Colouration matters in dull toads: ultraviolet adornment for ladies and agrochemicals fading effects

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Abstract :

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

Keywords : Amphibians, Honest signal, AMPA, Nicosulfuron, Sexual dichromatism

INTRODUCTION

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

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

system (Galván and Alonso-Alvarez 2009; Cheron et al. 2022), and the major groups of

animal pigments exhibit antioxidant activity (McGraw 2005) suggesting a trade-off

between the expression of colouration and resistance to pollutants (Arellano-Aguilar

and Macías Garcia 2008). Pollutants have been related to enhanced traits coloured by

black melanin (Lifshitz and St Clair 2016; Goiran et al. 2017), reduced carotenoid

colouration (Baatrup and Junge 2001; Alonso-Alvarez and Galván 2011; Shenoy 2012),

as well as modified hue and saturation of individuals (Larramendy 2017; Ujhegyi and

Bókony 2020). These impairments could result from negative effects of pollutants on

gonad function (McCoy et al. 2008; Hayes et al. 2010), leading to sex steroid disruption

(Trudeau et al. 2020). Testosterone, for instance, can affect chromatophore expression

and xanthophores dispersion in amphibian (Richards 1982; Tang et al. 2014). Despite

their potential higher susceptibility to contaminants, due to a lack of protective

epidermal structures and permeable skin (Wells 2007), only a handful of studies have

been conducted on the effects of pollutants on colouration in amphibians (Larramendy

2017; Ujhegyi and Bókony 2020). Agrochemicals are mostly sprayed in spring, when

many amphibians' species from temperate areas reproduce (Wells 2007; Berger et al.

2013; Lenhardt et al. 2015), and animals may be exposed to these compounds through

inhalation, skin permeability, and consumption of contaminated food as well as water.

In this study, we investigated skin colour variations and UV signalling according to

sex and considering different body parts (throat, ventral and dorsal areas) in a

widespread amphibian species, the spiny toad (*Bufo spinosus*), characterized by an

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).

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MATERIAL AND METHODS

Study species

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

Animal care and housing

Between 21-Sep-2020 and 27-Oct-2020, 81 free-ranging spiny toads (32 males and 49 females) were captured by hand on the roadside to the south of the Deux-Sèvres department (western France) and brought back to the laboratory. Toads were housed in a thermally controlled (17°C) room under natural photoperiod (12:12 h cycle), in individual plastic boxes (80×40×16 cm) lined with paper towels, with a shelter (halved PVC tubes) and a petri dish for water. Twice a week, in quantities adapted to their size, toads were fed crickets, meal worms and earth worms dusted with a mixture of $CaCO_3$ and multivitamin powder (Repti Calcium, Zoo Med Laboratories, Inc). Once a week, boxes were cleaned and paper towels were replaced.

Agrochemical exposure

AMPA (aminomethylphosphonic acid) is the primary metabolite of glyphosate, and is 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 ± 2.08 SE, AMPA males: 59.93 mm ± 3.87 SE, Nicosulfuron males: 59.98 mm ± 2.49 SE) or females (linear model: Sum Sq=231.43, F-value=1.703, p-value=0.193, Control females: 72.81 mm ± 8.33 SE, AMPA females: 73.81 mm ± 8.66 SE, Nicosulfuron females: 78.22 mm ± 7.43 SE). AMPA was administered by dissolution in drinking water (dechlorinated tap water) at 4 µg.L^{-1} (crystalline powders, 99% purity, ACROS ORGANICS™), and Nicosulfuron was administered by dissolution in drinking water (dechlorinated tap water) at 1 µg.L^{-1} (crystalline powders, 99% purity, ACROS ORGANICS™). 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.

