrupted by agrochemicals. Pigments
342 involved in trait colouration, such as melanin, may be physiologically costly to
343 produce (Jawor and Breitwisch 2003), and could be traded off with the costs of
344 agrochemical exposure (e.g. reduced growth and increased susceptibility to disease
345 (Baker et al. 2013). Additionally, agrochemicals, including AMPA, can cause damage
346 to organisms by producing free radicals that overwhelm the antioxidant system
347 (Galván and Alonso-Alvarez 2009; Cheron et al. 2022). Thus, pigments may rather be
348 allocated to immune- or antioxidant-system (detoxification mechanisms, (McGraw
349 2005)), rather than to ornamentation in exposed individuals (Faivre et al. 2003;
350 Arellano-Aguilar and Macías Garcia 2008; Lifshitz and St Clair 2016). Exposure to
351 agrochemicals and other pollutants results in reduced carotenoid colouration (Baatrup
352 and Junge 2001; Alonso-Alvarez and Galván 2011; Shenoy 2012; Lifshitz and St Clair
353 2016), probably because of the allocation of dietary carotenoids to counteract oxidative
354 stress (Arellano-Aguilar and Macías Garcia 2008), and also to a decrease in the
355 expression of brown melanin and an increase in black melanin (Lifshitz and St Clair
356 2016).

357

358 Interestingly, we show that, in AMPA-exposed males, throat yellow-orange
359 colouration was more vivid than that of control males, probably indicating higher
360 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**

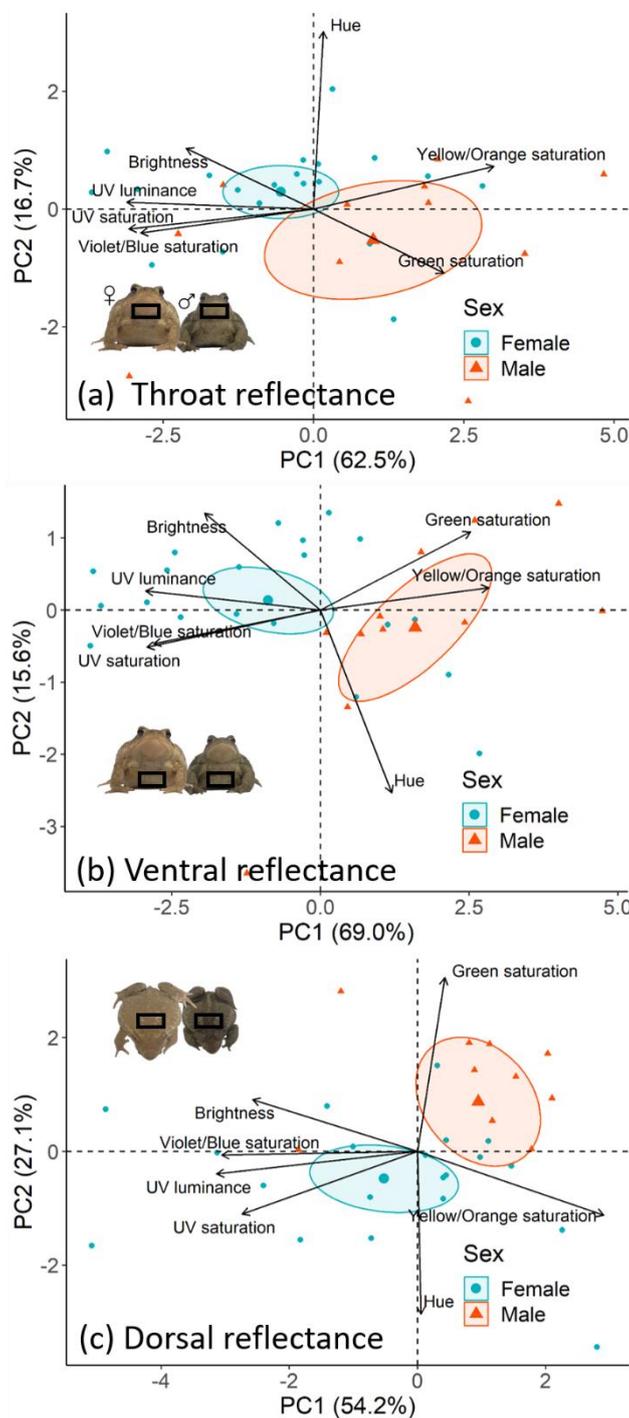
797 **Fig. 1: Ordination plot from reflectance principal component analysis (PCA) scores**
798 **grouped by sex in non-exposed spiny toads *Bufo spinosus*, for (a) throat reflectance,**
799 **(b) ventral reflectance and (c) dorsal reflectance.** PC1 and PC2 values show the
800 contribution of the axes 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.