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(body size) and log(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

presented in Appendix B. Second, we used linear models (LMs) to test 1) the effects of

sex and 2) the effects of body condition on reflectance (PC 1 and PC 2 for each body

part). Sex, body condition and their interaction were defined as explanatory variables

and reflectance PC1 and PC2 for each body part as response variables. We tested these

relationships in control individuals only, to test for this effect without the effect of

contaminant exposure. Third, we tested the effects of the treatment (Control, AMPA,

or Nicosulfuron) on reflectance (PC 1 and PC 2 for each body part) in each sex

separately, as PC1 and PC2 values were highly different between males and females

(LM models: all p-values<0.029, except for the comparisons in PCA 2 throat coloration

between sexes [p-value=0.159], see also **Fig. 1**). Finally, we tested the effects of body

condition, sex and their interaction on reflectance in AMPA and Nicosulfuron exposed

individuals only. These variables were selected by backward stepwise selection, and

only the last retained variables are presented in the final models. All analyses were

performed with R v.4.0.5 (R Core Team 2019).

RESULTS

Reflectance sexual dimorphism in control individuals

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

Effect of agrochemical exposure on reflectance and body condition

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

DISCUSSION

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

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

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

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

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The mate choice hypotheses can be strengthened by the fact that colouration is often

an honest signal of individuals' quality and ability to reproduce (Baatrup and Junge

2001; Clutton-Brock 2009; Shenoy 2012; Blévin et al. 2014). In birds, UV colouration is

already known to reflect individual condition and quality (Prum 2006), and to be

related to age, sex, and morphology in lizards (Martin et al. 2013). In our study, we

show that females with higher UV reflectance were in better body condition, which is

consistent with an honest signal of quality in female spiny toads. The relationship

between colouration and quality is common in various species, as only individuals in

good condition can afford to allocate pigments for trait colouration without

compromising other functions (Hamilton and Zuk 1982). UV colouration might be

costly to produce (Senar 2006), and its production could be traded off with many other

functions. Colouration can be influenced by immunocompetence and parasite load

(Molnár et al. 2012; Olsson et al. 2013), testosterone levels (Cox et al. 2005, 2008), and

oxidative stress (Simons et al. 2012), which could all be influenced by exposure to

agrochemicals (Mann et al. 2009; Brandt et al. 2016; Cheron et al. 2022). Our results

thus suggest that UV colour could be an important female-condition signal for males

in amphibians. To understand the underlying mechanisms, we should further

investigate if UV colouration in this species is linked to immunity or hormone levels

(such as oestrogen), and additionally to fecundity or investment in reproduction, as it

has been shown in lizards (Kopena et al. 2020).

We were here able to show that spiny toads are sexually dichromatic, and this

dichromatism can be affected by agrochemicals. Numerous agrochemicals have

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

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

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 Superieur, 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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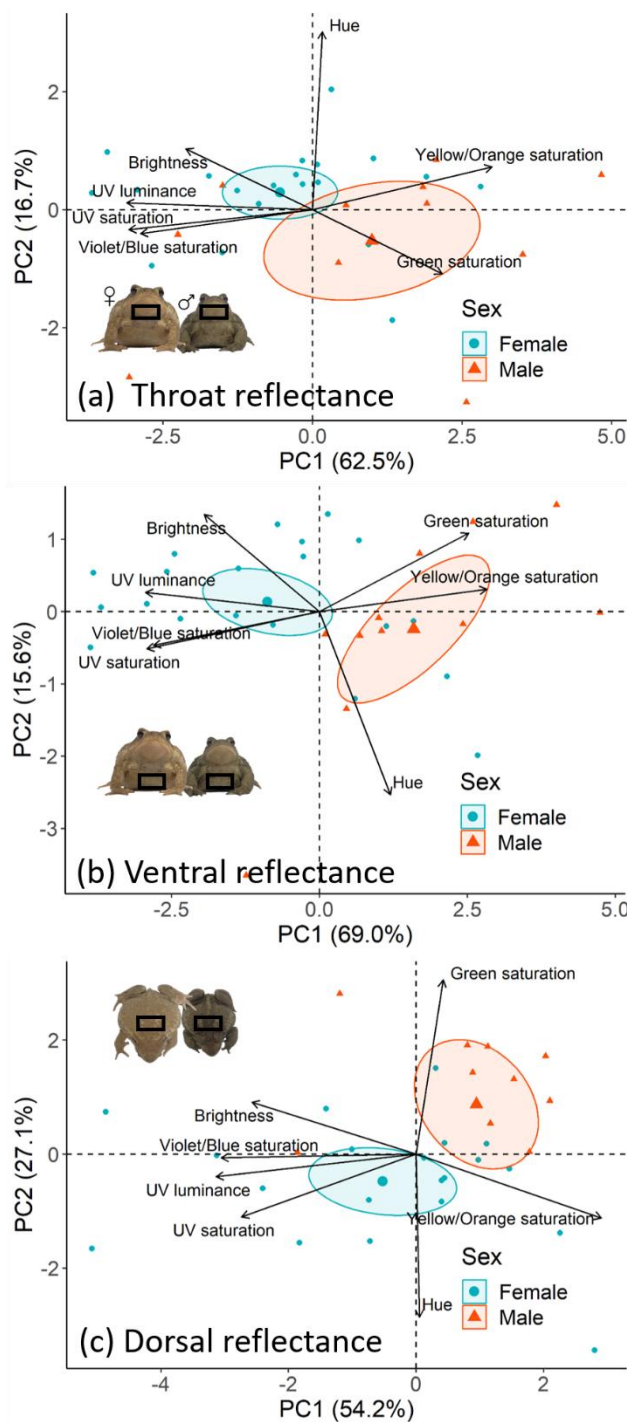795

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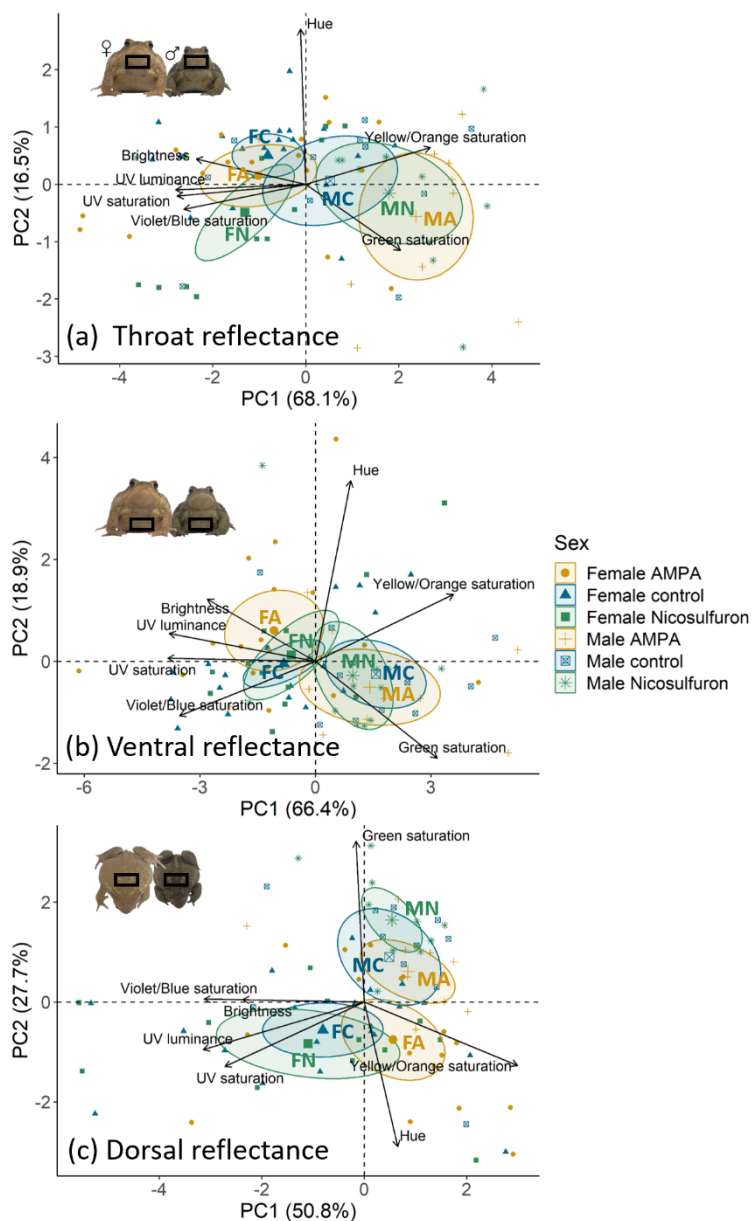
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Fig. 1: Ordination plot from reflectance principal component analysis (PCA) scores grouped by sex in non-exposed spiny toads *Bufo spinosus*, **for (a) throat reflectance, (b) ventral reflectance and (c) dorsal reflectance**. PC1 and PC2 values show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