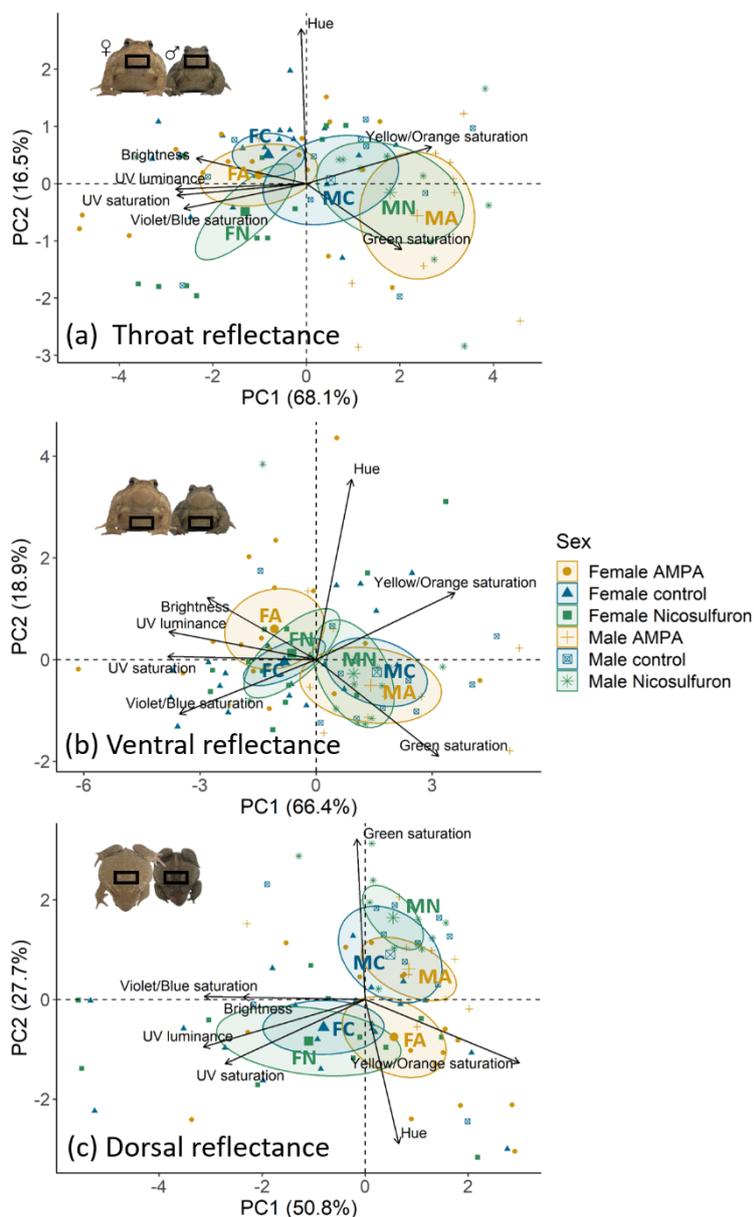
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 827 the centroid of PCA scores for that group.



828

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 836 **grouped by sex and agrochemical treatment in spiny toads *Bufo spinosus*, for (a)**
 837 **throat reflectance, (b) ventral reflectance and (c) dorsal reflectance.** PC1 and PC2
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 841 MC= male control), Nicosulfuron (FN= female Nicosulfuron, MN= Male
 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
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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	0.61 \pm 0.28	0.036
PCA 2	Sex	-0.41 \pm 0.29	0.174
<i>Ventral reflectance</i>			
PCA 1	Sex	1.11 \pm 0.33	0.002
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	1.05 \pm 0.3	0.002

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	0.84 \pm 0.38	0.036
	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	-0.92 \pm 0.31	0.004	-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**

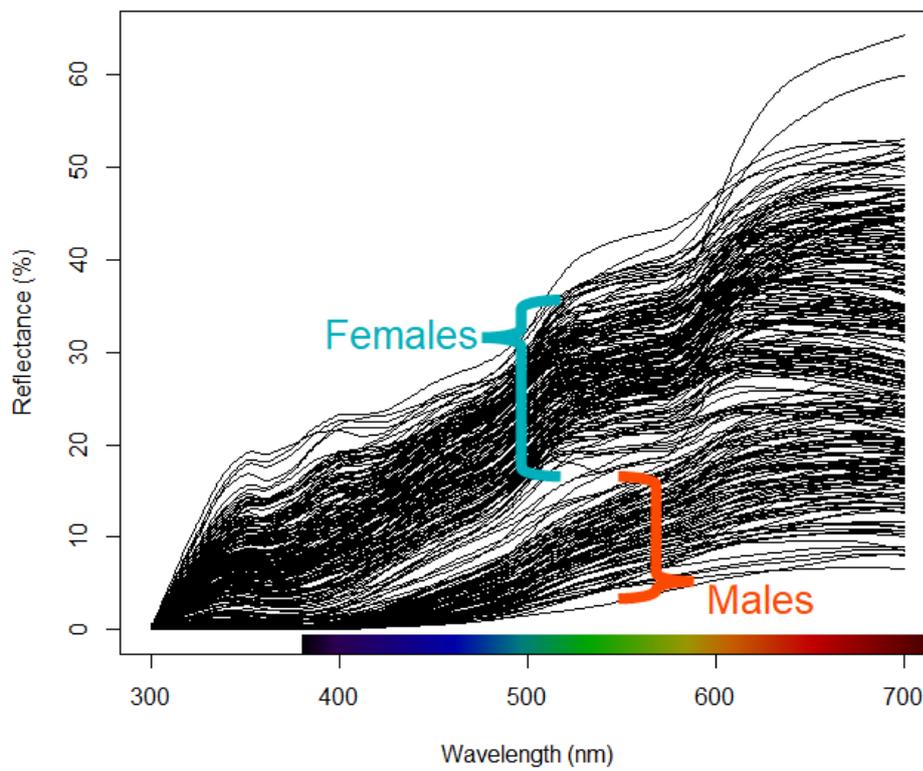
888 **Appendix A : Reflectance spectrum in female (turquoise bracket) and male (coral**
889 **bracket) spiny toads (*Bufo spinosus*, n=81).** The reflectance was measured in
890 duplicates on three body parts (throat, ventral and dorsal areas) by using a USB-2000
891 spectrophotometer.

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
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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.



913

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
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 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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