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

834

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

Caption to tables

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

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

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

Response variables	Explanatory variable	Estimate ± SE	p-value
Throat reflectance			
PCA 1	Sex	**0.61 ± 0.28**	**0.036**
PCA 2	Sex	-0.41 ± 0.29	0.174
Ventral reflectance			
PCA 1	Sex	**1.11 ± 0.33**	**0.002**
PCA 2	Sex	-0.18 ± 0.29	0.539
Dorsal reflectance			
PCA 1	Sex	0.69 ± 0.38	0.082
PCA 2	Sex	**1.05 ± 0.3**	**0.002**

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

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

Caption to Appendices

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

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

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

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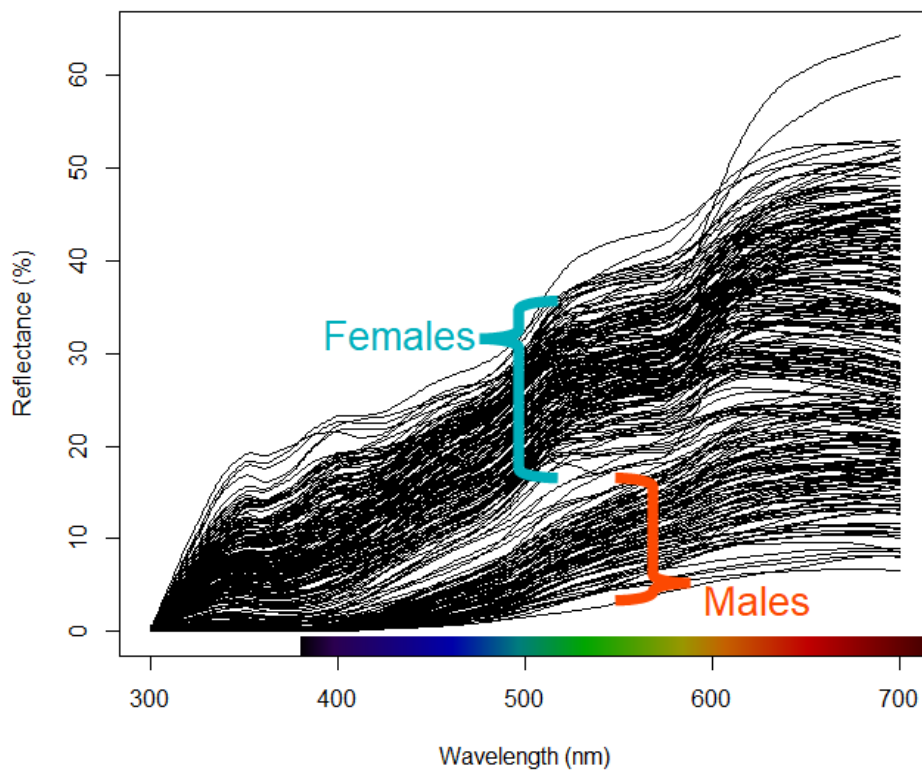
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Appendix B: Correlations between the Principale Components (PCs) 1 and 2, and the different fractions of reflectance included in the PCA: hue, UV-saturation, UV-luminance, total brightness (B1 300-700), yellow-orange saturation, violet-blue saturation,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

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

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